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Pestana**

**Risco de predação *versus* exposição a pesticidas em
invertebrados aquáticos**

**Predation risk *versus* pesticide exposure: effects of
fear and loathing on aquatic invertebrates**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação científica de Donald J. Baird, Research Professor, Department of Biology, University of New Brunswick.

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palavras-chave

stressores múltiplos, imidacloprid, interações predador-presa, invertebrados de água doce, truta, pesticidas

resumo

Os organismos aquáticos apresentam respostas plásticas à presença de predadores que são induzidas por pistas químicas presentes na água. Estas respostas além de minimizar os efeitos de predação, têm também custos associados que podem incluir alterações na tolerância às variações ambientais tais como contaminação por pesticidas. Os efeitos dos pesticidas em sistemas naturais resultam assim da toxicidade mas também do efeito nas interações predador-presa. Apesar de extremamente relevante nomeadamente para análises de risco, as possíveis interações entre contaminação por pesticidas e pressão predatória, não estão ainda esclarecidas nem tão pouco os mecanismos comportamentais e fisiológicos que as medeiam.

O principal objectivo da investigação aqui apresentada foi assim avaliar o efeito de um insecticida, imidacloprid, em diferentes espécies de invertebrados aquáticos sujeitos a diferentes níveis de risco de predação. Numa primeira fase o efeito do Imidacloprid nas comunidades naturais foi avaliado com base em estudos de mesocosmos conjuntamente com ensaios ecotoxicológicos baseados em respostas individuais. Pulsos de concentrações relevantes de imidacloprid revelaram-se extremamente tóxicas para insectos aquáticos. A alimentação e metabolismo de uma espécie de plecóptero, *Pteronarcys comsctocki*, mostraram-se como respostas sub-letais sensíveis e assim ferramentas válidas em termos de biomonitorização dos efeitos do pesticida. Usando um clone de *Daphnia magna* foi também avaliada a relativa importância das substâncias de alarme e de kairomonas de truta (*Salmo trutta*) como mediadores de respostas anti predação em *Daphnia*. A combinação de kairomonas e substâncias de alarme mostrou ser necessária para uma resposta mais forte induzindo alterações no comportamento, fisiologia e história de vida de *Daphnia*. Esta combinação de sinais químicos pode ser assim usada para avaliar efeitos de intimidação ou de risco de predação. Utilizando metodologias ecotoxicológicas padronizadas avaliaram-se em laboratório as respostas de *D. magna*, *Chironomus riparius* e *Sericostoma vittatum*, expostos a concentrações sub-letais de Imidacloprid bem como diferentes níveis de risco de predação. Observaram-se efeitos significativos de concentrações relevantes de Imidacloprid na fisiologia e comportamento dos insectos enquanto que *D. magna* se mostrou muito mais tolerante respondendo apenas a concentrações bastante altas de imidacloprid. A exposição combinada a ambos os stressores foi avaliada considerando mecanismos de acção ecotoxicológicos dos dois stressores e usando diferentes abordagens nomeadamente modelos de referência para misturas químicas e análises de variância. No caso dos insectos, foram observados efeitos aditivos na maioria dos parâmetros testados sendo que a exposição a concentrações sub-letais de Imidacloprid inibiu algumas das respostas anti predatórias com potenciais efeitos em termos de mortalidade devido a predação em campo. Para *D. magna* a exposição simultânea aos dois stressores mostrou desvios relativamente aos modelos de referência que incluem sinergismo para algumas dos parâmetros testados. Os resultados obtidos mostram que invertebrados sujeitos a elevada pressão predatória são mais afectados por concentrações sub-letais de pesticidas. Dado que muitas espécies de invertebrados são simultaneamente sujeitas ao risco de predação e à contaminação por pesticidas, o estudo do efeitos combinados destes dois factores nas comunidades aquáticas, é extremamente importante para melhor prever e interpretar os efeitos ecológicos da contaminação por pesticidas em sistemas naturais.

Keywords

multiple stressors, imidacloprid, predator-prey interactions, freshwater invertebrates, trout, pesticides.

Abstract

Aquatic species rely on plastic traits to defend themselves against predators. The induction of these antipredator defences is mediated by water-borne chemical cues. These induced defences have associated costs which can include decreased tolerance to future environmental change such as pesticide contamination. The effects of pesticides in natural systems can be a result not only of their direct toxicity but also of their indirect effects on predator-prey interactions. Despite their relevance for risk assessment, the interactive effects of pesticide contamination and predation stress are poorly understood because the underlying behavioural and physiological mechanisms are largely unstudied.

The main objective of this thesis was therefore to assess the toxic effects of imidacloprid, a widely-used insecticide, on different invertebrate species under different levels of predation risk. First, the effects of Imidacloprid on freshwater communities were assessed using field-deployed mesocosms and organism level bioassays. Pulses of environmentally-relevant concentrations of imidacloprid were highly toxic to non-target aquatic insects. Feeding and metabolic responses of a stonefly species *Pteronarcys comstocki*, proved to be a good early warning indicator of pesticide detrimental effects. Using a clone of *Daphnia magna*, the relative importance of alarm cues and fish kairomones (*Salmo trutta*) were studied as initiators of *Daphnia* antipredator defences. Although behavioural responses could be triggered by single cues, a combination of alarm cues and predator-specific compounds induced stronger responses and were necessary to induce complete antipredator responses (behavioural, physiological and in the life-history) of daphnids. A combination of both types of chemical cues could thus be used as surrogate for the effects of intimidation posed by predators. Using standardized laboratory toxicological methodologies different responses of *D. magna*, *Chironomus riparius* and *Sericostoma vittatum* to sublethal concentrations of imidacloprid were assessed under different levels of predation risk. Significant effects of environmentally-relevant concentrations of imidacloprid were observed on insect behaviour and physiology. Higher concentrations of imidacloprid were necessary to elicit effects on *D. magna*, relative to the other species tested. Considering the ecotoxicological mode of action of imidacloprid and perceived predation risk, the effects of simultaneous exposure to both stressors were assessed using different approaches with additive reference models and analysis of variance. Both stressors acted additively for most responses tested in the different insect species with exposure to sublethal concentrations of imidacloprid also inhibiting antipredator responses with potential negative consequences in terms of mortality from predation in the field. For *D. magna*, exposure to both stressors showed significant deviations from the reference models results including synergism for some endpoints tested. The results obtained provide insight into the conditions under which interactions between these stressors are likely to occur: invertebrates such as daphnids, midges and caddisflies living under fish predation stress will be more affected by sub-lethal concentrations of Imidacloprid than populations at low predatory pressure. Since predation risk and pesticides can be considered 'similarly-acting' stressors in an ecological sense, studying both types of stress within the same mechanistic framework can be of great value providing compatible data for modelling and allowing improved interpretation of ecological effects of relevant stressors.

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“In Biology the notions of meaning and intelligence are replaced by those of function and natural selection”

John Maynard Smith (1999). The idea of information in Biology *The Quarterly Review of Biology*, 74, 4, 395-400.

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Chapter I

General introduction

Predation and inducible antipredator responses. Effects on population dynamics, community structure and ecosystem function.

Predator–prey interactions have been intensively studied in terms of consumption and density interactions (i.e. changes in predator and prey population dynamics), which have been the basis for much of our understanding of species interactions and community dynamics (Sih *et al.* 1985; Begon *et al.* 1996). However, predators can also trigger changes in prey traits that reduce the effects of predation. A wide variety of antipredator responses has been demonstrated across prey species, illustrating the pervasiveness of predatory stress and the strength of the evolutionary pressures to reduce it (Lima & Dill 1990; Kats & Dill 1998; Lass & Spaak 2003a).

Ecological theory and empirical studies have demonstrated that organisms under predation risk engage in behavioural adaptive responses such as reduced activity or habitat shifts in order to reduce the probability of consumption by predators (Sih 1986; Lima & Dill 1990; Skelly & Werner 1990; Pijanowska & Kowalczewski 1997b; Baumgartner *et al.* 2002; Sih & McCarthy 2002; Rose *et al.* 2003). This avoidance behaviour may lead to a decrease in foraging activity and feeding and, consequently, prey behavioural decisions will reflect a trade-off between energy gain and mortality risk (Kohler & McPeck 1989; Ball & Baker 1996; Lima & Bednekoff 1999; Noonburg & Nisbet 2005)

Some organisms can also show alterations in their morphology (Appleton & Palmer 1988; McCollum & Leimberger 1997; Arnqvist & Johansson 1998; Van Buskirk & Relyea 1998; Barry 2000; Laforsch *et al.* 2004), physiology, development and reproduction rates (Reede 1995; Dahl & Peckarsky 2003b; Stoks *et al.* 2005b; Beckerman *et al.* 2007), to minimize or counterbalance the effects of predation and escape risky situations more rapidly, by maturing earlier or at smaller sizes. (Abrams & Rowe 1996; Reede 1997; Riessen 1999; Dahl & Peckarsky 2003a; Relyea 2007)

Under predation risk, fitness of prey organisms is influenced by conflicting demands between survival, maintenance, growth, reproduction and defence. Time and resource allocation trade-offs in prey mediate the benefit of reducing the risk of being eaten by predators and the costs of reduced energy intake and growth, changes in development and/or reproductive fitness (Abrams & Rowe 1996; McPeck & Peckarsky 1998; Boersma *et al.* 1999; Van Buskirk 2000; Dahl & Peckarsky 2003b; Preisser *et al.* 2005). The

different antipredator defences can thus be considered sublethal effects of predation (Peacor & Werner 2001) and can lead to indirect effects such as changes in competitive ability (Kohler & McPeck 1989; Relyea 2004a), increased vulnerability to other predators (Nesbitt *et al.* 1996; Hoverman & Relyea 2007) and reduced tolerance to future environmental change (Hanazato 2001; Rohr *et al.* 2004).

These costs and benefits have been demonstrated in the laboratory with a number of organisms including crustaceans (Tollrian 1995; Stibor & Navarra 2000; Boeing *et al.* 2005), insects (Peckarsky *et al.* 2001; Dahl & Peckarsky 2003b), and amphibians (Van Buskirk 2000; Relyea 2002; Rohr *et al.* 2004). However, obtaining empirical evidence for the existence of such costs has proved elusive, as it is difficult to accurately measure the benefits and costs of antipredator defences in natural populations under complex environmental regimes. Light, temperature, oxygen, availability of food and different predators are often highly unpredictable and can confound measurement of costs and benefits (Relyea 2002; Boeing *et al.* 2006). Moreover, without multiple traits being assessed, it is difficult to have a complete picture on the costs and benefits since the effects of specific traits on fitness can have alternative pathways and interaction among traits can also influence fitness (Relyea & Auld 2004; Ajie *et al.* 2007).

Since antipredator defences can carry significant costs, they are often plastic and inducible. Plastic, in the sense that prey only express those defences when needed, avoiding costs when predators are absent, and inducible, since they require external stimuli for activation. Inducible defences have been described in all main groups of animals as well as in plants, and have become a major study object during recent years (reviewed by (Tollrian & Harvell 1999). The evolution of inducible behavioural, physiological, morphological, and life-historical antipredator responses suggests that many defences while conferring a clear benefit to prey (i.e. reduced predation risk) can be too costly (see above) to maintain in the absence of predators (DeWitt *et al.* 1998; Stibor & Navarra 2000).

Inducible defences are thus examples of adaptive phenotypic plasticity which can be defined as “*the potential for an organism to produce a range of different, relatively fit phenotypes in multiple environments*” (DeWitt *et al.* 1998). Therefore, the ability to adapt their phenotypes to changing environments allows organisms to exploit a broader range of environmental conditions, and in this way predator-induced responses can reduce the risk

of being consumed by predators. Organisms showing phenotypic plasticity in their response to predation stress also incur other costs: the costs of being plastic. Factors such as costs of maintenance of the sensory and regulatory mechanism of plasticity, production costs of plastic genotypes that exceed those paid by fixed genotypes, information acquisition costs, e.g. sampling information under risky situations, costs of developmental instability, and genetic costs, such as negative pleiotropic effects of plasticity genes on traits other than the plastic ones, and costs of epistasis where regulatory loci producing plasticity may modify the expression of other genes have been identified as important costs of plasticity that limit the plasticity of genotypes to heterogeneous environments (DeWitt *et al.* 1998).

Therefore phenotypically plastic genotypes have both costs (lower fitness for a given trait value) and constraints (lower trait values) compared to fixed genotypes (DeWitt *et al.* 1998; Relyea 2002) which limit the evolution of phenotypic plasticity and also the degree of plasticity that organisms can show. As a result, the evolution of inducible defences over permanent or fixed defences is favoured when there is unpredictability of strong risk such as temporally and spatially variable presence of a predator, when there are non-fatal reliable cues, such as chemical cues, that indicate the enemy presence, when there is a clear benefit and effectiveness of the defence and also when there are costs involved with the defence in the absence of the predator (Tollrian & Harvell 1999; Relyea 2002).

All the costs, limits and conditions necessary for the evolution of inducible defences emphasize their ecological consequences. Inducible defences may have presented the best solution for organisms to adapt to their changing environments and track environmental variation which occurs at a time scale too fast to be tracked by evolutionary change. Therefore, adaptive plastic traits can facilitate short-term persistence of populations suffering environmental changes such as changes in predation risk before genetic adaptation can occur (Pigliucci 2005; Latta *et al.* 2007). Predator induced responses allow prey to be better defended and thus not so vulnerable to predation. Since predators co-evolving with prey also show counter adaptations to prey (induced defences), phenotypic plasticity and inducible responses of prey and predators can affect species co-existence as well as population dynamics and stability (Miner *et al.* 2005), and ultimately have important evolutionary consequences since they can mediate changes in selective pressures (Pigliucci 2005; Fordyce 2006).

It is clear that predators can affect prey populations both through direct consumption, i.e. density-mediated effects, and by inducing costly defensive traits or “trait-mediated effects” (Preisser *et al.* 2005). These trait mediated effects are the adaptive changes in prey behaviour, physiology, phenotype etc., in response to the presence of a predator. Both mortality caused by predators and the induction of behavioural, morphological, developmental and physiological antipredator responses act simultaneously and have effects along food webs through top-down or bottom-up trophic cascades, affecting prey resources (Beckerman *et al.* 1997; Moran & Scheidler 2002; Peacor 2002; Werner & Peacor 2003; Schmitz *et al.* 2004; Preisser *et al.* 2005), competitors as well as other predators (Peacor & Werner 1997). For example, predators can indirectly favour producers by reducing herbivore density and also by inducing herbivores to forage less (Hanazato 2001; Werner & Peacor 2003; Schmitz *et al.* 2004). Density and trait mediated effects of predation can thus cause indirect effects controlling population and communities dynamics (Peacor & Werner 2001).

Theoretical and experimental studies have revealed that trait-mediated direct and indirect interactions are common. Moreover, it has been hypothesized that effects caused by trait mediated mechanisms can be of similar if not higher magnitude than effects caused by density-mediated mechanisms (Lima & Dill 1990; Peacor & Werner 2001; Werner & Peacor 2003; Peacor & Werner 2004; Schmitz *et al.* 2004; Preisser *et al.* 2005).

Since trait-mediated interactions (direct and indirect) are influenced by the inducible defences of prey which are in turn induced by fear, i.e. by the perceived risk of predation (Kats & Dill 1998; Preisser *et al.* 2005), we need a better understanding of mechanisms of predation risk assessment and to investigate and measure prey induced responses as well as the costs and benefits associated with those antipredator responses. This will increase our ability to predict the effects of predation on population dynamics, nutrient cycles, community structure, biodiversity and ecosystem function (Vet 1999; Miner *et al.* 2005).

Predation risk assessment in freshwater environments and the ecological role of infochemicals.

Animals use extrinsic sensory information to gain knowledge of their local environment. This information includes cues on the availability of food, abiotic factors

such as light, temperature or shelter and of the presence of competitors and predators (Dicke & Grostal 2001). Recent studies have illustrated the proximate mechanisms involved in predator-prey interactions focusing on mechanisms of risk assessment and chemical cues as important stimuli that mediate antipredator responses (Kats & Dill 1998; Dicke & Grostal 2001). Chemical cues in aquatic environments are particularly important, given a general low light intensity, coupled with the relatively poor performance of auditory and visual senses. (Kats & Dill 1998; Burks & Lodge 2002; Lass & Spaak 2003a). Water is also an ideal medium for the evolution of chemical signal interactions allowing chemical persistence and gradients to render directional information to organisms (Burks & Lodge 2002).

Antipredator responses mediated by infochemicals have been shown to occur in a variety of freshwater organisms (Lima & Dill 1990; Kats & Dill 1998; Tollrian & Harvell 1999; Lass & Spaak 2003a), and it is hypothesized that the majority of intra- and interspecific interactions between aquatic organisms are mediated by infochemicals (Dicke & Grostal 2001; Burks & Lodge 2002). Therefore, the information carried along food webs by these chemicals can have complex indirect effects and, as it flows independently of trophic interactions, it is in fact an important factor shaping and controlling food webs (Vet 1999; Dicke & Grostal 2001; Werner & Peacor 2003).

The infochemicals that mediate species interactions can be classified considering the costs and benefits of the induced response of organisms involved in those interactions (Dicke & Sabelis 1988). An infochemical can be defined as a chemical that in the natural context conveys information about an interaction between two individuals, evoking in the receiver a behavioural or physiological response that is adaptive either to one or both the interactants. Infochemicals can be divided into two groups, pheromones and allelochemicals. Pheromones mediate intraspecific interactions and can benefit the origin-related organism, the receiver or both organisms. Allelochemicals mediate interspecific interactions and can be further divided into allomones if they evoke responses benefiting the sender, kairomones if they evoke responses that benefit the receiver or synomones if they evoke responses that benefit both the sender and the receiver (Dicke & Sabelis 1988).

Chemical cues produced by the predators that induce adaptive responses on prey organisms are thus termed kairomones and can be contained in the exuvia, eggs, excreta, pheromones or any other product of the predator (Turner *et al.* 1999; Stibor & Navarra

2000; Dicke & Grostal 2001; Iyengar & Harvell 2002; Ruther *et al.* 2002; Slusarczyk & Rygielska 2004). The fact that kairomones benefit the prey in terms of risk assessment and adaptive responses poses the question of the ecological role and benefits of these chemicals for the emitters (predators) and suggests that kairomone release might not be fully suppressed either because the emission of signals creates a larger total benefit (in another information context), or because emission is unavoidable, as is the case for excretory products (Burks & Lodge 2002).

Aquatic organisms can also assess predation risk through the use of indirect chemical signals. These chemicals may include pheromones from alarmed, injured or dead conspecifics (Pijanowska 1997; Wisenden *et al.* 1997; Chivers & Smith 1998; Wisenden *et al.* 2001; Jacobsen & Stabell 2004) but also allelochemicals from heterospecifics (Chivers *et al.* 1996; Wisenden *et al.* 1997; Huryń & Chivers 1999; Schoepner & Relyea 2005).

Considering the costs, limits and benefits of antipredator responses (DeWitt *et al.* 1998; Relyea 2002), prey have to rely on the available information in the local environment to cue the magnitude and specificity (number and combination of traits that can be induced) of antipredator responses (Burks & Lodge 2002). If we consider that during any predation event both kairomones and alarm cues are produced, there is a complex chemical environment that needs to be interpreted by prey to correctly assess the level of threat and deploy appropriate defences. Predators' kairomones are useful to prey species to assess the density or proximity of predators but give no information on the current predation regime (e.g. predators' preferences might vary over time). Similarly, alarm cues inform only that there is a non-specific threat but not which predator is present (Schoepner & Relyea 2005). Since alarm cues from conspecifics or heterospecifics alone can only provide general information about predation risk, rather than identifying a specific predator, they would not be reliable enough to induce costly antipredator responses. However, recognising such nonspecific cues can be advantageous, for example in terms of early warning of danger posed by unfamiliar predators (Pijanowska 1997). On the other hand, prey organisms require chemical cues that are specific to the type of predators (kairomones) because different predators can induce different and sometimes opposing antipredator responses.

In natural systems prey organisms face a complex chemical environment, filled with potential information on predatory stress (Chivers & Smith 1998). Infochemicals from

different predators, or from damaged, injured or consumed conspecifics and heterospecifics induce a variety of morphological, physiological, behavioural and life-history adaptations as defences against predation (Slusarczyk 1999; Schoeppner & Relyea 2005). Since prey defences vary in direction or magnitude according to the type of predator(s) present (e.g. invertebrate vs. vertebrate) and to the level of predation risk they face, the information provided by different cues is critical to assess the level of risk, deploy effective antipredator responses and avoid maladaptive responses (Weber & Declerck 1997; Dahl 1998; Turner *et al.* 1999; Barry 2000; Weber 2003; Hulsmann *et al.* 2004).

Chemical characterisation of chemicals inducing antipredator responses.

For a comprehensive understanding of the finely-tuned mechanisms of predation risk assessment as well as the ultimate explanations leading to the rich diversity of phenotypes and adaptations that we observe in a variety of organisms, it is essential to identify the infochemicals that mediate predator prey interactions (Burks & Lodge 2002; Pohnert *et al.* 2007). So far, only a few attempts have been made to identify kairomones from predators. The most commonly used approach for the identification of these cues involves fractionation of water samples or enriched extracts of predator kairomones using chromatographic techniques and then test the activity of fractions using bioassays that are chosen based on a specific trait showed by a specific prey organism as an antipredator response.

It has been reported that fish kairomones are water soluble and non-volatile with a low-molecular-weight (<500 Dalton), stable to extreme pH and temperature conditions within the range of pH 0.8 to pH 14 and -20°C to $+120^{\circ}\text{C}$ (Loose *et al.* 1993). It has further been shown that this compound is unlikely to be a protein due to its resistance to protease enzymes and that it easily loses its activity due to microbial degradation (Loose *et al.* 1993). These characteristics were assessed with a behavioural assay (diel vertical migration, DVM) with *Daphnia magna*. Von Elert and Loose (1996) using the same bioassay further demonstrated that kairomones produced by different fish species (*Leucaspis delineatus*, *Carassius carassius* and *Rutilus rutilus*) showed similar retention times of the activity by HPLC, indicating that the DVM-inducing kairomones from different fish species are chemically similar. The chemical cues were of anionic nature,

with low lipophilicity, quantitatively extractable by C₁₈-solid phase extraction, reversibly inactivated by acetylation and with no amino groups. The authors suggested that probably an hydroxyl group was essential for biological activity (vonElert & Loose 1996). Both these investigations showed that fractioning of the vertical migration-inducing extract by HPLC yielded only one active fraction for *D. magna* whereas a more recent investigation showed that activity that induced life history shifts in the same clone of *D. magna* was confined to two different adjacent fractions (Von Elert & Stibor 2006). This can mean that the induction of different responses (vertical migration and life-history shifts) can rely on different chemical signals released by fish that share some chemical characteristics. Different active fractions may support the work by Weber (Weber 2003) where differences in the responses of *Daphnia* to kairomones from different fish species were observed. Weber hypothesized that although the possibility of species specific kairomones cannot be excluded, a “fish kairomone cocktail” rather than a single compound may be responsible for the induction of antipredator responses. This can mean that different combinations of the different components of the kairomone cocktail can account for differences in prey responses (Weber 2003).

Chemical characteristics of fish kairomones are in part shared by invertebrate kairomones which also are small, heat-stable, water soluble, non-peptide and polar compounds (Tollrian & vonElert 1994). However, the fact that the responses of organisms such as *Daphnia* to both types of predators are different and sometimes contradictory suggests that it is unlikely that kairomones from fish and invertebrate predators are similar. Tollrian and von Elert (1994), using similar methodologies have shown that the kairomone produced by *Chaoborus*, an invertebrate predator that feeds on *Daphnia*, is probably a carboxylic acid (Tollrian & vonElert 1994).

Moreover, it has been shown that kairomones and alarm cues, alone or in combination, can induce differently the suite and/or magnitude of anti predator responses, suggesting that they are chemically different (Stirling 1995; Pijanowska & Kowalczewski 1997a; Wudkevich *et al.* 1997; Wisenden *et al.* 1999; Wisenden & Millard 2001; Iyengar & Harvell 2002; Schoeppner & Relyea 2005; Laforsch *et al.* 2006; Turner *et al.* 2006). Because the experiments performed by von Elert and Stibor (2006) were performed with extracts of fish kairomones, produced when fish were feeding on *Daphnia*, fractions

inducing responses can be in fact from different chemical signals such as alarm cues from consumed conspecifics and kairomones from fish (Von Elert & Stibor 2006).

Based on the different results reported in the literature, it is clear that this area requires further investigation, particularly focusing on the robustness and ambiguities of the bioassays used to test the activity of individual components of the kairomone. Furthermore, if the activity of the kairomone is due to a mixture of different fractions and not to an individual component, a reliable bioassay might be difficult to achieve because different fractions can induce differently the different antipredator responses and traits.

Chemical identification of the compound(s) inducing antipredator responses can be even harder to achieve because bacteria may also play a pivotal role in chemical signalling in predator prey interactions. It has been suggested that bacteria and not fish are involved in the production of factors that induce vertical migration in *Daphnia* (Ringelberg & Van Gool 1998), or that bacteria from predators can act as an activating factor for latent alarm cues of prey (Stabell *et al.* 2003; Jacobsen & Stabell 2004). This would explain the similar chemical characteristics and effects of cues from different fish species due to the ubiquitous distribution of bacteria. Since microbial degradation of chemical cues from predators has also been demonstrated (Loose *et al.* 1993; Beklioglu *et al.* 2006) further investigation will be crucial to elucidate the role of bacterial communities in predator prey interaction and on trophic cascades.

Nevertheless, the pursuit of the chemical identity and structure of infochemicals (kairomones and also alarm cues) as well as the role of microbial communities in predator prey interactions is essential for understanding the physiological and ecological role of these substances, the metabolic costs involved in their production, their species specificity and the molecular and physiological mechanisms of prey induced responses. Chemical characterization of infochemicals will allow the evaluation of their distribution in natural systems, and a better control of laboratory and *in situ* experiments (with synthetically produced analogs). This will in turn increase our ability to model the results and to monitor and predict the effects of predation risk in natural communities (Burks & Lodge 2002; Pohnert *et al.* 2007).

Variation in chemically induced antipredator responses.

Although chemical induced antipredator responses are continuously being demonstrated in a variety of freshwater organisms, there is a considerable variation in the magnitude, direction and number of responsive traits measured in laboratory experiments.

Different levels of predation risk can be associated with predator density or proximity, and it has been shown that chemically induced defences are also dependent on the concentrations of infochemicals which is a clear demonstration that prey can assess different level of predation risk and consequently exhibit different responses that differ in their magnitude according to the concentration of these chemicals (Reede 1995; Anholt *et al.* 1996; von Elert & Pohnert 2000; Holker & Stief 2005; Schoeppner & Relyea 2005; Castro *et al.* 2007a; Schoeppner & Relyea 2008)

Specific antipredator responses have been shown when organisms are exposed to different predator species (Chivers & Smith 1998; Turner *et al.* 1999; Weber & Van Noordwijk 2002; Dzialowski *et al.* 2003; Relyea 2003a). Vertebrate and invertebrate predators due to different size selectivity can induce different antipredator responses and sometimes life history adaptations in opposite directions. Daphnids, for example, in response to visually hunting vertebrates, such as fish, which select larger individuals, shift their resource allocation in response to fish cues towards reproduction, maturing earlier at a smaller size as well as producing more and smaller neonates. In contrast, when in the presence of gape-limited predators (such as invertebrates like *Chaoborus*) which select smaller individuals, daphnids invest into somatic growth, maturing later and at a larger size (Riessen 1999). The foraging strategies of predators might also induce different prey responses (Wooster & Sih 1995; McIntosh & Peckarsky 1999; Turner *et al.* 1999; Dzialowski *et al.* 2003; Weber 2003; Hoverman & Relyea 2007). In natural systems the responses of prey to multiple predators may vary depending on predator-predator interactions and the potential conflicting antipredator responses to multiple predators which can decrease or enhance the risk of predation (Sih *et al.* 1998).

Chemically induced antipredator responses have been shown to be stronger in responses to chemical cues from feeding predators (Stabell *et al.* 2003; Schoeppner & Relyea 2005, 2008), and predator diets also influence these chemically-induced responses (Kats & Dill 1998; Slusarczyk 1999; Stabell *et al.* 2003; Jacobsen & Stabell 2004;

Schoeppner & Relyea 2005). Prey organisms can differentiate the diet of predators and adjust the magnitude of responses. For instance, it has been shown that organisms exhibit stronger responses to cues from predators feeding on conspecifics or phylogenetically-related organisms than to chemical cues from starved predators or fed distantly related prey (Huryn & Chivers 1999; Slusarczyk 1999; Schoeppner & Relyea 2005; Brodin *et al.* 2006; Beketov & Liess 2007). This specificity of responses towards type (species?) and diet of predators allows prey organisms to adjust the level and direction of induced defences based on the information provided by kairomones from predators and alarm cues from conspecifics and heterospecifics. This shows that kairomones alone (i.e. from starved predators), alarm cues alone (macerated prey) or in combination (predators feeding on prey) can produce a range of unique antipredator responses. (Stirling 1995; Pijanowska & Kowalczewski 1997a; Wudkevich *et al.* 1997; Wisenden *et al.* 1999; Wisenden & Millard 2001; Iyengar & Harvell 2002; Schoeppner & Relyea 2005; Laforsch *et al.* 2006; Turner *et al.* 2006).

There are also genetic and population differences in the plasticity and in the magnitude of induced defences. The specific response of a population to predator's chemical cues is influenced by the evolutionary history with respect to the presence of a particular predator in a particular habitat. Therefore, populations which co-occur with predators show local adaptation to habitat-specific predation regimes and stronger responses towards predators than naïve prey (Boersma *et al.* 1999; Gyssels & Stoks 2006). Different genotypes can also respond differently to the presence of predators, and this difference relies not on the presence/absence of a response but on different combinations and magnitudes of the different traits showing that different responses can be uncoupled and evolve independently (Boersma *et al.* 1998).

The availability and dynamics of food can also interfere with the responses of prey to chemical cues from predators (Slusarczyk 2001; Beketov & Liess 2007; Gliwicz & Maszczyk 2007), and the size selectivity of predators determine the conflicting or additive pattern of responses to both factors (Weber 2001). Food availability can additionally influence antipredator responses due to higher foraging activity and consequently higher predation risk in organisms experiencing food limitation (Anholt *et al.* 1996; Anholt & Werner 1998; Benard 2004; Holker & Stief 2005; Gliwicz & Maszczyk 2007).

The induced responses that prey organisms show to predators or to predation risk are also influenced by the temporal and spatial pattern of predation risk (Van Buskirk & Relyea 1998; Sih & McCarthy 2002; Slos & Stoks 2006), density of conspecifics (Anholt & Werner 1998; Peacor 2003; Turner 2004; Bolnick & Preisser 2005) and abiotic factors such as temperature (Weetman & Atkinson 2002; Lass & Spaak 2003b), light intensity (Brewer *et al.* 1999), refuge or substrate (Frandsen & Dolmer 2002; Baumgartner *et al.* 2003).

The sheer number of factors found to influence perception of predation risk clearly demonstrate the difficulty of studying their interacting roles. However, it is important to consider also that as each antipredator response can be associated with costs, chemical cues from predators can influence a variety of traits differently (in number, combination and magnitude) that although influenced by different factors represent a compromise between costs and effectiveness of the antipredator response (Boersma *et al.* 1998; De Meester & Weider 1999; Sakwinska & Dawidowicz 2005). Additionally, different experimental protocols have been applied in laboratory experiments (e.g. assessing different prey responses to predation risk posed by starved predators versus predators fed conspecifics), without considering different environmental contexts especially changes in resource level and temporal and spatially variation of multiple predators (Sih *et al.* 1998; Van Buskirk & Relyea 1998).

Nevertheless, assessing the inducible responses to predator infochemicals in the laboratory is a good starting point to measure the non-lethal effects of predation and a useful tool to understand how trait mediated interactions can influence natural communities.

Studying the combined effects of different stressors: predation risk and exposure to pesticides.

With the increasing usage of pesticides many surface water bodies are now contaminated. Pesticides found in most ecosystems are now major selective forces causing the decline of non-target species and the impairment of ecosystem functions (Tilman *et al.* 2001; Rohr *et al.* 2006). In order to conserve structure and function of these natural

communities it is necessary to assess the direct and indirect effects of pesticide contamination (Fleeger *et al.* 2003; Rohr *et al.* 2006).

The assessment of pesticide effects has been focused on lethal concentrations rather than sub-lethal effects of lower concentrations. This is surprising since although pesticide concentrations found in natural habitats can be high enough to kill certain organisms during, for example, chemical spills, generally only low concentrations are found, i.e. below the threshold for lethal or major sublethal effects. Such low concentrations of pesticides can affect a wide range of individual traits including changes in behaviour (including reduced foraging and changes in swimming ability), physiology, reproduction and morphology and also on neurotransmitters, hormones and immune response (Walker *et al.* 2001). Moreover, stress ecology studies tend to focus on the effects of single factors, and often ignore the reality of stressor interactions (Relyea & Hoverman 2006). Despite the variety of ecotoxicological tests that have been designed to assess the effects of pesticides on organisms, populations and communities, most of these assessment tools do not take into account that aquatic organisms in the natural environment are often exposed to multiple contaminants and natural stressors with possible antagonistic, additive, and at other times synergistic effects. Natural stressors such as food shortage, oxygen deficiency, high temperature, competition and predation may interact with pesticides, and because standard toxicity tests are performed under favourable conditions they can fail to accurately predict the effects of pesticide contamination in natural systems (Hanazato 1998, 1999, 2001; Rohr *et al.* 2004; Relyea 2005a; Relyea *et al.* 2005; Rohr & Crumrine 2005; Rohr *et al.* 2006; Pereira & Goncalves 2007).

We need a better understanding of how multiple stressors interact with each other, to facilitate the assessment of possible additive or more-than-additive effects, and thus better inform risk assessment practices (Van Straalen 2003; Eggen *et al.* 2004; Relyea & Hoverman 2006). The effects of pesticides are typically assessed with classical laboratory ecotoxicological studies which are designed to generate information to be used to determine water quality criteria and safe concentrations of pollutants (Walker *et al.* 2001). Analysing the effects of pesticides in the absence of other stressors may underestimate their effects on natural communities since it fails to consider possible interactions with those other stressors. The goal of increasing the ecological relevance in toxicity studies is being achieved with respect to several abiotic factors such as temperature, pH, water

chemistry (Heugens *et al.* 2001), but ecotoxicologists tend to exclude from their investigations biotic factors such as parasitism, competition or predation. This will limit the extrapolation of laboratory results to effects on populations in the field (Hanazato 2001; Relyea 2005a; Rohr *et al.* 2006).

Predation and pesticide exposure can through lethal and sublethal effects alter natural communities through density and trait mediated effects (Fleeger *et al.* 2003; Relyea 2005a; Rohr *et al.* 2006). The effects of pesticides on aquatic organisms can be further influenced by the possible interaction between pesticide exposure and predator-prey interactions (Hanazato 1999, 2001; Fleeger *et al.* 2003; Relyea & Hoverman 2006; Lurling & Scheffer 2007). The strength of predator prey interactions can be modified by a change in density or traits (behaviour, physiology, reproduction, etc) in one or both species caused by lethal or sublethal concentrations of pesticides. Consequently, effects of pesticide exposure will also result in changes in the magnitude and possibly direction of density and trait mediated indirect effects of predation (Rohr *et al.* 2006). If the relative magnitude of mortality due to predation and effects on individual traits due to the perceived risk of predation can be difficult to assess and predict especially because they can occur simultaneously, sub-lethal effects of pesticides usually are observed at much lower concentrations than those causing acute effects. Thus, the relative magnitude of lethal versus sub-lethal effects of pesticides on individual traits depends on the dose–response curves of a given pesticide for a given species which can be easily generated by laboratory toxicity assays. On the contrary, assessing the effects of perceived predation risk will give an indication of only part of the total net effects of predation. Nevertheless, studies on predation risk assessment and prey inducible responses to different concentration of predator chemical cues show that it is feasible to assess the sublethal effects of predation in controlled conditions which in turn allows the assessment of the effects of pesticides to organisms under different levels of predation risk.

Combined effects of pollutants and predation risk may be important if the uptake or effect of the pollutant is altered by the presence of predators, or if pollutants compromise normal anti-predator responses. Pollutants can mimic the effects of predator chemical cues or inhibit the induction of defences and have therefore the potential to disturb predator-prey interactions (Dodson *et al.* 1995; Barry 1998; Hanazato 1999; Hunter & Pyle 2004). At the same time, induced responses of prey organisms in terms of behaviour, physiology

and life history in response to the presence of predators might reduce their tolerance to environmental stress and thus affect sensitivity to toxicants (Hanazato 2001; Kieu *et al.* 2001; Rose *et al.* 2001; Relyea 2003b; Rohr *et al.* 2004).

Investigations dealing with the effects of contaminants on predator-prey interactions have been shown that low concentrations of anthropogenic chemicals can disrupt the chemical communication between organisms, increase the costs of predator induced responses or even change the magnitude and direction of those responses increasing the risk of predation (Hanazato 1999; Relyea & Hoverman 2006). Several studies have been reporting variable effects of pesticide and predation stress at population or community level that are mediated by density effects of predation and by direct intraspecific and interspecific interactions (Hanazato 1998; Chang *et al.* 2005; Relyea 2005a; Rohr & Crumrine 2005).

Effects on prey behaviour caused by exposure to pesticides can also increase the risk of predation. For example, *Baetis* mayflies exposed to organophosphate and pyrethroids insecticides spend more time on top of rocks and thus became more visible to fish predators, experiencing higher predation rates (Schulz & Dabrowski 2001). Similarly, exposure to high concentrations of toxicants can induce maladaptive behaviours in zooplankton species such as spinning behaviour (Dodson *et al.* 1995) or differences in the swimming speed (Preston *et al.* 1999) which can make them more easily detected by fish. Effects of pesticides on prey behaviour can thus influence predator prey interactions by altering encounter rates and thus affect survivorship of prey organisms.

The mechanisms by which pesticide exposure can interact with predation risk remain unclear and more information on how responses to both types of stressors are modified by the exposure to the other is still scarce (Lurling & Scheffer 2007). Research on the combined exposure of aquatic prey organisms to pesticides and chemical cues from predators have demonstrated that the detrimental effects of pesticides can be influenced or modified solely by the perception of risk from predation due to combined physiological or behavioural stress with effects on survival (Relyea 2003b, 2004b; Maul *et al.* 2006), morphology (Barry 1998, 1999), growth and life-history (Rose *et al.* 2001; Relyea 2003b; Rohr *et al.* 2004; Campero *et al.* 2007), and that the induced antipredator responses can be affected by anthropogenic chemical substances (Barry 1999; Preston *et al.* 1999; Kieu *et al.* 2001; Sakamoto *et al.* 2006). These investigations show that both pesticide exposure

and predation risk have some similarities especially in terms of behavioural and physiological effects on organisms with consequences for their life history.

Ecotoxicological studies have shown that low levels of pesticides and other contaminants can impair food acquisition and can increase metabolic rates reducing the energy available for growth, alter allocation of energy to maintenance, growth or reproduction and can modify the action of specific biomolecules (e.g pesticides which inhibit specific enzymatic activities) (Walker *et al.* 2001). In turn, such effects can propagate through higher levels of organisation with consequences to populations, communities and ecosystems (Allen *et al.* 1995; Maltby 1999; Forrow & Maltby 2000; Maltby *et al.* 2002; Slijkerman *et al.* 2004). Moreover, laboratory and *in-situ* toxicity tests focused on organismal level responses (energy budget, feeding and respiration, growth) have been developed and intensively used with several organisms as sensitive assessment tools to monitor the sublethal effects of different types of contaminants in water and sediment (Widdows 1985; Maltby *et al.* 1990; Lam 1996b; Chappie & Burton 1997; Hatch & Burton 1999; Maltby 1999; McWilliam & Baird 2002a, 2002b; Verslycke *et al.* 2004).

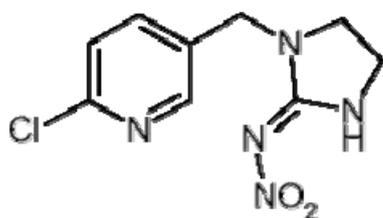
Similarly, prey organisms under predation risk also experience reduced energy intake (Kohler & McPeck 1989; Lima & Dill 1990; Peckarsky & McIntosh 1998; Rose *et al.* 2003; Holker & Stief 2005; Beckerman *et al.* 2007; Campero *et al.* 2007), increased metabolic demands (Bengtsson 1982; Beckerman *et al.* 2007; Campero *et al.* 2007) and alterations in the allocation of acquired resources to growth, reproduction or development (Stoks *et al.* 2005; McPeck *et al.* 2001, McPeck 2004; Stibor 2002; Beckerman *et al.* 2007). Recent studies have also demonstrated that molecular antipredator responses can be induced in organisms under predation risk including the production of heat shock proteins (Pijanowska & Kloc 2004; Pauwels *et al.* 2005), reduce energy storage levels (Stibor & Navarra 2000; Stoks *et al.* 2005b) or alteration of some enzymes (Campero *et al.* 2007).

Pesticides and predation risk can therefore be considered 'similarly-acting' in an ecological sense. Studying natural and anthropogenic stressors and their combined effects under the same framework with similar experimental approaches might be of great value for ecological risk assessments since it can provide compatible data for modelling, allowing improved interpretation of ecological effects within a broader ecosystem context (Barata & Baird 2000; Relyea & Hoverman 2006; Rohr *et al.* 2006; Barata *et al.* 2007).

Imidacloprid as a model pesticide.

Imidacloprid is a chloronicotinoid insecticide produced by Bayer Crop Science, belonging to the newest class of insecticides, the neonicotinoids, which are chemically related to the natural nicotinic acetylcholine receptor (AChR) agonists nicotine and epibatidine (Matsuda *et al.* 2001). Patented in 1985, Imidacloprid is a neurotoxic insecticide that acts via ingestion or direct contact, disrupting the nervous systems of insect pests by binding to postsynaptic nicotinic AChRs preventing acetylcholine from binding to the same receptor. Imidacloprid is not easily degraded by acetylcholinesterase and thus prolonged activation of the nicotinic AChRs by imidacloprid causes lack of muscular coordination, tremors, desensitization and blocking of the receptors, decreased activity, leading to modified feeding behaviour, and ultimately, death of insects (Moffat 1993; Matsuda *et al.* 2001; Tomizawa & Casida 2003).

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine] is the active ingredient of several commercial products such as Admire®, Confidor®, Gaucho®, Advantage®, Merit®, Intercept®, Maxforce IC®, Genesis®, Impower®, Provado®, Premier®, Premise® and Marathon®, which are used worldwide as systemic insecticides to control sucking and mining pests and with a wide range of uses such as seed dressing, soil and foliar treatments in different crops including rice, cotton, cereals, maize, sugar beet, potatoes, vegetables, citrus fruit, apples and pears, vineyards and stone fruits. Imidacloprid is also used as effective flea control agent for domestic pets and is also increasingly being used in urban areas for the control of turf pests in golf courses, parks and household lawns (Cox 2001; Fossen 2006; CCME 2007).



CAS: 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine
 FORMULA: C₉H₁₀CN₅O₂

Fig 1- Chemical structure of imidacloprid

Imidacloprid, as all neonicotinoids, shows selective toxicity for insects over vertebrates, which is in part attributable to a higher affinity for insect nAChRs compared

with their vertebrate counterparts and also because the nicotinic neuronal pathway is more abundant in insects (Tomizawa & Casida 2005). Neonicotinoids interact with nAChRs in a very different way than nicotine because neonicotinoids are not protonated but instead have an electronegative tip consisting of a nitro or cyano pharmacophore that is responsible for the potency and selectivity for insect receptors, i.e. the neonicotinoids are not ionized at physiological pH and selective for the insect nAChR, and the nicotinoids are ionized at physiological pH and selective for the mammalian nAChR (Tomizawa & Casida 2003, 2005).

The use of neonicotinoids is currently increasing faster than other insecticides such as carbamates and organophosphates (which inhibit acetylcholinesterases, AChEs) and pyrethroids (which prolong Na⁺ channel opening), mainly due to their excellent plant systemic activity and the decreased effectiveness because of resistance or increased restrictions due to toxicological considerations of the other neuroactive insecticides (Matsuda *et al.* 2001; Tomizawa & Casida 2005). Neonicotinoids at present account for 11%-15% of the total insecticide market (Tomizawa & Casida 2005).

The persistence of Imidacloprid in soils depends on several factors such as organic matter content, temperature and whether the field is cropped or not. Dissipation times (DT₅₀) vary from 80 days to 2 years (Cox 2001). Adsorption is the main fate process for imidacloprid in soils and the transformation products of imidacloprid in aerobic soil typically are imidacloprid urea, 6-chloronicotinic acid and 6-hydroxynicotinic acid, which will lead to the formation of carbon dioxide (CCME 2007).

The persistence in soil, high solubility and mobility (Table I) mean that imidacloprid is a potential contaminant of surface and ground waters via drift, dissolved runoff or leaching (Cox 2001; Gupta *et al.* 2002). Imidacloprid is increasingly being found in aquatic systems especially during rainfall events and in shallow wells, and there is a general concern on effects of low concentrations of imidacloprid in surface and ground waters to aquatic life (CCME 2007). Exposure to light, pH, temperature and microbial community influence the persistence of imidacloprid in the aqueous environment. The formulation of the imidacloprid product further influences persistence in the aquatic environment with higher half-life values for powder formulations than for liquid with increased persistence in high application rates (Sarkar *et al.* 1999).

Photolysis of Imidacloprid is generally fast (Table I) and hydrolysis half-life of imidacloprid varies from 33 to 44 days being dependent on formulation, pH and temperature (Sarkar *et al.* 1999). However hydrolysis half live greater than 1 year have been also reported (CCME 2007).

In the aqueous environment, imidacloprid is also metabolized by microorganisms with non-sterile conditions decreasing imidacloprid half lives with and without light (Table I). Phototransformation and biotransformation in the aqueous environment are thus the significant transformation routes of imidacloprid. The major transformation products resulting from incubation under the environmentally relevant non-sterile conditions and light exposure are desnitroimidacloprid (guanidine derivative), imidacloprid urea, 6-chloronicotinic acid and the olefin derivative (Bacey 2000; CCME 2007).

Imidacloprid with a low octanol-water partition coefficient has a low potential for accumulation in aquatic species. In vertebrates, imidacloprid undergoes rapid passage through the body. It is rapidly and almost completely absorbed by the gastrointestinal tract and quickly eliminated via urine and faeces. It is metabolized through hydroxylation at the imidazolidine ring, hydrolysis to 6-chloronicotinic acid and loss of the nitro group with formation of the guanidine. Research on worker bees and houseflies have demonstrated that imidacloprid is also readily excreted by insects (Suchail *et al.* 2003; Nishiwaki *et al.* 2004).

In insects, imidacloprid is metabolised by hydroxylation at the imidazolidine ring, and the main metabolites, the olefin derivative and hydroxyl-imidacloprid, can also show insecticidal activity and thus extend the toxicity of imidacloprid (Suchail *et al.* 2003; Nishiwaki *et al.* 2004). Although additional mechanisms might exist, P450-monooxygenases are key factors in imidacloprid detoxification and resistance development in insects (Wen & Scott 1997; Nauen *et al.* 2002; Liu *et al.* 2003).

In plants, imidacloprid is metabolized via loss of the nitro group, hydroxylation at the imidazolidine ring, hydrolysis to 6-chloronicotinic acid, and formation of conjugates. The main metabolites detected in plants are imidacloprid-guanidine olefin and imidacloprid-guanidine (CCME 2007).

The transformation products of imidacloprid appear to be considerably less toxic to invertebrates than the parent compound (CCME 2007) but the olefin metabolite, which is found in imidacloprid-treated plants, is more toxic to insects than imidacloprid itself.

Table I–Physical and chemical properties and aquatic environmental fate of imidacloprid
(Data with no references were taken from (EXTOXNET 1998))

Molecular weight	255.7	
Water solubility	514 mg/L (20 °C)	
Vapor pressure	1.5×10^{-9} mm HG (20°C)	
Henry's constant	6.5×10^{-11} atm m ³ /mole (20°C)	
Average application rate	28 –312 (g a.i. ha ⁻¹)	(CCME 2007)
K _{oc} (soil organic carbon water partitioning coefficient)	132-310	
Log K _{ow} (octanol-water partition coefficient)	0.57 (22 °C)	
Soil anaerobic half life	27.1 days	
Soil aerobic half life	997 days	
Soil photolysis half-life	38.9 days	
Aquatic photolysis half life	< 3 h (simulated sunlight, 30°C) 1.2 h (d H ₂ O, λ = 290 nm, 24°C) 2.1 h (Confidor; d H ₂ O, λ=280 nm)	(Kagabu & Medej 1995) (Moza et al. 1998) (Wamhoff & Schneider 1999)
Hydrolysis half life	> 30 days	(Sarkar <i>et al.</i> 1999)
Freshwater half life (sunlight)	22 days 4 days	(SERA 2005) (Sanchez-Bayo & Goka 2005)
(sterile/non-sterile) (dark)	28.4 / 4.19 days	(CCME 2007)
(sterile/non-sterile)	10-24 weeks 499 / 331 days	(Kagabu & Medej 1995) (CCME 2007)
Water and sediment half life	66 days 14.8 days (mesocosm)	(Sanchez-Bayo & Goka 2005) (CCME 2007)
Maximum detected aquatic concentrations	0.6 µg/L (Wilapa Bay, USA) 6,33 µg/kg in sediment (Wilapa Bay, USA) 14 µg/L (Lake Wales Ridge, USA) 6.7µg/L (groundwater) 6.4µg/L (groundwater) 0.5 - 11.9µg/L (agric runoff, Canada)	(Felsot & Ruppert 2002) (USGS 2003) (Fossen 2006) (CCME 2007) (CCME 2007)

Desnitroimidacloprid which is a major metabolite of imidacloprid from soil and water degradation, shows little neurotoxicity to insects but is more toxic than imidacloprid to mammals (Cox 2001).

Imidacloprid is acutely toxic to adult fish at relatively high concentrations but it is however extremely toxic at low concentrations to several species of aquatic invertebrates (Table II). Algae are also less sensitive (ca. three orders of magnitude) to imidacloprid than most insect species (CCME 2007). Therefore, even low concentrations of imidacloprid reaching surface waters can exert lethal and sublethal effects on aquatic non target species, and especially insects with potential impairment of macroinvertebrate communities and critical ecosystem functions (Kreutzweiser *et al.* 2007). In Canada, freshwater and marine water quality guidelines for the protection of aquatic life are available for imidacloprid. The freshwater quality guideline was based on a 28-day test with the midge *Chironomus riparius*, and resulted in an interim freshwater quality guideline of 0.23 µg a.i./L. The interim marine water quality guideline was based on a 48-hour acute test with larvae of the saltmarsh mosquito *Aedes taeniorhynchus*. A final value of 0.65 µg a.i./L was reached. These guidelines were based on toxicity data of technical grade imidacloprid and on the most sensitive species and parameters, and thus “are conservative and intend to protect all forms of aquatic life and all aspects of aquatic life cycles during an indefinite period of exposure to the water column” (CCME 2007)

The exposure to the active ingredient compared to commercial products can also yield different levels of toxicity to aquatic organisms. Formulants and inert ingredients present in a formulated product may themselves exert some toxicity, or may increase the toxicity of the active ingredient by affecting its uptake, metabolism or excretion (Cox 2001; Rohr *et al.* 2006). However, the few investigations dealing with toxicity of imidacloprid and of commercial products have produced mixed results as this could vary depending on the formulation of several products, endpoints and species tested (Cox 2001; CCME 2007; Jemec *et al.* 2007; Stoughton *et al.* 2008).

Table II –Toxicity of Imidacloprid to several non-target freshwater organisms.

Organism	Endpoint and toxicity values	references
Fish:	LC ₅₀ (96 hrs) = 211 mg/L	(Fossen 2006)
<i>Oncorhynchus mykiss</i>	LOEC behaviour(96hrs) = 64 mg/L LOEC growth(60 days) = 2.3 mg/L	(CCME 2007)
<i>Lepomis macrochirus</i>	LC ₅₀ (96 hrs) = 105 mg/L	(CCME 2007)
<i>Leuciscus idus</i>	LC ₅₀ (96 hrs) = 237 mg/L	(CCME 2007)
Aquatic Invertebrates:		
<i>Daphnia magna</i>	LC ₅₀ (48 hrs) = 85 mg/L LC ₅₀ (48 hrs) = 10.4 mg/L (27°C) LC ₅₀ (48 hrs) = 17.36 mg/L (20°C)	(EXTOXNET 1998) (Song <i>et al.</i> 1997)
	LOEC _{rep} (21 days) = 2.5 mg / L LC ₅₀ (48 hrs) = 64.8 mg / L EC _{50 imm} (96h) = 6.029 mg / L	(Jemec <i>et al.</i> 2007) (Sanchez-Bayo & Goka 2006b)
<i>Hyallorella azteca</i>	LC ₅₀ (96 h) (juveniles) = 0.0654 mg/L NOAEC _{mortality} (96 h) = 0.00035 mg /L	(CCME 2007)
<i>Lumbriculus variegatus</i>	EC _{50 imm} (96h) = 0.0062 mg/L LC ₅₀ (96 h) = 0.044 mg/L	(Alexander <i>et al.</i> 2007) (Song <i>et al.</i> 1997)
<i>Aedes aegypti</i>	LC ₅₀ (96 h) = 0.0105 mg /L;	(SERA 2005)
<i>Chironomus tentans</i>	NOAEC (96 h) = 0.00317 mg /L NOAEC _{growth} (10 day) = 0.00067 mg/L	(Stoughton <i>et al.</i> 2008)
<i>Chironomus riparius</i>	EC ₅₀ (reduced emergence) = 0.00311 mg/L	(CCME 2007)
<i>Epeorus longimanus</i>	LC50 (24h) = 0.0021 mg/L LC50 (96 h) = 0.00065 mg /L	(Alexander <i>et al.</i> 2007)

Predator-prey models used.

Research on chemically induced responses of aquatic invertebrates to predators has been performed in a variety of species with a strong emphasis on planktivorous fish-*Daphnia* systems (Lass & Spaak 2003a). In this thesis it was decided to study the responses of different invertebrate species, the cladoceran *Daphnia magna*, Strauss, the

midge *Chironomus riparius*, Meigen and the caddisfly *Sericostoma vittatum* Rambur, to fish predation risk using chemical cues from Brown Trout (*Salmo trutta*, Linnaeus).

Members of the genus *Daphnia* have a set of characteristics that makes them suitable species for ecotoxicity studies as well as for studies on predator induced responses. Daphnids are small planktonic crustaceans that occupy a key position in lake and pond food webs since they are often the most significant herbivore determining water quality through their selective consumption of algae. At the same time they provide a major dietary component for several fish species because of their large size, high rate of reproduction, and lack of an effective escape response. They have short life cycles; can be easily cultured in the laboratory and under favourable conditions they reproduce parthenogenetically. Several different toxicity tests (laboratory and *in situ*) are widely used for a variety of behavioural, physiological and life-history endpoints (Baird *et al.* 1991b; OECD 1998, 2000; McWilliam & Baird 2002a, 2002b). *Daphnia*, as all planktonic organisms, due to their weak swimming abilities, are constrained in escaping behaviour and refuge being under strong predation pressure (Lass & Spaak 2003a). *Daphnia magna* has shown responses to both vertebrate and invertebrate chemical cues and it has been intensively used in toxicity studies.

In the presence of chemical cues from fish, daphnids usually show responses that mimic that predation selection. Thus, under the perceived risk of fish predation, *Daphnia* usually respond with life history shifts like maturing earlier at a smaller size as well as producing more and smaller neonates (Reede 1995, 1997; De Meester & Weider 1999; Spaak *et al.* 2000; Declerck & Weber 2003; Hulsmann *et al.* 2004; Gliwicz & Maszczyk 2007). This will increase fitness because it increases the probability of reproduction before being eaten by visually hunting, positive-size selective predators like fish (Lass & Spaak 2003a; Hulsmann *et al.* 2004).

Morphological responses of daphnids such as modifications of body and head shape (Stabell *et al.* 2003) and the induction of longer tail spines (Dzialowski *et al.* 2003) have been also observed in response to fish infochemicals, although the adaptive value of these responses is much clearer when induced by the presence of invertebrate predators that feed on small prey (Tollrian 1995). Nevertheless, morphological defences are effective when the handling time for predators is increased and thus also the survivorship of the prey population (Kolar & Wahl 1998).

Behaviour and physiological responses to fish infochemicals have generally short induction times and thus represent the first defence mechanisms for prey (De Meester & Cousyn 1997). Avoidance behaviour (Pijanowska & Kowalczewski 1997b; Roozen & Lurling 2001), changes in phototactic behaviour (Ringelberg & vanGool 1995; De Meester & Cousyn 1997), changes in swimming behaviour (Weber & Van Noordwijk 2002), aggregation and alertness (Pijanowska & Kowalczewski 1997b; Brewer *et al.* 1999) are common responses of daphnids in response to fish chemical cues. While behavioural responses reduce the probability of encounters with fish predators (Lass & Spaak 2003a), they can also reduce energy intake (Rose *et al.* 2003; Beckerman *et al.* 2007). The presence of predators has also been shown to place higher metabolic demands on prey organisms, manifested in elevated rates of oxygen consumption, reflecting an increased cost of vigilance under predation risk (Bengtsson 1982; Pijanowska 1997; Woodley & Peterson 2003; Beckerman *et al.* 2007).

Chironomidae (Insecta, Diptera) are extremely relevant in freshwater systems since they often dominate the benthic communities of lotic and lentic environments in number and biomass (Merrit & Cummins 1996). Sediment-dwelling larvae of the genus *Chironomus* occur in the benthos of small rivers, lakes, and ponds facilitating the bioturbation and bioirrigation of the sediment (Matisoff & Wang 1998; Stief & de Beer 2002) with significant effects on habitat structure and sediment microbiology (Stief & Holker 2006) and on nutrient cycling (Svensson & Leonardson 1996). *Chironomus* short life cycle includes aquatic stages (eggs, four larval stages and pupae stage) and an aerial stage (adult stage), which, together with high reproduction outputs allows for the maintenance and culture in the laboratory. Because of the direct contact with the sediment that larval stages experience, chironomids such as *C. riparius* and *C. tentans* have been used as model organisms for laboratory and *in situ* sediment toxicity studies with larval behaviour, growth, emergence, and survival often used as endpoints with different protocols (Chappie & Burton 1997; OECD 2001; Soares *et al.* 2005; Faria *et al.* 2006; Domingues *et al.* 2007; Faria *et al.* 2007).

Chironomids are also a major component of fish diets, having been also used to investigate predator impacts in invertebrate communities. With weak locomotor and swimming capabilities, the direct contact with the sediments and drifting behaviour are strategies to avoid predators. Chironomids appear to respond to chemical cues from

predators and reductions in activity levels (Holker & Stief 2005), growth and development (Ball & Baker 1996; Noonburg & Nisbet 2005) have been shown for chironomids in response to nonlethal cues from fish.

The caddisfly *Sericostoma vittatum* Rambur, (Trichoptera: Sericostomatidae) is an endemic species of the Iberian Peninsula. The aquatic larvae of *S. vittatum* occur all year round, they feed on conditioned leaves, and are among the most abundant and conspicuous consumers in many streams of this region (Feio & Graça 1997). For this reason, they can play a key role in the fragmentation of allochthonous organic matter of streams (Feio & Graça 2000; Gonzalez & Graça 2003). *S. vittatum* have relatively long life cycles (Gonzalez & Graça 2003) and, like chironomids, they have several aquatic stages (eggs, larval stages and pupae) and a terrestrial adult stage.

Caddisflies have been previously used in ecotoxicological studies (Schulz & Liess 1999, 2000, 2001; Berra *et al.* 2006), and are also important food items for several fish species. Along with their burrowing capabilities and drifting behaviour, the mineral case produced by the larvae is an important antipredator trait, since it can reduce detection, change palatability, and increase the handling time for predators (Wissinger *et al.* 2006). At the same time mineral cases are a constraint in terms of locomotor ability (Boyero *et al.* 2006). Caddisflies can also use chemical cues from predators to assess predatory threat and behavioural responses, including reductions of activity and feeding, have been demonstrated for caddisflies responding to predation risk (Wissinger *et al.* 1999; Kuhara *et al.* 2001; Boyero *et al.* 2006).

Fish are among the most important predators of zooplankton and insect larvae, and several investigations studied the various effects of fish predators in insect and crustacean prey. Although the exact chemical nature of fish kairomones is to date unknown (see above), planktivorous and non-planktivorous fish infochemicals have been shown to have similar chemical characteristics and induce similar responses (Loose *et al.* 1993; vonElert & Loose 1996). This makes the use of different fish species possible. Fish chemical cues are quickly released into the medium; they are water-soluble, non-volatile and stable over a wide temperature range (Loose *et al.* 1993). Because fish kairomones are thus suitable to be used under controlled conditions in laboratory experiments, it was decided to use brown trout, *Salmo trutta* as a source of nonlethal cues of fish predation. Brown trout are native to Europe and prefer cold, well-oxygenated upland waters. They are mainly diurnal, feeding

on aquatic and terrestrial insects, crustaceans and small fish. Daphnids, chironomids and caddisflies are some of the most important items in the diet of brown trout (Penczak & Formigo 2000). Moreover, several studies have previously demonstrated prey responses to trout chemical cues in several different prey species (Huryn & Chivers 1999; Dahl & Peckarsky 2002; Peckarsky *et al.* 2002; Lass & Spaak 2003a).

Research objectives and thesis outline.

Since inducible antipredator responses have costs, and can lead to increased sensitivity to environmental stress, and because pesticides can also interfere with predator prey interactions namely by disrupting chemical communication or impose additional physiological stress, the research presented in this thesis aims to improve the understanding of effects of pesticides to aquatic invertebrates under different levels of predation risk. To address this, four separate chapters, following the general introduction, are organized focusing on different issues.

Imidacloprid is a relatively new pesticide and because studies using different aquatic organism are still scarce it was important to evaluate the potential impact of this contaminant to aquatic non-target species and evaluate effects on natural communities. In Chapter II, the effects of imidacloprid on macroinvertebrate benthic communities were assessed with structural and functional endpoints using artificial streams. Feeding and respiratory responses of the North American stonefly *Pteronarcys comstockii*, Smith, were simultaneously analysed to assess the responsiveness and relevance of organism-level responses to monitor stress caused by imidacloprid contamination. Although not used to monitor effects of imidacloprid under predation risk, the stonefly *P. comstockii* was chosen for this study because they were abundance in a nearby stream, and have a long life cycle (Merrit & Cummins 1996) which allows the use of similarly sized and aged organisms. *Pteronarcys* can feed on leaf litter playing an important role in the breakdown of leaf detritus in many North American freshwaters and have a relatively high sensitivity to pollutants (Merrit & Cummins 1996; Pesacreta 1997; Cole *et al.* 2001; Ruesink & Srivastava 2001).

Trying to demonstrate the different responses to different chemical cues that simulate the risk of predation, effects of exposure to kairomones from trout, to alarm cues from

macerated conspecifics and a to combination of both cues were assessed with behavioural, physiological and life-historical *D. magna* responses (chapter III). The results allow a comparison with investigations done with Daphnids and fish chemical cues.

The effects of Imidacloprid were then studied with *D. magna* (Chapter IV) and with two insect species, *S. vittatum* and *C. riparius* (Chapter V), under different levels of predation risk. Fish predation risk was simulated by exposure to a mixture of water conditioned by fish and alarm substances from macerated conspecifics. Because behaviour (feeding) and physiology (energy expenditure and allocation into growth or development) appear to mediate many responses of organisms to the perceived risk of predation and also to sub-lethal concentrations of pesticides, feeding (or growth) and metabolic rates (respiration) as endpoints were chosen together with some life history traits in the organisms with short live cycles (*Daphnia* and *Chironomus*). The objective was to test experimental procedures that are used for toxicity testing to assess the potential interaction between predation risk and imidacloprid exposure and try to understand if the different inducible defences observed for prey species with different life-history, behavioural and morphological characteristics can affect their sensitivity towards imidacloprid or if their antipredator responses are altered with exposure to the pesticide.

Finally, in Chapter VI, the implications of this work for understanding the effects of pesticides on inducible defences, predator-prey interactions and on natural communities are discussed while some possible directions for future research on combined effects of pesticides and natural stressors are also referred.

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Chapter II

Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms

Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms.¹

Abstract

Structural and functional responses of a benthic macroinvertebrate assemblage to pulses of the insecticide imidacloprid were assessed in outdoor stream mesocosms. Imidacloprid low (2 ug/L) and high (20 ug/L) concentrations, were chosen for this study with untreated streams as controls. Endpoints included benthic invertebrate abundance and composition, leaf litter degradation and feeding rate of caged stoneflies (*P. comstocki*). Laboratory experiments were also conducted to assess effects of imidacloprid on respiration of *P. comstocki*. Results indicated that pulses of imidacloprid caused important changes in macroinvertebrate abundance and community composition. In streams treated with imidacloprid, feeding rates of *P. comstocki* feeding on *Alnus* spp. leaf disks were significantly reduced compared to controls. These results correlated well with the effects of imidacloprid on leaf litter decomposition in artificial streams. Physiological stress and reduced activity of insects exposed to sublethal concentrations of imidacloprid was also demonstrated by the reduction in oxygen consumption of stoneflies with short exposure to sublethal concentrations of imidacloprid. Our findings suggest that leaf litter degradation and single species responses such as feeding rate of selected shredder species can be sensitive endpoints in terms of sublethal toxicity which can be used as early warning indicators and as *in situ* biomonitoring tools for pesticide contamination. The data generated illustrates the value of mesocosms experiments in environmental assessment and how the consideration of functional and structural endpoints of natural communities together with *in situ* single species bioassays can improve the evaluation and prediction of pesticide effects on stream ecosystems.

Keywords—Pesticides, mesocosms, community responses, sub-lethal effects, leaf decomposition , *in situ* feeding assay, *Pteronarcys comstocki*

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Introduction

The use of neonicotinoids such as imidacloprid is currently increasing faster than other insecticides such as carbamates, organophosphates and pyrethroids, mainly due to their excellent plant systemic activity coupled with the reduced effectiveness of other neuroactive insecticides (Matsuda *et al.* 2001; Tomizawa & Casida 2005).

Imidacloprid acts on the target species through ingestion or direct contact, blocking the nicotinergetic neuronal pathway. Nicotinergetic receptors are more abundant in insects than in warm-blooded animals and thus neonicotinoids exhibit selective toxicity towards insects (Tomizawa & Casida 2005). Imidacloprid is used worldwide as an agricultural insecticide to control sucking and mining pests, as a flea control agent for domestic pets, and is also used in urban areas for the control of turf pests in golf courses, parks and household lawns (Cox 2001; Fossen 2006; CCME 2007). Its persistence in soil, high solubility and mobility make it a potential contaminant of surface and ground waters through drift, dissolved runoff or leaching (Cox 2001; Gupta *et al.* 2002). Imidacloprid is increasingly being found in aquatic systems especially during rainfall events with levels in runoff from agricultural areas reaching concentrations of up to ca. 12 µg/L (CCME 2007).

Single species toxicity tests in the laboratory have already shown that imidacloprid is toxic to fish at relatively high concentrations (LC₅₀ higher than 100 mg/L) and can be extremely toxic at low concentrations (LC₅₀ of 0.65 - 65 µg/L) to several species of aquatic invertebrates, especially insects (CCME 2007). However, such laboratory single species assays are insufficient to fully assess ecological risks, for several reasons (Crane 1997): (i) it is difficult to extrapolate from laboratory results to conditions occurring in nature, which encompass a wider range of habitat conditions than those found in laboratory beakers; (ii) the responses of species tested in standard toxicity tests may not fully encompass the responses of the diversity of species found in natural systems; (iii) it is difficult to present the full range of relevant exposure routes in the laboratory especially for modern insecticides, which tend to be characterized by short half lives and are applied in low doses, although often highly toxic to non-target aquatic species (Crane 1997; Boxall *et al.* 2002; Caquet *et al.* 2007). It is thus important to consider higher-tier experiments to assess the effects of these substances on aquatic ecosystems (Boxall *et al.* 2002).

The use of controlled semi-field exposures, such as mesocosms, provides an opportunity to perform ecosystem-level research that can complement laboratory bioassays and therefore better predict effects of ecologically relevant exposures of different types of anthropogenic substances to natural communities (Hickey & Golding 2002; Culp *et al.* 2003; Van den Brink *et al.* 2005; Van den Brink 2006). Mesocosms are smaller and less complex than natural ecosystems, yet they allow the use of relevant biological conditions (ambient abundance of natural biota), while maintaining the statistical power through replication of treatments and precise regulation of pesticide concentrations and other physico-chemical variables (Crane 1997; Baird *et al.* 2001). Since sublethal, population-level responses are of great significance to natural ecosystems, it is important to consider the application of *in situ* bioassays to obtain reliable and sensitive measures of pesticide effects (Maltby 1999; Slijkerman *et al.* 2004). *In situ* bioassays deployed within semi field experiments have the advantage of testing selected species under realistic field conditions compared to laboratory tests and provide a mechanistic explanation of effects by linking organism level physiological or behavioural responses and community functional parameters (Hruska & Dube 2004; Coors *et al.* 2006; Caquet *et al.* 2007; Kreutzweiser *et al.* 2007). The use of mesocosm approach in combination with experimental tests covering functional and structural endpoints at various levels of biological organisation can thus help identify causal mechanisms responsible for direct and indirect effects of pesticide contamination on natural communities and ecosystem functions (Baird *et al.* 2001; Mills & Semlitsch 2004; Slijkerman *et al.* 2004; Relyea *et al.* 2005; Rohr *et al.* 2006).

This paper describes the effects of imidacloprid pulses on structural and functional measures of benthic invertebrate communities, as this best reflects the edge of field scenario for runoff of this substance (CCME 2007). This was achieved using field deployed artificial stream mesocosms inoculated with natural substratum and a portion of an extant riverine benthic community. Endpoints for the assessment included benthic invertebrate abundance and composition and leaf litter degradation. The sensitivity and relevance of organism-level responses to stress caused by pulses of imidacloprid were also assessed with the North American stonefly *Pteronarcys comstocki* Smith, through feeding bioassays deployed in mesocosms systems together with laboratory measures of oxygen consumption. Pteronarcid stoneflies have a long life cycle (1-3 years) (Merrit & Cummins 1996) which allows for the collection of similar size individuals in the field which can be

easily transported and acclimatised to laboratory conditions. Pteronarcids are detritivore shredders, feeding primarily on leaf litter and have a relatively high sensitivity to pollutants (Merrit & Cummins 1996; Pesacreta 1997; Cole *et al.* 2001; Ruesink & Srivastava 2001). Here we link the responses of the stonefly to results derived from a mesocosms experiment performed in August 2005 focused on pesticide mixtures. However, it should be noted that the present paper only considers results from a single substance exposure treatment with imidacloprid, as these are used to compare with the responses of the stonefly bioassay *in situ* and in the laboratory. The full results of the mixture experiment will be published elsewhere (Culp *et al* in prep).

Material and Methods

Mesocosms treatments and set-up

The mesocosm experiment was conducted for 20 days in August 2005 and included a treatment to examine the effects of pulse (24-h) concentrations of imidacloprid (0, 2, and 20 µg/L) every 7 days on the macroinvertebrate community. Mesocosms were deployed at the Agriculture and Agri-Foods Canada facility adjacent to the Saint John River, approximately 10 km southeast of Fredericton, New Brunswick, Canada. The partial flow-through mesocosms were designed to simulate lotic habitats by encompassing a portion of substratum and of the benthic invertebrate community collected in the Nashwaak River, New Brunswick, Canada (46°14294'N, 66°36722'W) in semi-field conditions (Culp *et al.* 2003).

Each mesocosm table represented one treatment and contained eight replicate streams with a planar area of 0.065 m² and a 10-L volume (Fig 1). Groundwater from the extensive Saint John River aquifer was used to provide water to the mesocosms. Imidacloprid solution was mixed in a stock tank by diluting a 1-mL aliquot of 240 g/L Admire® (Bayer CropScience; Calgary, AB, Canada). The insecticide mixture was delivered to a treatment reservoir in each mesocosm table by positive displacement pumps (Viking Pumps; Pulsefeeder 25-H duplex pump, Cedar Falls, IA, U.S.A.). Wastewater from the mesocosms was passed through carbon filters (Culligan Inc.; activated carbon filter cylinder, Moncton, NB, Canada) to remove all contaminants before any water was discharged to the environment. Water was continuously pumped and was completely exchanged every 7

min. Each replicate stream was screened with 400 μm Nitex® mesh (Aquatic Ecosystems Inc.; Apopka, FL, U.S.A.) to prevent nymphs from escaping as well as to capture emerging insects. Water velocity in the stream was 11–12 cm/s which is similar to the typical substratum–water interface in rivers (Culp *et al.* 2003).

Prior to initiating the experiment, benthic substrata composed of fine and coarse substratum obtained from the invertebrate sampling site on the Nashwaak River were introduced into each replicate. Cobblestones (7–10 cm) were also collected from this site with five stones randomly assigned to each replicate stream. Cobble and gravel were gently washed to remove any attached invertebrates while maintaining the periphyton community. This procedure established a lotic substratum consisting of a 2–3 cm layer of gravel–cobble plus surface stones that were covered with periphyton.

Once inoculated with cobble and after deploying the leaf bags (see below), the streams were allowed to equilibrate for 2 days prior to the addition of benthic invertebrates. Benthic invertebrates were collected upstream of the gravel collection site on the Nashwaak River with U-nets (area $\frac{1}{4}$ 0.06 m²). Five U-nets were collected, pooled and subsampled into four equal portions twice obtaining 16 replicate samples. These invertebrate assemblage subsamples were inoculated into replicate streams (one per treatment table). Thus, a similar invertebrate assemblage was inoculated in the same replicate stream on each mesocosm table. Five U-nets were used to slightly increase (c. 10%) the ambient density of aquatic invertebrates in the streams offsetting mortality because of transport from the river to the mesocosm test site. Once inoculated with invertebrates, the streams were permitted to equilibrate for 1 day before beginning the insecticide exposures. At the end of the 20 days the contents of replicate streams were sieved and water samples and invertebrates were collected from each replicate stream.

Chemical analysis determined the actual concentrations of imidacloprid in each treatment. Chemical analyses were conducted at an Environment Canada laboratory in Saskatoon, SK, Canada on a Micromass Quattro Ultima liquid chromatography mass spectrometer (LC-MS) equipped with a stainless steel column (100 · 2.2 mm; MS Xterra C-8; 100 · 2.2 mm; Waters, Milford, MA, U.S.A.). Samples for imidacloprid analyses were taken from each mesocosm table at the onset, during and at the end of the imidacloprid pulse. These samples were collected in 1 L, amber vials (EPA vials; Fisher scientific; Fair Lawn, NJ, U.S.A.) and stored at 4 °C until shipment to the laboratory.



Fig. 1 - Detailed view of the mesocosm tables, artificial streams with leaf bags, outflow containers with *in situ* bioassays and of the cages for the stonefly feeding bioassay.

Macoinvertebrate community responses

At the end of experimental period, i.e. after 20 days, benthic invertebrates were collected by sieving the entire contents of each stream using a 250-mm sieve, and were preserved in 10% formalin. Macroinvertebrates were counted and identified to family level (Ephemeroptera, Plecoptera, Trichoptera, Diptera and Coleoptera) or Order (Oligochaeta, Nematoda and Collembola). Emerged insects found in the emergent traps were collected every other day with an aspirator and preserved for identification.

The family biotic index (FBI), including tolerance values for non insect macroinvertebrates (Barbour *et al.* 1989; Mandaville 2002) was calculated to assess its discriminatory sensitivity towards pesticide contamination. FBI was calculated as:

$$FBI = \sum \frac{x_i t_i}{n}$$

where x_i is the number of individuals in the “ith” taxon, t_i is the tolerance value of the “ith” taxon, and n is the total number of organisms in the sample. Simpson’s diversity index (DI) calculated as:

$$DI = 1 - \sum_{i=1}^s (p_i)^2$$

where p_i is the proportion of individuals of family I and s the total number of families, was also calculated as a measure of heterogeneity and gives the probability of two individuals, chosen at random and independently from the population, belong to different families.

The Community Loss Index (CLI) measures the loss of benthic taxa in a study site with respect to a reference site. Values increase from 0 as the degree of dissimilarity between the sites increases (Mandaville 2002). CLI was calculated as:

$$CLI = \frac{d - a}{e}$$

where a is the number of taxa common to both replicate streams, d is the total number of taxa present in the reference stream, and e is the total number of taxa present in the pesticide treated stream. In this study, CLI was determined by comparing the total number of taxa present in each stream replicate of each imidacloprid concentration (“ e ”) to the number of taxa present in each of the respective stream replicate of the control treatment (“ d ”). The average value for the different streams in the control was calculated comparing all four streams of the control with each other in all possible combinations. This was done to account for the variation of the subsampling strategy utilized. We have not considered the insect pupae or the unidentified early instars of organisms for the calculation of biotic indexes or richness measures.

Leaf litter degradation

Alder leaves (*Alnus sp.*) were collected autoclaved and then conditioned by keeping them for 20 days at 20°C in aerated stream water and leaf litter collected from the Nashwaak River. 10 × 10 cm leaf bags were constructed by placing 1 ± 0.01 g of air dried leaves in coarse (mesh size 1 cm) for determination of decomposition of leaf material from invertebrate feeding and microbial activity. Fine mesh plastic bags (mesh size 0.250 mm) were used to assess microbial decomposition of leaf material. One coarse and one fine leaf bags were placed in every stream after inoculation of the substratum (Fig. 1). After 20 days leaf bags were collected, returned to the laboratory in plastic bags and stream water. Invertebrates and particulate organic debris were gently removed from the leaf material with distilled water and a soft brush. The leaf material was dried at 60 °C for 96 h and weighed. Five extra leaf bags were used to determine initial air dry weight to oven dry weight (four days at 60 °C) conversion factor. The leaf oven dry mass remaining was used to calculate the exponential decay coefficient (k ; i.e., decomposition rate) (Petersen & Cummins 1974).

In situ feeding bioassay

P. comstocki nymphs of similar size were collected from Nashwaak River, New Brunswick, Canada and were acclimated to laboratory conditions: 20 °C, 14L/10D light regime in aerated groundwater used in the mesocosm experiment and fed *ad libitum* with alder leaves for one week prior to the experiment.

For each treatment 15 *P. comstocki* nymphs (average length 20.00 mm +/- 1.79 SD) were allocated to individual cylindrical cages (5 cm x 5 cm) capped in both ends with 1mm² mesh containing food in the form of 5 alder leaf discs of known dry weight and a pebble in order to provide a hard surface for organisms to cling on.

The *in situ* cages were deployed in the outflow of the correspondent mesocosm table in plastic containers thus receiving the same water and experiencing similar physical characteristics (temperature, luminosity) of artificial streams (Fig 1).

Leaf discs used in feeding experiments were autoclaved and then conditioned by keeping them for 20 days at 20°C in aerated stream water and leaf litter collected from the Nashwaak River. They were dried for 4 day at 60°C and weighted. Leaf discs were soaked in groundwater used in the mesocosm experiment prior to the feeding trials. After the 20 days of exposure period animals and the remaining food were removed, dried at 60 °C for 4 days and reweighed. Feeding rate was calculated as the difference between the initial and final leaf discs dry mass (mg) and divided by elapsed time (20 days). Five control cages per treatment with leaf discs but no animals were used to correct for weight change due to factors other than feeding.

Laboratory measurements of oxygen consumption

Oxygen consumption was determined by simple static respirometry, using larvae held for 4 hrs in 50-ml gastight syringes (Hamilton, USA). To measure oxygen consumption 10 replicates were used each holding one organism of approximately similar size that were previously exposed to different concentrations of Imidacloprid for 20 hrs (0, 2, 10 and 20 µg/L). Syringes were filled with the appropriate test solutions and organisms, the air remaining was expelled from each syringe and they were left in the dark in a water bath (20°C). Initial oxygen concentrations were measured with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow). After 4 hours, the final oxygen concentrations were measured in the same way. Organisms were weighted for 60°C for 4 days. Oxygen consumption was determined by the differences in the oxygen content of water before (T_0) and after ($T_{\text{final}}= 4\text{hrs}$) the exposure period, and the respiration rate was expressed as µg oxygen consumed per mg of organism per hour.

Due to the limited number of syringes available, this experiment was performed in two runs over two consecutive days using half the replicates for every treatment each day

(randomised block design). Organisms were exposed in laboratory tanks to the appropriate pesticide solutions and no food for 20 hours before the respiration trials giving a total of 24 hours of exposure.

Data analysis

One-way analysis of variance, with individual stream mesocosms as replicates, was used for analysis of all variables. One-way analysis of variance was also used to compare feeding and respiration of *P. comstocki* among the different imidacloprid concentrations. For respiration rates, day of measurement was blocked as a random factor. Whenever significant differences were observed Dunnett post hoc test was used for multiple comparisons to determine which treatments were significantly different from the control. Invertebrate density data were log (x+1) transformed to stabilise variances across treatments (Zar 1996), although untransformed data were used for the calculation of biotic indices. Analysis of variance were performed with the MINITAB 13.0 statistical package (Minitab 2000).

Results

The mean pulse concentration of imidacloprid over 3 pulses (± 1 SE) was 1.63 $\mu\text{g/L}$ (± 0.15) for the low concentration and 17.60 $\mu\text{g/L}$ (± 1.82) for the high concentration. Average temperatures (± 1 SE) were 14.7°C (± 0.23) for control table, 14.5°C (± 0.24) for the treatment with low imidacloprid concentration and 14.9°C (± 0.24) for the treatment with high imidacloprid concentration. The feeding bioassay results for the control treatment were obtained using cages deployed in the outflow of a different table (a second control treatment), with slightly higher mean daily temperature: 15.5°C (± 0.41) due to problems on a peristaltic pump. The replicates from this mesocosm treatment were not included in the analysis of macroinvertebrate community or leaf decomposition.

Effects of imidacloprid on macroinvertebrate community

By day 20, the macroinvertebrate community was dominated by insects in all treatments (more than 92% in all streams). From these, Heptageniidae Ephemeroptera, Lepidostomatidae, Hydropsychidae and Helicopsychidae Trichoptera, chironomids and

dipteran pupae and Elmidae beetles were all present at high densities in untreated control streams. The non-insect taxa were of only minor importance except for oligochaetes which made up less than 3 % of the total numbers of organisms in the control treatment.

Imidacloprid pulses reduced species' densities and community diversity within the benthos in experimental streams compared to control streams (Table I). The community loss index showed a significant increase which reflects a significant reduction in diversity caused by imidacloprid contamination (Table I). The family biotic index, Simpson's index, total richness and EPT (Ephemeroptera, Plecoptera, and Trichoptera) richness did not show any statistical significant increases or decreases.

Imidacloprid had an adverse effect on benthic communities in experimental streams with ca. of 5 and 42 % reductions in the abundance of invertebrates exposed to pulses of low (0.63 µg/L) and high (17.60 µg/L) imidacloprid concentrations (Table I, Fig. 2). In the high concentration treatments there was a clear reductions of total benthic insects (ANOVA $F_{2,11}= 6.40$, $p=0.019$). Average reductions in abundance for Plecoptera and Ephemeroptera were 55 and 67.7% (ANOVA $F_{2,11}=6.52$, $p=0.018$ and ANOVA $F_{2,11}= 6.86$, $p=0.016$) respectively in streams exposed to high concentrations of imidacloprid giving an overall average reduction in abundance of EPT taxa of 69 % (ANOVA $F_{2,11}=6.40$, $p=0.019$). At the high imidacloprid concentration treatment, the average abundance of Trichoptera was reduced by 70%, but was not statistically significant (ANOVA $F_{2,11}=2.52$, $p=0.135$).

Coleoptera had no significant effects at the high imidacloprid concentration treatment, but was reduced by 28.6 % (ANOVA $F_{2,11}=0.63$, $p=0.554$) and no significant effects of imidacloprid were observed for dipterans collected from the streams after 20 days (ANOVA $F_{2,11}=1.62$, $p=0.251$). Oligochaetes also showed high sensitivity to these high imidacloprid concentrations with a 75 % reduction in density in the high concentration treatments (ANOVA $F_{2,11}=13.12$, $p=0.002$). Although all the parameters tested were not significant for the lowest concentration of imidacloprid, there was a reduction of 30% on EPT taxa and caddisfly abundance (Fig. 2). Considering benthic and emerged insects we observe a similar pattern with a reduction in abundances of all insects Orders but this time with a significant decrease in the abundance also for dipterans with exposure to pulses of high concentrations of imidacloprid (ANOVA $F_{2,11}=5.04$, $p=0.034$).

Effects of imidacloprid on leaf litter

There was no evidence that imidacloprid at either test concentration inhibited microbial decomposition rates. Mass loss in fine mesh bags at all test concentrations did not differ significantly from controls (ANOVA $F_{2,11}=0.80$, $p=0.478$; Fig. 3). In contrast, total decomposition of leaf material in coarse mesh bags (a combination of invertebrate feeding and microbial decomposition) was reduced in treated streams being significantly different in the highest imidacloprid concentrations, compared to control streams (ANOVA $F_{2,11} = 4.95$, $p=0.035$; Fig.3). Organisms found in the leaf bags were collected and combined with benthic samples from the respective stream replicates.

Table I- Comparison of benthic invertebrate community endpoints (mean \pm SE) in response to different imidacloprid concentrations.

	Imidacloprid concentrations			Df	F	p
	0	1.63 $\mu\text{g/L}$	17.60 $\mu\text{g/L}$			
Density (n° /stream)	310 \pm 45	296 \pm 94	177 \pm 19 *	2,11	8.26	0.009
Total richness	21 \pm 1	19 \pm 2	16 \pm 1	2,11	3.24	0.087
EPT richness	12 \pm 1	12 \pm 1	9 \pm 1	2,11	3.31	0.084
EPT / chironomids	1.65 \pm 0.45	0.97 \pm 0.20	0.49 \pm 0.13	2,11	3.90	0.060
Simpson's DI^a	0.74 \pm 0.05	0.72 \pm 0.05	0.55 \pm 0.09	2,11	2.74	0.118
mFBI^b	4.77 \pm 0.52	5.44 \pm 0.19	6.23 \pm 0.40	2,11	3.43	0.078
CLI^c	0.22 \pm 0.03	0.22 \pm 0.04	0.47 \pm 0.04*	2,19	10.00	<0.001

^a Simpson's DI: Simpson diversity index

^b FBI: modified Hilsenhoff's family biotic index

^c CMI: Community loss index

* Treatments significantly different from control

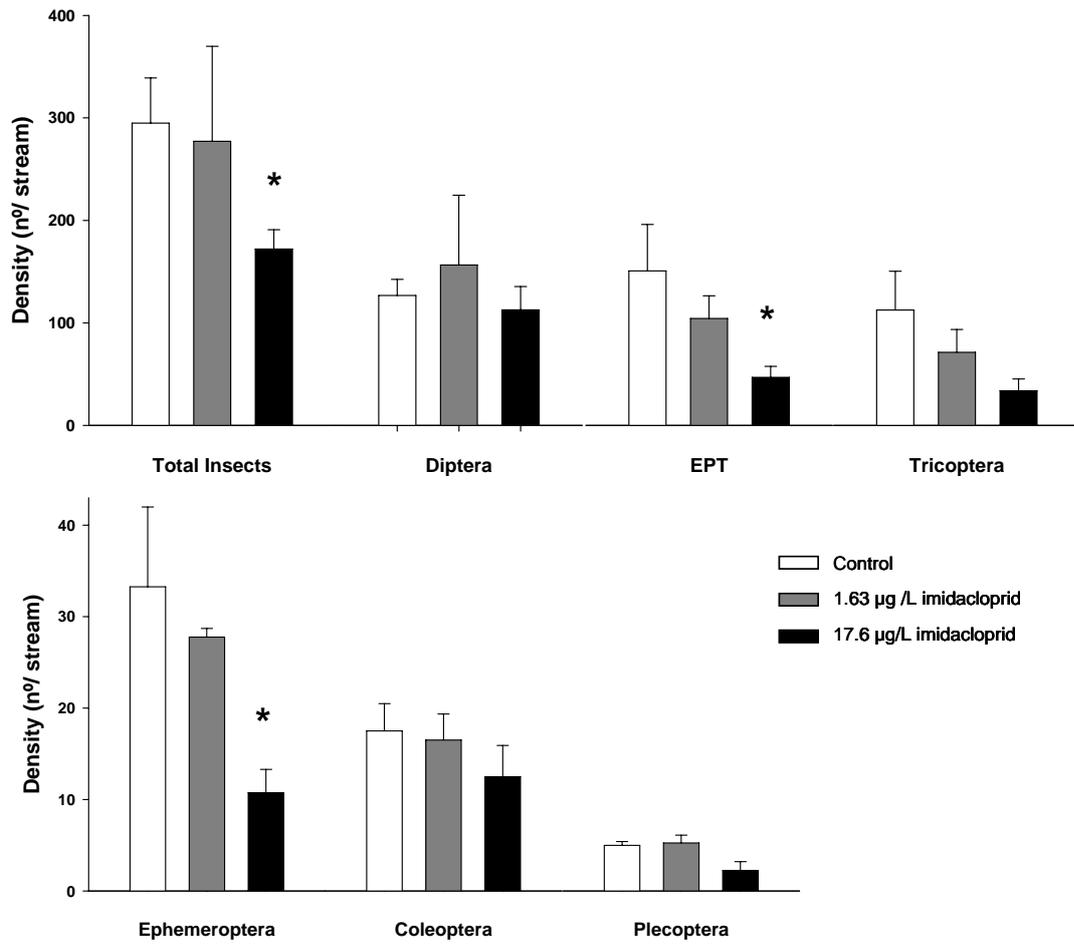


Fig. 2 - Comparison of mean densities (+ SE) of benthic insects after 20 days in stream mesocosms. An asterisk indicates a significant difference between pesticide treatments levels and control (Dunnett test $p < 0.05$). EPT: Ephemeroptera, Plecoptera and Tricoptera

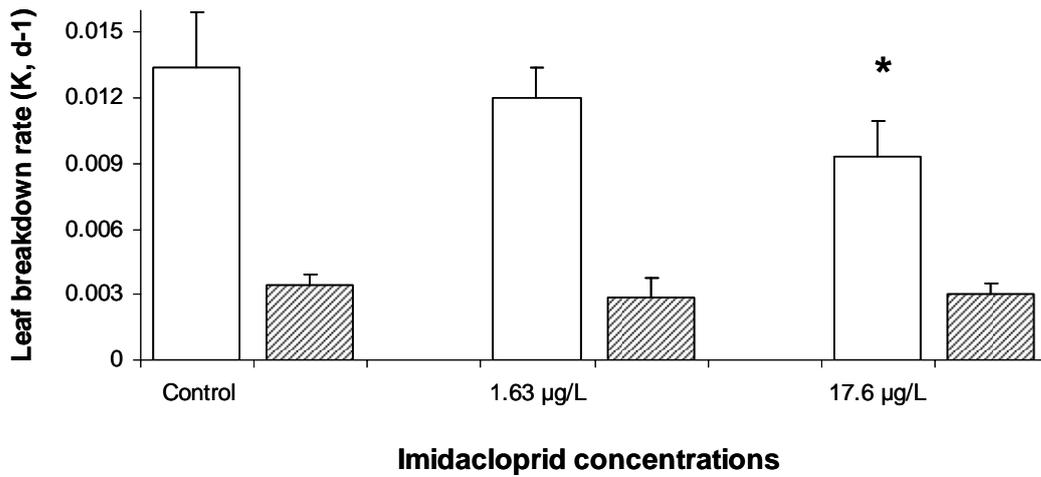


Fig. 3 - Comparison of leaf breakdown rates (mean + SE) in coarse (empty bars) and fine (shaded bars) after 20 days in stream mesocosms. Asterisks denote imidacloprid treatments that were significantly different from the control treatment (Dunnett test , $p < 0.05$).

Effects of imidacloprid on stonefly in situ and laboratory bioassays

In the feeding and respiration experiments, observed mortality was always below 10%. The feeding rate of *P. comstocki* deployed in the outflow of the mesocosm systems was reduced in treated tables (27 and 71% in low and high imidacloprid concentration pulses respectively), but was only significant in the high concentration treatment (ANOVA $F_{2,44}=14.34$, $p < 0.001$; Fig.4).

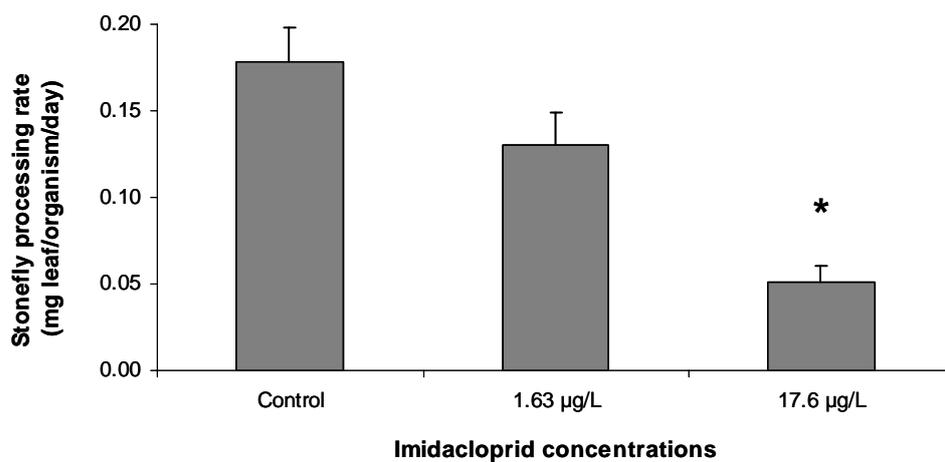


Fig. 4 - *In situ* *P. comstocki* processing rate (mean + SE) measured over 20 days in stream mesocosms. Asterisks denote imidacloprid treatments that were significantly different from the control treatment (Dunnett test , $p < 0.05$).

P. comstocki showed reductions in oxygen consumption when exposed in the lab for 24 hours to imidacloprid, but this was significant only at nominal concentrations of 10 $\mu\text{g/L}$ or above (ANOVA $F_{3,39}=33.66$, $p=0.008$; Fig. 5). No significant effects were observed for day of measurement (ANOVA $F_{1,39}=4.41$, $p=0.126$) or interaction of both factors (ANOVA $F_{3,39}=2.14$, $p=0.114$).

Actual concentrations of imidacloprid although not measured are not expected to differ considerably from the nominal concentrations because we have used the same stock solution used for mesocosm experiments and the oxygen consumption experiments were performed in the dark which substantially reduces the degradation of imidacloprid.

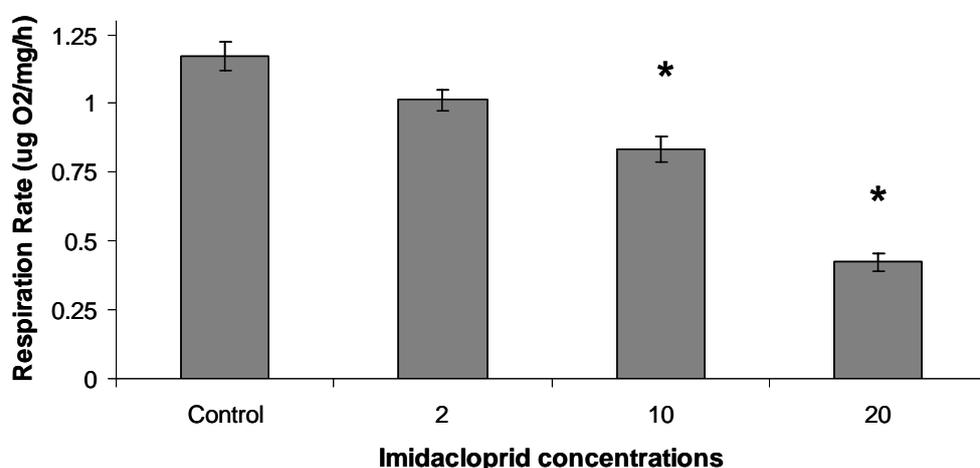


Fig. 5 - *P. comstockii* laboratory respiration rates (mean + SE) measured. Asterisks denote imidacloprid treatments that were significantly different from control (Dunnett test, $p<0.05$).

Discussion

Risk assessments of chemicals rely on a combination of predicted environmental concentration (the exposure level) and no-effect concentrations (the effects threshold) which are usually obtained from laboratory acute and/or chronic responses. Model ecosystem studies provide critical information on the ecological risks posed by highly toxic chemicals with short half lives that are most likely to be at effect-concentrations during brief exposure periods (e.g. pulses during runoff events). Such evidence can help to minimise the uncertainty of extrapolation from laboratory results to natural ecosystems (Van den Brink 2006).

Our study assessed the effects of multiple pulses of different concentrations of imidacloprid on benthic invertebrates. The use of a natural benthic community and substratum and the assessment of structural and functional endpoints allowed a more ecologically relevant estimate of long term and sub-lethal effects of imidacloprid, compared with traditional laboratory toxicity tests. By including data from an *in situ* ecotoxicological bioassay to complement information on the structure of the macroinvertebrate community and functional parameters, the methodology presented here can help making a mechanistic link between effects at various levels of organization and thus more precisely assess the direct and indirect effects of pesticide contamination on ecosystem functioning. Moreover, using *in situ* assays together with mesocosm experiments has the advantage of simultaneously generating ecotoxicological data for several species under semi-field conditions (Hruska & Dube 2004; Slijkerman *et al.* 2004; Hruska & Dube 2005; Slijkerman *et al.* 2005; Coors *et al.* 2006).

Concentrations of imidacloprid in the range of 5-50 µg/L have been previously shown to be acutely toxic to several non-target freshwater insects such as dipterans and caddisflies (Song *et al.* 1997; Pestana *et al.* 2008a; Stoughton *et al.* 2008) and mayflies such as *Epeorus longimanus* have an extremely low 96 hrs-LC₅₀ of 0.65 µg/L (Alexander *et al.* 2007). Some non-insect taxa are can also be sensitive to low concentrations of imidacloprid such as *Hyallorella azteca* with a 96 hr-LC₅₀ of 17-65 µg/L (Stoughton *et al.* 2008) and the oligochaete *Lumbriculus variegatus* with a 96 hr-EC₅₀ of 6.2 µg/L (Alexander *et al.* 2007).

Our study showed significant effects of pulses of imidacloprid on abundances of invertebrates, namely EPT taxa and oligochaetes at concentrations of 17.60 µg/L. Coleoptera seem to be in general more tolerant to imidacloprid than other insects. Much higher concentrations have been previously shown to cause significant declines in abundance of aquatic insects in controlled field experiments (rice paddies) (Sanchez-Bayo & Goka 2006a). Although benthic dipteran larvae densities were not significantly different among treatments an analysis on the emerged insects showed that a greater number of dipterans had emerged in control streams. This highlights a possible effect of imidacloprid (delayed maturation) already observed for chironomids (Pestana *et al.* 2008a) and at the same time stresses the importance of insect adult biomass or insect emergence as a functional parameter to be used in field deployed mesocosms (Schulz & Liess 2001; Culp

et al. 2003). Mayflies for instance, have been previously shown to respond to lower concentrations of imidacloprid by maturing earlier (at the cost of size) (Alexander *et al.* 2008) and thus lower concentrations of imidacloprid could impair reproductive fitness of sensitive species. Not considering the adults and insect emergence could thus underestimate the effects of imidacloprid on natural systems. In our experiment an analysis of the numbers of emerged adults revealed only different results for dipterans, possibly as a consequence of the range of concentrations tested.

It was also clear that some diversity and biotic indices usually calculated in rapid bioassessment protocols can fail to discriminate effects of pesticide contamination on natural benthic communities. The fact that certain diversity indices place relatively little weight on rare taxa and more weight on common taxa (Rosenberg & Resh 1993; Mandaville 2002) and the fact that tolerance values with respect to organic pollution used for the family biotic index may not be in concordance with the sensitivity of organisms to pesticides can represent a disadvantage for the use of these measures as indication of contamination and especially of effects of toxic insecticides in insect dominated communities (Rosenberg & Resh 1993). Chironomids made a significant contribution to these indices (abundance and tolerance values) because chironomid larvae were present in high densities even in the high imidacloprid concentration replicate streams. This was surprising considering some results of laboratory tests that show low tolerance of chironomids to imidacloprid (Pestana *et al.* 2008a; Stoughton *et al.* 2008).

The community loss index, which only considers taxa richness and not abundance, was however significantly affected in the high concentration treatment showing a significant reduction on the number of taxa present in imidacloprid treated streams compared to control streams. This index was chosen to assess the effects of imidacloprid accounting for the variability arising from the sub sampling procedure. In this way we can say that effects of imidacloprid on community composition were significant above the range of “natural variability”.

The fact that these indices were only calculated to benthic communities (no adults), the low number of replicates used and the fact that organisms were identified only to family level or higher could have also contributed to the lack of sensitivity of these measures.

Neurotoxic insecticides such as imidacloprid have been shown to induce several behavioural responses such as uncontrolled muscular contractions and reduced activity of

insects (Moffat 1993; Matsuda *et al.* 2001). Our results demonstrated that pulses of imidacloprid impair feeding by *P. comstocki* on leaf material. The feeding rates observed in our study are relatively low compared to other experiments using pteronarcid nymphs (Cole *et al.* 2001). Although usually considered as shredders (Merrit & Cummins 1996; Ruesink & Srivastava 2001; Kreutzweiser *et al.* 2007), pteronarcid stoneflies are known to rely on other food sources and furthermore the use of dried leaf material and the confinement in *in situ* cages could have can affect their feeding rates (Freilich 1991; Ruesink & Srivastava 2001). In any case, the feeding bioassay with this species deserves further refinement in order to increase the feeding rates of caged organisms, for example through a longer conditioning or soaking of leaves thus increasing the magnitude of response and consequently the power of statistical analysis.

Feeding inhibition has already been reported in tricopteran species (Pestana *et al.* 2008a), daphnids (Pestana *et al.* 2008b); ephemeropterans and oligochaetes (Alexander *et al.* 2007), as responses to imidacloprid in laboratory and in pteronarcids and tipulids; in mesocosm experiments (Kreutzweiser *et al.* 2007).

In the laboratory assay, imidacloprid exposure also induced a reduction in the respiratory rates of *P. comstocki* possibly as a consequence of reduced activity or through disruption of ventilatory movements (“push up behaviour”), characteristic of these organisms under static flow conditions (Ericksen *et al.* 1996; Genkai-Kato *et al.* 2000). The same effect of reduced oxygen consumption as a response to exposure to imidacloprid has been observed for caddisflies using similar experimental procedures (Pestana *et al.* 2008a).

Reductions in activity can therefore lead to reduce foraging efficiency and thus reduced energy intake of aquatic invertebrate species with consequences for growth, reproduction and survival. In our case it was not possible to relate feeding rates of *P. comstocki* deployed *in situ* with abundances within the benthic communities inoculated because several stoneflies were removed from invertebrate samples to avoid predation within artificial streams. Due to the size of many pteronarcid nymphs they were wrongly considered as predators and systematically removed from invertebrate sub samples being almost absent in artificial streams.

The results from leaf decomposition further suggest that imidacloprid can affect leaf shredding since a significant reduction in decomposition rates was observed for coarse

mesh bags deployed in the artificial streams receiving pulses of high concentration of imidacloprid. This also indicates that the functional role played by *P. comstocki* in the studied system correlated well with leaf processing by the natural community. By identifying invertebrates only to Family level it is difficult to determine the functional feeding role of the majority of the invertebrate community present in artificial streams, however the effects of imidacloprid on leaf decomposition observed in coarse leaf bags may be related to the abundance and feeding behaviour of lepidostomatid tricopterans (shredders) and chironomids. Chironomids, although generally considered collector-gatherers, have been shown to feed also on coarse particulate organic matter (Callisto *et al.* 2007). The invertebrate colonisation of leaf material in coarse mesh bags was not considered due to the relatively small area of artificial streams and also to some problems in the collection of leaf bags which might have introduced some uncertainties concerning the contribution of organisms to leaf shredding.

Although we did not measure microbial community structure or activity directly, leaf decomposition in fine leaf bags was not significantly different among the imidacloprid treatments. Moreover, no effects of higher concentrations of imidacloprid on microbial activity and decomposition have been already shown in artificial systems (Kreutzweiser *et al.* 2007). Together, these results show that imidacloprid does not seem to affect the microbial community directly and for this reason, the feeding inhibition by leaf-shredding insects was less likely due to a reduced microbial conditioning of leaves. Thus, although microbial colonization and conditioning of leaf litter is critical for the diet of shredders (Graça 2001), sublethal concentrations of imidacloprid most likely affect leaf litter breakdown, through impairment of the feeding behaviour of leaf-shredding invertebrates.

Conclusion

In this study the effects of pulses of imidacloprid were assessed under controlled conditions of exposure that mimicked field conditions. Moreover, functional and structural endpoints were used to better understand the mechanisms of imidacloprid toxicity at ecosystem level. The concentrations of imidacloprid tested here are expected to be within realistic environmental concentrations and previously shown to occur in agricultural runoffs. Our data suggest that significant adverse effects on aquatic macroinvertebrate

community composition would be expected where imidacloprid concentrations in water reach or exceed 17 µg/L even if for short periods. Imidacloprid reaching streams or rivers at these or even lower concentrations can exert lethal and sublethal effects on aquatic non-target organisms (feeding inhibition, delayed maturity, size at emergence) with potential impairment of insect reproductive fitness, thus influencing critical ecosystem properties and functions.

Plant litter decomposition was tested as an indicator of imidacloprid effects in insect dominated benthic communities, and coarse and fine mesh bags, have shown to be suitable for application in mesocosms studies to distinguish the effects of pesticides on aquatic fungi and macroinvertebrates, the two main decomposers. In this way, a useful functional endpoint can be used to complement pesticide risk assessment programs based on structural parameters providing an indication of the possible impairment in the functioning of the stream ecosystems. Feeding assays are used as chronic assays in laboratory and in the field with a wide range of organisms (Lam 1996a; Hatch & Burton 1999; Maltby *et al.* 2002; McWilliam & Baird 2002a; Irving *et al.* 2003) and constitute sensitive biomonitoring tools to assess effects of low levels of pesticides. *In situ* single species feeding assays can also be used with an array of invertebrate species in mesocosms studies as early warning indicators of pesticide contamination. This study highlights the importance of combining fine-resolution studies of individual species with model ecosystem studies and the integration of functional and structural endpoints for a better understanding of the mechanisms that link organism level responses to population- and community-level processes and to improve our prediction of possible sub-lethal as well as indirect effects of pesticides.

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Chapter III

Predator threat assessment in *Daphnia magna*

Straus: the role of kairomones versus alarm cues

Predator threat assessment in *Daphnia magna* Straus: the role of kairomones versus alarm cues.²

Abstract

Using a clone of *Daphnia magna* Straus we studied the relative importance of alarm cues and fish kairomones as initiators of *Daphnia* antipredator defences. By exposing *D. magna* to infochemicals that simulated the presence of an active fish predator, we observed cue-specific responses in terms of altered behavioural, physiological and life-history traits.

Results were consistent with the hypothesis that *D. magna* processes information from alarm cues from macerated conspecifics and from predator kairomones to assess the level of predation risk, adjusting the magnitude of their responses to the different levels of threat perceived. Behavioural and physiological responses were triggered by single cues (fish kairomones or alarm cues), whereas life history shifts were unaffected by exposure to alarm cues alone. Simultaneous exposure to both cues elicited stronger behavioural, physiological and life-history responses indicating that both the concentration and nature of infochemicals influence *D. magna* responses to predation risk, avoiding unnecessary costs or even maladaptive responses. Based on our results and those of others, we hypothesize that a combination of prey-specific alarm cues and predator-specific substances is necessary to trigger the full deployment of antipredator responses in daphnids.

Key words: fish-daphnia interactions, chemical cues, predation risk assessment, inducible responses, Brown trout.

²J.L.T. Pestana, D. J. Baird and A.M.V.M. Soares, *submitted to the journal Oecologia*.

Introduction

Animals use extrinsic sensory information to gain knowledge of their local environment. This information includes cues on the availability of food, abiotic factors such as light, temperature or shelter and the presence of competitors and predators (Dicke & Grostal 2001). Chemical cues in aquatic environments, are particularly important, given a general low light intensity, coupled with the relatively poor performance of auditory and visual senses (Kats & Dill 1998; Lass & Spaak 2003a). For this reason, it is hypothesized, the majority of intra- and interspecific interactions between aquatic organisms are mediated by infochemicals (Dicke & Grostal 2001; Burks & Lodge 2002). These chemically-mediated interactions are receiving greater attention because of their potentially important role in population dynamics, structuring of communities and ecosystem function (Vet 1999; Turner *et al.* 2000; Burks & Lodge 2002).

Of the different types of chemical cues available in aquatic environments, those that can signal the presence of predators are important because, in addition to direct predator-induced mortality, simply by their presence within a system, predators can exert sub-lethal effects on prey which can affect their abundance, morphology, physiology, life history and behaviour (Lima & Dill 1990; Kats & Dill 1998; Lass & Spaak 2003a). Antipredator responses have associated costs because they represent a compromise between energy gains from foraging (and thus evolutionary fitness) and energy losses arising from the deployment of an effective antipredator strategy (Abrams & Rowe 1996; Boersma *et al.* 1998; DeWitt *et al.* 1998; Sakwinska & Dawidowicz 2005). Thus, the specificity and reliability of the chemical cues are critical to allow prey organisms to correctly assess predation risk and maximise the benefit of the anti-predator responses (Tollrian 1995; De Meester & Weider 1999).

Aquatic organisms use direct and indirect sources of chemical cues to assess predation risk. Cues produced by predators that induce adaptive responses in prey are termed kairomones, and can be contained in the exuvia, eggs, excreta, pheromones or any other substance released by the predator (Turner *et al.* 1999; Stibor & Navarra 2000; Dicke & Grostal 2001; Iyengar & Harvell 2002; Ruther *et al.* 2002; Slusarczyk & Rygielska 2004). Indirect chemical cues can also inform prey organisms about the presence of predators. These chemicals may include pheromones from alarmed, injured or dead conspecifics

(Pijanowska 1997; Wisenden *et al.* 1997; Chivers & Smith 1998; Wisenden *et al.* 2001; Jacobsen & Stabell 2004) and also allelochemicals from heterospecifics (Chivers *et al.* 1996; Wisenden *et al.* 1997; Huryn & Chivers 1999).

Predator kairomones are useful to prey species in assessing the density or proximity of predators but give little information on instantaneous predation risk (since predators' feeding motivation varies over time), similarly, alarm cues indicate only that there is an unspecified threat, but do not provide information on the identity of the predator (Schoeppner & Relyea 2005). During any predation event both kairomones and alarm cues can be liberated, resulting in a complex set of chemical signals to be interpreted in order to correctly assess the level of threat and make the appropriate defensive decisions (Slusarczyk 1999; Schoeppner & Relyea 2005). Interpretation of the information contained in each cue can be crucial to avoid maladaptive responses since prey defences (behavioural, morphological and in the life history) can vary in direction or magnitude according to the type of predator present (invertebrate *vs.* vertebrate) and to the level of predation risk posed (Weber & Declerck 1997; Dahl 1998; Turner *et al.* 1999; Barry 2000; Weber 2003; Hulsmann *et al.* 2004).

Research on chemically-induced responses of aquatic invertebrates to predators has been performed in a variety of species with a strong emphasis on planktivorous fish-*Daphnia* systems (Lass & Spaak 2003a). In the presence of chemical cues from fish, daphnids normally show responses that mimic predation selection. Thus, in response to fish infochemicals, *Daphnia* usually respond with life-history shifts such as precocious maturation as well as the production of more and smaller neonates, (Reede 1995, 1997; De Meester & Weider 1999; Spaak *et al.* 2000; Declerck & Weber 2003; Hulsmann *et al.* 2004; Gliwicz & Maszczyk 2007). This will tend to maximise fitness by increasing the probability of reproduction under strong mortality risk from visual, positively-size selecting predators and at the same time compensate the predation related mortality (Lass & Spaak 2003a; Hulsmann *et al.* 2004).

Morphological responses of daphnids such as modifications of body and head shape (Stabell *et al.* 2003) and the induction of longer tail spines (Dzialowski *et al.* 2003) have been also observed in response to fish kairomones, although the adaptive value of these responses is clearer in the presence of invertebrate predators that feed on small prey (Tollrian 1995). Nevertheless, morphological defences are effective when the handling

time for predators is increased, thus reducing average instantaneous mortality risk within the prey population (Kolar & Wahl 1998).

Behavioural and physiological responses to fish infochemicals have generally short induction times and thus represent the first defence mechanisms for prey (De Meester & Cousyn 1997). Avoidance behaviour (Pijanowska & Kowalczewski 1997b; Roozen & Lurling 2001), changes in phototactic behaviour (Ringelberg & vanGool 1995; De Meester & Cousyn 1997), changes in swimming behaviour (Weber & Van Noordwijk 2002), aggregation and alertness (Pijanowska & Kowalczewski 1997b; Brewer *et al.* 1999) are common responses of daphnids to fish chemical cues. While behavioural responses reduce the probability of encountering fish predators (Lass & Spaak 2003a), they can also reduce energy intake (Rose *et al.* 2003; Beckerman *et al.* 2007). The presence of predators has also been shown to place higher metabolic demands on prey organisms, manifested in elevated rates of oxygen consumption, reflecting an increased cost of vigilance under predation risk (Bengtsson 1982; Pijanowska 1997; Beckerman *et al.* 2007).

Since numerous methods have been used to prepare chemical stimuli from different predators and because a range of endpoints are studied (see above), literature is equivocal concerning the relative importance of the various cues daphnids use to assess predation risk. *Daphnia* have been shown to respond to kairomones from predators alone (Walls & Ketola 1989); to alarm cues from conspecifics alone (Pijanowska 1997; Laforsch *et al.* 2006), or to mixtures of both cues (Slusarczyk 1999; Stabell *et al.* 2003). It has also been noted in some studies that daphnids fail to respond to alarm cues or kairomones alone (Stirling 1995; Slusarczyk 1999; Slusarczyk & Rygielska 2004). However, most studies fail to control for the effects of alarm substances, and in most cases predators are fed conspecifics of the experimental organisms during kairomone solution preparation.

The aim of this work is to understand the relative importance of alarm cues and fish kairomones for *D. magna* antipredator defences. Since alarm cues and kairomones may not induce all traits in the same way or with the same magnitude it is important that behaviour, physiology and life history are simultaneously studied when prey organisms are exposed to different concentrations of different chemical stimuli. Thus, responses of *D. magna* exposed to trout kairomones (i.e. starved predators), to alarm cues from macerated conspecifics and to a combination of both cues were evaluated, testing two main hypotheses: (1) *D. magna* should be able to show responses to trout kairomones alone and

in combination with alarm cues and adjust those responses accordingly with the stimuli concentration and (2) the exposure to the combination of both cues should induce stronger responses.

Material and Methods

Experimental animals

D. magna, clone F (*sensu* (Baird *et al.* 1991a) was cultured under a 16:8 light dark photoperiod and $20^{\circ} \pm 2^{\circ}\text{C}$. The culture medium was ASTM hard water enriched with an organic extract and animals were fed the green alga *Chlorella vulgaris* Beijerinck, at a concentration of 3.0×10^5 cells ml^{-1} . Medium and food were renewed every other day keeping a density of 18-22 adults L^{-1} . Neonates from the 3rd or 4th clutch of cultured animals were employed in all experiments.

Brown trout, *Salmo trutta*, Linnaeus was used as a model vertebrate predator. Brown trout feed on aquatic and terrestrial insects, small fish and crustaceans including daphnids (Penczak & Formigo 2000). *D. magna* has been shown to respond to chemical cues from different fish species including trout (Lass & Spaak 2003a, 2003b). Young brown trout were obtained from a fish farm, and transferred to laboratory where they were kept at $17 \pm 1^{\circ}\text{C}$ in 60L plastic tanks with aerated artificial pond water (APW) (Naylor *et al.* 1989), and fed once every day with commercial fish food and once a week with daphnids and chironomids. After one month, healthy fish were selected to be used in the preparation of fish chemical cues (kairomones).

Preparation of chemical cues

To prepare the kairomone solution, 20 brown trout (4 - 6 cm) were held in 10 L aerated ASTM for 24 hrs. After that period, the water, containing trout kairomones, was filtered (0.45 μm Whatman acetate cellulose filter) and frozen at -20°C . Fish were not fed for 3 days prior to, and during this 24 hour period to reduce the level of ammonia and faeces in our fish conditioned water. For the production of *D. magna* alarm cues, 50 adult *D. magna* were macerated in 100 mL of ASTM, the solution was filtered (0.45 μm Whatman acetate cellulose filter) and frozen at -20°C .

These stock solutions of trout kairomones and *Daphnia* alarm cues were thawed at room temperature in the day they were used in the experiments. Different concentrations of chemical cues were used to simulate three levels of perceived risk of predation: low, medium and high assuming that higher density of predators, i.e. higher concentration of kairomones is an indication of higher predation risk which could result in higher mortality with higher concentration of alarm cues. In the same way lower concentrations of alarm cues simulate lower predation risk and thus correspond to a lower concentration of trout chemical cues. Thus, kairomones, alarm cues and a combination of both were diluted in ASTM to prepare the following experimental treatments:

- **(CTR)**, Control = ASTM, no chemicals cues added
- **(K), Low** Kairomone = 0.05 fish L⁻¹
- **(K), Medium** Kairomone = 0.1 fish L⁻¹
- **(K), High** Kairomone = 0.2 fish L⁻¹

- **(AC), Low** Alarm cue = 1 crushed daphnia L⁻¹
- **(AC), Medium** Alarm cue = 2 crushed daphnia L⁻¹
- **(AC), High** Alarm cue = 4 crushed daphnia L⁻¹

- **(K+AC), Low** Kairomone + Alarm cue = (0.05 fish + 1 crushed daphnia) L⁻¹
- **(K+AC), Medium** Kairomone + Alarm cue = (0.1 fish + 2 crushed daphnia) L⁻¹
- **(K+AC), High** Kairomone + Alarm cue = (0.2 fish + 4 crushed daphnia) L⁻¹

Feeding experiments

The effects of the different chemical cues on the feeding rates of *D. magna* were assessed using 150 ml jars with 100 ml of ASTM. For each chemical cue treatment five replicates were used with five 4 day-old *D. magna* juveniles plus three blank replicates, i.e. with no organisms present. To check if algal concentration changed during the assay Daphnids were exposed to the different treatments for 24 hours at 20° ± 1°C, in the dark (to minimize algal growth) and fed *C. vulgaris* at a concentration of 3.0 x10⁵ cells ml⁻¹. At the end of the test, individuals were removed and algae concentration was determined by spectrophotometry (in a Jenway 6505 UV/Vis. Spectrophotometer). Individual feeding

rates (cells/organism/hour) were determined as the change in cell density during 24 h according to the method given by Allen, Calow & Baird (1995) First instar daphnids (<12 hours) were isolated and maintained in ASTM with *C. vulgaris*, without organic extract until they reached the 4th day.

Measurement of oxygen consumption

The O₂ consumption experiment was performed using standard respirometry methods using 50-ml gastight syringes (Hamilton, USA). Three syringes were employed per treatment with five 4 day-old *D. magna* juveniles in each. Syringes were filled with 30 ml of the appropriate test solutions (without food) and organisms, the air remaining was expelled from each syringe, which was placed in a water bath (20°C) for 24 hours, with light excluded. After 2 hours, initial O₂ concentrations were measured with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow, UK). After 22 hrs, the final O₂ concentrations were measured in the same way. The oxygen consumption was given by the differences in the oxygen content of water before (T₀= 2h) and after (T_{final}= 24h) the exposure period and the respiration rate is expressed as and µg O₂ consumed per organism per hour. In a separate experiment three blank controls (syringes with no organisms) were employed for every treatment to correct for ambient O₂ depletion due to factors other than organism's respiration. The depletion in the oxygen content on these blank controls was used as a correction factor for the appropriate treatments. For the respiratory experiments first instar daphnids (<12 hours) were isolated and maintained in ASTM, with *C. vulgaris* and organic extract, until the start of experiments.

Life table experiment

A 14-day life table experiment was initiated with first instar daphnids (<12 hours), under the same temperature and photoperiod described for the culturing procedures. Each treatment was replicated 10 times with one organism per replicate. Test solutions (50 ml per replicate) were renewed every other day and *C. vulgaris* was supplied every day in a concentration of 3.0 x10⁵ cells ml⁻¹. We measured size at maturity, neonate size, age at first reproduction, total number of offspring per female, size at the end of experiments and also the intrinsic rate of population increase (*r*).

Body length of test organisms was measured at maturity (the day the first eggs were visible in the brood pouch) and at day 14. To assess neonate size, 5 neonates of the first clutch of every replicate were pooled and 20 drawn at random for measurement. Body length measurements were performed using a stereomicroscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. Body length of neonates was measured by measuring length (taken in the central axis) of the 1st exopodite of the 2nd antennae, measured in the moult carapaces (Soares 1989).

Data analysis

For every endpoint (feeding, respiration and life history parameters), effects of the different treatments were analysed using a general linear model ANOVA. Since the experiment did not conform to a full factorial design, kairomones, alarm cues and their mixture were considered as 3 independent variables and thus no interactions were analysed. Dunnett's post-hoc test was employed to determine differences relative to the control treatment (Zar 1996).

The intrinsic rate of population increase (r) was calculated iteratively using the Euler's equation:

$$1 = \sum_{x=0}^n e^{-rx} l_x m_x,$$

where r is the rate of population increase (day^{-1}), x is the age class in days (0...n), l_x is the probability of surviving to age x , and m_x is the fecundity at age x . Replicate pseudo values for r were generated using the jackknife procedure (Meyer *et al.* 1986).

Results

Life history

D. magna exposed to trout kairomones only (K), and to a combination of alarm cues and trout kairomones (K+AC) showed increased fecundity, a reduction in age at first reproduction, a reduction in size at maturity, a reduction in neonate size and an increase in the intrinsic rate of population increase (r) relatively to the control treatment (Fig.1, Table I).

Daphnids exposed to a combination of both cues (K+AC treatments) showed stronger responses in every parameter with significant effects observed for offspring, neonate size, age at first reproduction, size at maturity and intrinsic rate of population increase (r) (Fig.1, Table I). Treatment K also induced significant responses in offspring, neonate size, age at first reproduction, and intrinsic rate of population increase (r). (Fig.1, Table I). Results also show that daphnids exposed to kairomones (K) or to kairomone and alarm cues (K+AC) adjust their responses according to the strength of the predation signals. Exposure to low predation risk levels revealed no significant effects for any of the parameters or cues tested. (Fig.1)

Exposure to alarm cues alone (AC) had no significant effects on any of the life history parameters although slightly higher fecundity was observed with exposure to the highest concentration of alarm cue. (Fig1, Table I)

Feeding

Exposure to increasing concentrations of different types of chemical cues caused reductions in the feeding of *D. magna* (Fig. 2). Feeding rates were significantly different in the high level of predation risk (higher concentrations of cues) for kairomones (K) and alarm cues alone (AC) and in combination (K+AC). The effects of combined exposure to both types of chemical cues (K+AC) were stronger in every level of predation risk, with significantly lower feeding rates also observed for the medium concentration of kairomones and alarm cues (K+AC). *D. magna* showed varying responsiveness to the different types of cues with $AC < K < K+AC$ in terms of effects on feeding rates (Fig. 2, Table I).

Respiration

Exposure to alarm cues (AC), kairomones (K) and combined cues (K+AC) also affected the respiration of *D. magna*, with significantly elevated respiration rates with increasing concentration for all combinations of cue (K, AC, K+AC) (Fig. 3, Table I). Combined exposure to kairomones and alarm cues (K+AC) resulted in more pronounced effects compared to single cues with significantly different respiration rates observed at the low concentration treatment (Fig. 3, Table I).

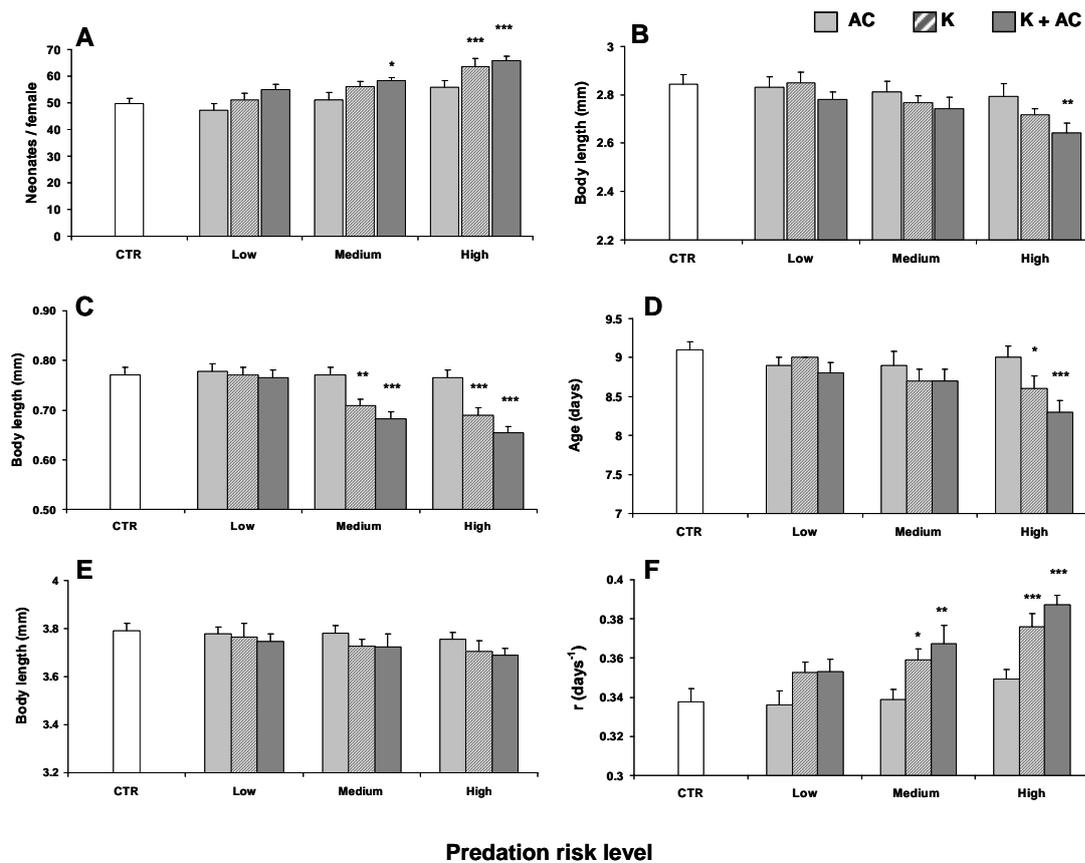


Fig.1 - *D.magna* life history parameters (mean + SE). A- total number of neonates produced per female; B- size at maturity; C- neonate size (first clutch); D- Age at first reproduction; E- Size at day 14; F- rate of population increase (r). Asterisks denote differences statistically different relatively to the control (* P < 0.05, ** P < 0.01 and *** P < 0.001).

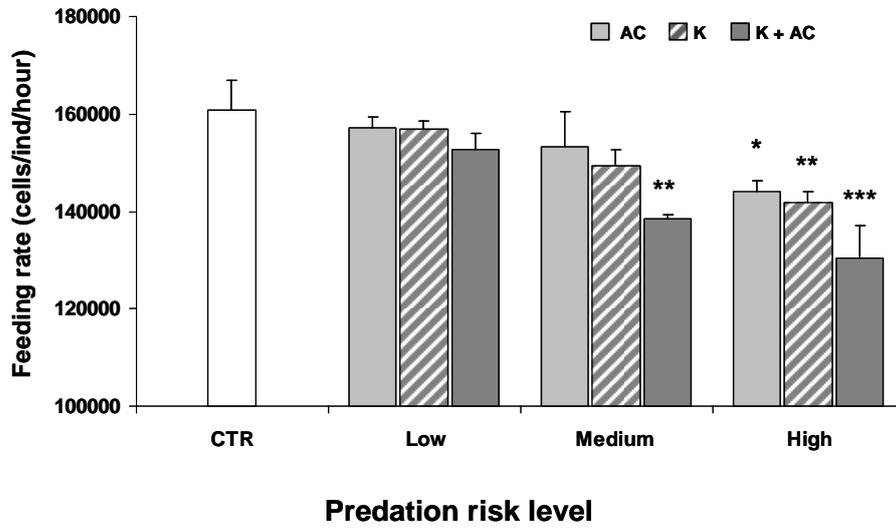


Fig 2 - *D. magna* feeding rates (mean + SE). Asterisks denote differences statistically different relatively to the control (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

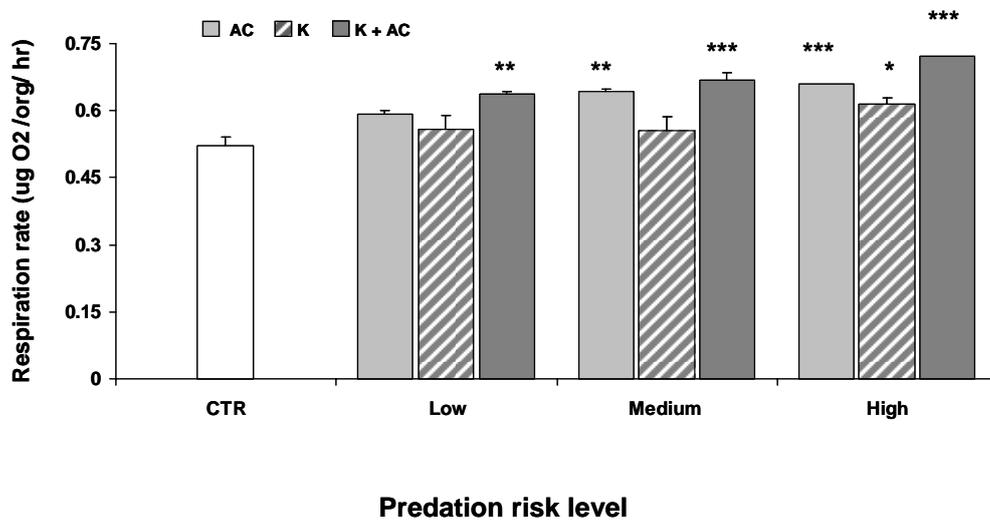


Fig 3 - *D. magna* respiration rate (mean + SE). Asterisks denote differences statistically different relatively to the control (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Table I: ANOVA results for the different parameters analysed in *D. magna* exposed to the different chemical stimuli. AC- alarm cue from macerated daphnia; K – trout kairomones; K+AC- kairomones + alarm cues

Factor	<i>df</i>	<i>F</i>	<i>p-value</i>
Offspring			
AC	3	2.44	0.07
K	3	7.81	<0.001
K+AC	3	8.98	<0.001
Size at maturity			
AC	3	0.30	0.825
K	3	2.49	0.065
K+AC	3	4.40	0.006
Neonate size			
AC	3	0.15	0.931
K	3	8.49	<0.001
K+AC	3	16.18	<0.001
AFR			
AC	3	0.49	0.693
K	3	3.00	0.035
K+AC	3	5.78	0.001
Size at day 14			
AC	3	0.14	0.934
K	3	0.93	0.429
K+AC	3	1.27	0.291
Rate of population increase (<i>r</i>)			
AC	3	0.82	0.484
K	3	6.35	0.001
K+AC	3	11.14	<0.001
Feeding			
AC	3	3.04	0.040
K	3	4.11	0.012
K+AC	3	10.94	<0.001
Respiration			
AC	3	8.50	0.001
K	3	3.19	0.046
K+AC	3	15.82	<0.001

Discussion

The results of our study indicate that *D. magna* can use information available from alarm cues from macerated conspecifics and from predator kairomones to assess different levels of predation risk and adjust the magnitude of their responses accordingly to the levels of perceived threat.

D. magna responded to chemical cues in a concentration-dependent manner, i.e., responses were stronger when exposed to higher concentration of the different cues, (kairomones, alarm cues and kairomones mixed with alarm cues). This implies that daphnids can detect cue concentrations, leading to perceived differences in levels of predation risk and consequently to differences in the magnitude of responses in different traits. This is in accordance with empirical results showing that daphnids species adjust the level of responses to the perceived level of predation risk (Reede 1995; Castro *et al.* 2007b).

D. magna showed significantly lowered feeding rates and at the same time significantly higher rates of oxygen consumption when exposed for 24 hours to all types of chemical cues tested. Feeding reductions and increased metabolic demands have already been reported for daphnids exposed to fish infochemicals (Rose *et al.* 2003; Beckerman *et al.* 2007). Similarly, incomplete and non-specific information given by alarm cues alone can be sufficient for the induction of reversible behavioural defences such as increased vigilance, alertness, decreased activity or changes in the swimming speed (Pijanowska 1997). Because alarm cues only provide information with respect to the mortality risk and no information on the predator present, feeding and metabolic responses of *D. magna* observed in the treatments with alarm cues only are probably responses to a general threat. This suggests that alarm cues from conspecifics are indeed employed as an early indicator of danger with even low concentrations of alarm cues inducing immediate and non-specific antipredator responses in *D. magna*.

The feeding and respiratory responses of *D. magna* were stronger when exposed to a mixture of kairomones from starved predators and alarm cues from macerated conspecifics compared to exposures to single cues. This additive response of *D. magna* to a combination of information on the identity of the predator (kairomones) and on predator's

diet (alarm cues), suggests that prey organisms can interpret complex chemical signals and adjust the magnitude of their response to a perceived level of predation risk.

The life history responses of *D. magna* exposed to trout kairomones and to trout kairomones combined with alarm cue from macerated conspecifics are in accordance to the general response of daphnids exposed to infochemicals from fish (Reede 1995; Pijanowska *et al.* 2006) representing adaptive responses to visually positive size-selective predators like trout. The fact that exposure to alarm cues alone did not induce a response in any of life history traits probably means that *D. magna* requires specific information in terms of predator identity before committing to a more complete and costly antipredator response. Since antagonistic life history responses have been shown in daphnids under vertebrate or invertebrate predation risk (Weber & Declerck 1997; Dzialowski *et al.* 2003), information on the predator species is likely to be critical for the onset of life history responses and to avoid maladaptive responses. Stronger responses in the life history of *D. magna* were also observed in treatments where a mixture of trout kairomones and alarm cues was used as surrogate for predation risk thus suggesting that as with the other traits, information on both a predator's identity and its current diet is used by *D. magna* to more precisely assess the level of predation risk.

Despite the extensive literature on predation risk assessment using daphnids as prey organisms, the majority of laboratory studies are performed with infochemicals from predators that were previously fed test organisms, making these "diet cues" a mixture of kairomones from predators and alarm cues from consumed conspecifics.

Only a few studies have dealt with the relative importance of kairomones (from starved predators) and alarm cues for the assessment of predation risk in *Daphnia*. While some previous investigations have revealed no effects of alarm cues on behaviour (Stirling 1995) and morphology of daphnids (Walls & Ketola 1989), short exposures to alarm cues have been shown to influence escape and aggregation behaviour of *D. magna* (Pijanowska 1997). The author demonstrated the adaptive value of rapid recognition of alarm cues, since organisms exposed to alarm cues more efficiently avoid fish predator attacks than naive organisms, suggesting that signals resulting from alarm cues and a predator cue would be important for prey organisms to assess the local scale of predation risk. Unfortunately, only high concentrations of alarm cues from crushed conspecifics were tested and not the effects of kairomones alone on the same behavioural responses

(Pijanowska 1997). Stirling (1995) has shown that *Daphnia* respond behaviourally to fish kairomones alone, but that some clones require additional cues from damaged conspecifics to respond to the cue of unfamiliar predators. The possibility of assessment of risky environments posed by unfamiliar predators can also represent an adaptive value of recognition of alarm cues (Stirling 1995).

In contrast, Slusarczyk (1999) found no responses of *D. magna* in terms of ephippial egg production with infochemicals from crushed *Daphnia* alone. Results were only observed when alarm cues were added in combination with water conditioned by fish fed daphnia or chironomids. Slusarczyk also did not find evidence for effects in offspring production of daphnids exposed to alarm cues alone whereas mean clutch size was higher when daphnia were exposed to kairomones alone (i.e. from fish fed with chironomids and no alarm cues from conspecifics) (Slusarczyk 1999).

Antagonistic life history responses were also observed for *D. magna* exposed to high concentrations of kairomones and alarm cues with effects of chemical cues from different copepod predators feeding on *Daphnia* significantly different from the effect of chemicals from crushed conspecifics (Pijanowska & Kowalczewski 1997a). The authors suggested that the mere presence of cues from macerated *Daphnia* can indeed induce 'prudent' behaviour and represent a non-specific response to predation risk (Pijanowska & Kowalczewski 1997a).

A recent study on morphological responses of daphnids to predation risk indicated that alarm cues from macerated conspecifics could alter the plastic morphology of daphnids (Laforsch *et al.* 2006). The authors used a much higher concentration of alarm cues compared to our study and they still observed a much more pronounced effect of exposure to chemical cues from predators fed with conspecifics or heterospecifics, on the same morphological responses (Laforsch *et al.* 2006). In contrast, our results indicate that low concentrations of alarm cues can induce behavioural and physiological responses but do not elicit life-history responses. We have also confirmed that for every parameter tested, prey responses to a combination of kairomones and alarm cues were more pronounced than with exposure to single cues.

Stabell *et al.* (2003) observed morphological alterations in *Daphnia* exposed to alarm cues and kairomones from fish which had been previously fed conspecifics and close related heterospecifics, but failed to observe these effects through exposing daphnids to

kairomones from fish fed with earthworms. The authors proposed latent alarm cues resulting from the digestion of conspecifics and/or activated by bacteria in fish or in water were responsible for the response to infochemicals (Stabell *et al.* 2003). Latent alarm cues could be an explanation for the effects of alarm cues combined with trout kairomones in our experiments (more bacteria from the fish water would accelerate the process and induce a stronger response), but does not seem to be consistent with the lack of life-history responses in *D. magna* exposed to alarm cues alone, nor the responses observed for *D. magna* exposed to kairomones from starved fish.

Collectively, these results and ours suggest that although daphnids can detect and respond to single chemical cues (kairomones and alarm cues), exposure to both types of infochemicals will induce stronger, more complex responses. Moreover, there is evidence that a combination of kairomones and alarm cues from crushed conspecifics induce weaker prey responses than those induced when predators consume prey. In our experiments, it was necessary to maintain the same concentration of kairomone and alarm cues across treatments, and thus we did not feed trout with *D. magna* when preparing the kairomone stock solution. We can only hypothesize that conditioned water by fish fed with conspecifics would induce even stronger responses, compared to a mixture of macerated conspecifics and kairomones. Keeping in mind that *D. magna* can recognize and respond to kairomones alone, digestive enzymes or digested prey tissues could further activate alarm cues from consumed conspecifics (Stabell *et al.* 2003). Together with kairomones from predators, these alarm cues are likely responsible for the differences in signal strength observed for responses to cues from consumed or macerated conspecifics observed for *Daphnia* and other taxa (Slusarczyk 1999; Schoeppner & Relyea 2005).

Considering that plastic antipredator responses imply costs, limits and benefits (DeWitt *et al.* 1998), prey organisms rely on the amount of information available in the environment to determine the magnitude and specificity (number and combination of traits that can be induced) of antipredator responses (Burks & Lodge 2002). As with our results, the more reversible and immediate responses, with lower costs, should be the most affected by exposure to non-specific information, i.e., to alarm cues alone. This is likely true for increases in vigilance that affected feeding and oxygen consumption rates of *D. magna* in our experiments. More costly defences such as morphological or life-history responses of

daphnids probably require more reliable cues for their induction, explaining why they are less often observed as responses to single cues.

We hypothesise that a combination of prey-specific alarm cues and predator-specific compounds can be necessary to induce a more complete set of antipredator responses in daphnids. This hypothesis is based on three main ideas that arise from investigations on predation risk assessment:

1- Specific antipredator responses have been shown when prey are exposed to different predator species (Turner *et al.* 1999; Dzialowski *et al.* 2003; Weber 2003) and to different predators' diets (Slusarczyk 1999; Stabell *et al.* 2003; Jacobsen & Stabell 2004; Schoeppner & Relyea 2005)

2- Kairomones and alarm cues alone or in combination can produce a range of unique antipredator responses, suggesting that they are chemically distinct (Stirling 1995; Pijanowska & Kowalczewski 1997a; Wudkevich *et al.* 1997; Wisenden *et al.* 1999; Wisenden & Millard 2001; Iyengar & Harvell 2002; Schoeppner & Relyea 2005; Laforsch *et al.* 2006; Turner *et al.* 2006).

3- Investigations on their chemical properties suggest that kairomones from invertebrates seem to be different from kairomones of vertebrates (Loose *et al.* 1993); that the chemical nature of fish kairomones is similar across species (vonElert & Loose 1996; Von Elert & Stibor 2006) being a mixture of substances with different fractions inducing different antipredator responses in *Daphnia* (Von Elert & Stibor 2006).

Conclusion

While behavioural and physiological responses in *D. magna* could be triggered by single cues, in this case either fish kairomones or alarm cues, life-history shifts were not induced by alarm cues alone. The responses observed were more pronounced when higher concentrations of cues were tested. Simultaneous exposure to both cues elicited stronger behavioural, physiological and life-history responses showing that the concentration and specific nature of infochemicals determine the ability of *D. magna* to correctly assess predation risk, and thus avoid unnecessary costs or maladaptive responses.

In natural systems prey organisms face a complex chemical environment. Infochemicals from different types of predators and from damaged, injured or consumed

conspecifics and heterospecifics filled with potential information induce a variety of behavioural, physiological and life-history adaptations as defences against predation.

In future, chemical characterisation of kairomones and alarm cues is essential to truly understand the finely-tuned mechanism of predation risk assessment. This will in turn contribute to a better understanding of how prey organisms make key decisions under different levels of predation pressure.

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Chapter IV

Interactions between pesticide exposure and inducible antipredator responses in the zooplankton grazer *Daphnia magna* Straus

Interactions between pesticide exposure and inducible antipredator responses in the zooplankton grazer, *Daphnia magna* Straus.³

Abstract

Prey responses to predatory chemical cues have been previously shown to be modified under pesticide exposure. Here, we study the effects of the pesticide Imidacloprid on the responses of *Daphnia magna* to a combination of predator-release kairomones from trout and alarm substances from conspecifics, simulating different levels of perceived predation risk. Predatory cues induced more rapid growth, higher fecundity and smaller size at maturity in *D. magna*, while Imidacloprid exposure resulted in reductions in growth, fecundity and delayed maturation. Moreover, there were significant interactions between stressors for most life-history parameters. Both imidacloprid and predatory cues impaired feeding and increased metabolic costs in *D. magna*, resulting in enhanced effects of imidacloprid exposure under predation risk. Our results demonstrate that pesticide exposure can increase the costs of inducible antipredator defences and impair life-history responses of daphnids under fish predation pressure. Since trait-mediated effects are well-known to play a key role in population dynamics, the combined direct and indirect effects of sublethal concentrations of pesticides could induce maladaptive responses in zooplankton populations, reducing population viability. The joint effects of simultaneous exposure to both types of stressors were assessed using different approaches: conceptual models usually used for the evaluation of contaminant mixture exposures and analysis of variance. The importance of risk assessment of chemicals under ecologically relevant scenarios such as the presence of predators is discussed.

Key words: Predator-prey interactions, imidacloprid, Brown trout, infochemicals, multiple stressors.

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Introduction

Pesticide contamination of lake ecosystems is an ongoing environmental concern, particularly in areas of intense agricultural activity, often at concentrations which can potentially affect natural communities and ecosystem functioning. Thus, lake zooplankton populations often have to cope with both natural and anthropogenic structuring forces acting simultaneously: e.g. fish predation and contaminant exposure (Hanazato 2001).

Predators not only have direct effects on prey populations through a reduction of prey density (direct consumption), as they also induce costly defensive traits that can affect population dynamics (Preisser *et al.* 2005). These predator-induced phenotypic changes can be developmental, morphological, physiological, or behavioural (Lass & Spaak 2003a; Werner & Peacor 2003). In response to the presence of fish predators daphnids can show changes in behavioural traits such as predator avoidance (Pijanowska & Kowalczewski 1997; Roozen & Lurling 2001), changes in phototactic behaviour (Ringelberg & vanGool 1995; De Meester & Cousyn 1997), changes in swimming behaviour (Weber & Van Noordwijk 2002), aggregation and alertness (Pijanowska & Kowalczewski 1997; Brewer *et al.* 1999). Fish predation can also typically induce life-history changes such as early maturation at smaller size and an increase in fecundity with more but smaller neonates (Reede 1995, 1997; De Meester & Weider 1999; Spaak *et al.* 2000; Declerck & Weber 2003; Hulsmann *et al.* 2004; Gliwicz & Maszczyk 2007). The presence of predators has also been shown to reduce energy intake (Rose *et al.* 2003; Beckerman *et al.* 2007) place higher metabolic demands on daphnids (Pijanowska 1997; Beckerman *et al.* 2007) and also induce molecular mechanisms to cope with this predatory pressure (Pijanowska & Kloc 2004; Pauwels *et al.* 2005).

Pesticides that enter aquatic systems can also affect zooplankton populations by exerting lethal and sub-lethal effects at the organism level. Effects on communities and ecosystem processes are further influenced by competition, sensitivity of species, chemical properties and timing of application of pesticides (Hanazato 1998, 2001; Fleeger *et al.* 2003). The toxicity of pesticides on zooplankton can be modified by the presence of other contaminants or by natural stressors that can exert additive, antagonistic or synergistic effects. Oxygen deficiency (Hanazato & Dodson 1995), food shortage (Takahashi & Hanazato 2007) high temperature (Song *et al.* 1997; Hanazato 1998) and predation risk

(Hanazato 1995, 1999; Barry 2000) have been shown to modify the toxicity of pesticides to daphnids. Combined effects of pollutants and predation risk may be important if the uptake or effect of the pollutant is altered by the presence of predators due to combined physiological or behavioural stress or if pollutants compromise normal anti-predator responses. The combined effects of these two stressors can vary depending on the mechanisms of action of chemicals and also food-chemical interaction (Rose *et al.* 2001).

Pollutants can mimic the effects of predator chemical cues or inhibit the induction of defences and have therefore the potential to disturb predator-prey interactions. (Dodson *et al.* 1995; Barry 1998; Hanazato 1999; Hunter & Pyle 2004) At the same time, induced responses of daphnids in terms of behaviour, physiology and life history as a consequence of the presence of predators might reduce daphnid tolerance to environmental stress and in turn affect their sensitivity to toxicants (Hanazato 2001; Kieu *et al.* 2001; Rose *et al.* 2001).

Some pesticides have been shown to induce morphological changes in daphnids similar to those induced by invertebrate predators (Hanazato & Dodson 1995; Barry 1998; Hanazato 1999). Pesticides have also been shown to cause changes in the behavioural (Dodson *et al.* 1995) and life history (Hanazato & Dodson 1992; Barry 2000) responses of daphnids to predators.

Nevertheless, laboratory studies remain focused on the effects of single factors, and generally ignore possible interactions with other stressors (Relyea & Hoverman 2006). Focused research in this area is clearly needed to improve our understanding of how multiple stressors interact with each other, and to facilitate the assessment of possible additive or more-than-additive effects. In this way, we can improve risk assessment practices, by introducing more realism, in a systematic fashion (Van Straalen 2003; Eggen *et al.* 2004; Relyea & Hoverman 2006). Moreover, the assessment of pesticide effects on predator-prey interactions and the incorporation of lethal and sub-lethal endpoints such as behaviour, physiology and life history would permit a more precise extrapolation of the effects of sublethal concentrations of pesticides to natural populations (Hanazato 2001; Fleeger *et al.* 2003; Relyea 2005; Relyea & Hoverman 2006).

Here, we examine how a pesticide, imidacloprid, interacts with behavioural, physiological and life-history responses of *Daphnia magna* to predatory infochemicals from fish. Imidacloprid is a systemic insecticide, belonging to a class of chloronicotinyl

insecticides, acting on the nicotinic acetylcholine receptors (nAChRs) which are common to many invertebrate taxa, and insects in particular (Tomizawa & Casida 2003). Prolonged activation of the nAChRs by imidacloprid causes desensitization and blocking of the receptors, leading to decreased activity, lack of muscular coordination, tremors and ultimately, death (Moffat 1993). Pesticide exposure has been shown to impair feeding in a variety of organism including daphnids and *D. magna* exposed to sublethal concentrations of imidacloprid show delayed maturation and reduced fecundity (Jemec *et al.* 2007) which contrasts with the shift towards more neonates and increased development rates induced by the presence of fish. Thus, we hypothesize that exposure to imidacloprid can affect daphnia-fish interactions by causing alterations in the direction or magnitude of induced responses to fish predation risk.

Material and Methods

Experimental animals

D. magna, clone F (*sensu* (Baird *et al.* 1991) was cultured under a 16:8 light dark photoperiod and $20^{\circ} \pm 2^{\circ}\text{C}$. The culture medium was ASTM hard water enriched with an organic extract and animals were fed the green alga *Chlorella vulgaris* Beijerinck, at a concentration of 3.0×10^5 cells/ml. Medium and food were renewed every other day keeping a density of 18-22 adults/L. Neonates from the 3rd or 4th clutch were used in all the experiments.

Brown trout, *Salmo trutta* Linnaeus, was used as our model vertebrate predator. Brown trout feeds on aquatic and terrestrial insects, small fish and crustaceans including daphnids (Penczak & Formigo 2000). *D. magna* has been shown to respond to chemical cues from different fish species including trout (Lass & Spaak 2003a, 2003b). Young brown trout were obtained from a fishfarm and transferred to laboratory where they were kept at $17 \pm 1^{\circ}\text{C}$ in 60L plastic tanks with aerated artificial pond water (Naylor *et al.* 1989). After approximately one month fish with healthy appearance were selected to be used in the preparation of fish chemical cues.

Preparation of chemical cues

It was decided to use a combination of kairomones from brown trout and also alarm cues (hereafter termed predation cues) from macerated *Daphnia* to prepare the following experimental treatments:

- No risk of predation = No predation cues added
- Low perceived risk of predation = (0.05 fish + 1 crushed daphnia) L⁻¹
- High perceived risk of predation = (0.2 fish + 4 crushed daphnia) L⁻¹

These treatments were chosen based on previous work assessing the responses of this *D. magna* clone to chemical cues from trout and alarm substances from conspecifics were assessed and where details on fish maintenance and preparation of chemical cues are also described in detail (Pestana *et al.* 2008b).

Test chemical

Confidor® 200 SL was purchased from Bayer CropScience AG (Monheim, Germany) and was used to prepare the appropriate stock solutions of imidacloprid with distilled water. Different stock solutions were used in the different experiments. The concentrations determined by HPLC-UV of stock solutions were 427 mg/L which was used in the acute tests, 444 mg/L for the feeding and oxygen experiments and 438 mg/L used throughout the chronic 21-day test. Stock solutions were stored at 4 °C protected from light. Tests solutions were prepared by adding an appropriate amount of stock solution in ASTM hard water.

Acute toxicity experiments

Acute lethality (OECD 2000) was estimated to gauge the inherent sensitivity of *D. magna* to the pesticide, and also to establish a range of sub-lethal concentrations to be used in the feeding and respiration experiments. Test solutions of Imidacloprid (Confidor®) were prepared in ASTM. There were ten replicates with five organisms each per treatment. Organisms were exposed in glass vials containing 100 ml of pesticide solutions and no food. After 48 h exposure, mortality was determined by immobilisation. The mortality assessment was also conducted in the presence of predator exudates (high perceived

predation risk treatment only), to find out if these altered the lethal sensitivity of *D. magna* to imidacloprid.

Feeding experiments

Three concentrations of imidacloprid (2.2, 4.4 and 8.8 mg/L) plus control were studied across three predation risk treatments (zero, low and high perceived risk of predation) in a total of 12 experimental treatments with five replicates each. The feeding experiments were performed in the dark with twenty five 4-day old juveniles per treatment in 150-ml glass vials containing 100 ml of solution plus *C. vulgaris* at 3×10^5 cells ml according to standard methodologies (Allen *et al.* 1995; Taylor *et al.* 1998; Barata *et al.* 2000) and described in detail elsewhere (Pestana *et al.* 2008b).

Measurement of oxygen consumption

Two concentrations of imidacloprid (2.2 and 4.4 mg/L) plus control were tested in full cross design with the 3 different levels of predation risk (zero, low and high) in a total of 9 experimental treatments. The O₂ consumption experiment was performed using standard respirometry methods with 50-ml gastight syringes (Hamilton, USA). Three syringes were used per treatment and were filled with 30 ml of the appropriate test solutions (no food added) and with five 4-day old juveniles of *D. magna*, the remaining air was expelled from each syringe, which were left in the dark in a water bath (20 °C) for 24 hrs. The oxygen consumption was given by the differences in the oxygen content of water before and after the exposure period and the respiration rate is expressed as and $\mu\text{g O}_2$ consumed per organism per hour. The methodologies used in the oxygen consumption experiments are based on previous work with *D. magna* where a more complete description of methods can be found (Pestana *et al.* 2008b).

Life table experiment

Life table experiment was initiated with first instar daphnids (<12 hours) and under the same temperature and photoperiod described for the culturing procedures. A 21 day life time experiment was performed (OECD 1998), testing three concentrations of imidacloprid (2.2, 4.4 and 8.8 mg/L) plus control in full cross design with 3 different levels of predation risk (zero, low and high) in a total of 12 experimental treatments. Ten replicates were run

for each treatment with one organism per replicate. Test solutions (50 ml per replicate) were renewed every other day and *C. vulgaris* was supplied every day in a concentration of 3.0×10^5 cells/ml. Age and size at first reproduction, number of broods, total number of offspring per female, and also the intrinsic rate of population increase (r) were the life-history parameters measured. Size of experimental organisms was also measured in the start (30 randomly selected neonates) and at the end of the experimental period (21 days) to assess effects on somatic growth. Body length measurements were performed using a stereomicroscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer.

Data analysis

EC50 values for mortality (measured as immobilization) were calculated using the probit method. For feeding, oxygen consumption and somatic growth (measured over 21 days), two-way ANOVAs were performed using predation cues and imidacloprid concentrations as fixed factors. Life historical data (intrinsic rate of natural increase (r), number of neonates produced by female, age at maturity, size at first reproduction and number of broods) was analyzed with factorial MANOVA followed by separated ANOVAs all with imidacloprid concentration and predation risk as fixed factors. Whenever a significant interaction was observed we also performed one-way ANOVAs testing for the effects of predation cues in the different imidacloprid treatments. All statistical analysis were performed with the Minitab statistical software (Minitab 2000).

All data were analysed for homogeneity of variance, normality and residuals were checked. The intrinsic rate of population increase (r) was calculated iteratively using the Euler's equation:

$$1 = \sum_{x=0}^n e^{-rx} l_x m_x,$$

where r is the rate of population increase (day^{-1}), x is the age class in days (0...n), l_x is the probability of surviving to age x , and m_x is the fecundity at age x . Replicate pseudo values for r were generated using the jackknife procedure (Meyer *et al.* 1986). Somatic growth rate was calculated according to Burns (Burns 1995) as $g = (\ln BL_t - \ln BL_0) / \Delta t$,

where BL_0 and BL_t are body length of organisms at day 0 and day 21 respectively, and time $\Delta t = 21$ days.

To address the effects in the mixtures experiments, the observed effect on feeding, respiration, offspring production and intrinsic rate of population increase (r) were compared to the expected effects of mixtures calculated from effects of single compound exposures. This procedure is usually based on already described conceptual models: concentration addition (CA) and independent action (IA) (e.g. Backhaus *et al.* 2003; Altenburger *et al.* 2004; e.g. Jonker *et al.* 2004; Jonker *et al.* 2005). Although these two conceptual models take into consideration the information available on chemicals' mode of action, it has been suggested to consider the ecotoxicological mode of action of chemicals when considering effects at the whole organism level such as physiological and life historical responses (Barata *et al.* 2007).

In this study both conceptual models were fitted to feeding and respiration data to check the suitability of the different approaches. Although the mechanism of action of predatory cues is unknown, imidacloprid and predation risk can impair food acquisition and increase the respiration rates due to effects on behaviour of daphnids. Thus, they share a common ecotoxicological mode of action (*sensu* Barata & Baird 2000).

For the IA conceptual model the fit to our data was made using equation 1:

$$Y = \mu_{\max} \prod_{i=1}^n q_i(C_i) \quad (\text{equation 1})$$

where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, $q_i(C_i)$ the probability of non-response, μ_{\max} the control response for the selected endpoint and \prod the multiplication function.

$$\sum_{i=1}^n C_i / EC_i = 1 \quad (\text{equation 2})$$

For the CA model fit, equation 2 was applied, where C_i is the dose used for stressor i in the mixture and EC_{xi} is the effect dose of stressor i that produces the same effect ($x\%$) as the whole mixture.

The procedure analysis suggested by Jonker *et al.* (2005) was followed for the analysis of feeding and oxygen consumption experiments since it permitted significance testing of

model fit for both the independent action model and the concentration addition model and also because the analysis takes into account different nonlinear dose–response characteristics of stressors (Jonker *et al.* 2005). Deviations from the reference models such as synergism/antagonism, dose-ratio and dose level were assessed by additional parameters used in the mathematical models and are tested within a nested framework, more details in Jonker *et al.* (2005). The models are fitted to the data using a maximum-likelihood method. The best fit is chosen using a Chi-square test which minimizes the objective function based on the log likelihood. The biological interpretation of these additional deviation parameters are described in Table II.

Life history responses of daphnids exposed to neurotoxic pesticides are known to be driven by a reduction in resources acquired from food whereas chemical cues that simulate predation risk elicit responses also through a direct action on the allocation of energy to growth and development (Beckerman *et al.* 2007). Since the response of daphnids to fish chemical cues is generally characterized by an increase in neonate production and a shorter development time with a consequently increase on intrinsic rate of population increase (r), imidacloprid and fish chemical cues cause contrasting effects. It was therefore decided to apply the IA conceptual model to analyse the effects of mixtures of both stressors on offspring production and intrinsic rate of population increase (r). The analysis procedure for reproductive endpoints was different because the approach described by Jonker *et al.* (2005) relies on dose response curves and it is not suitable to estimate joint effects if the different stressors in the mixture cause contrasting effects (stimulation *vs.* inhibition). Because imidacloprid and fish cues also show differences in terms of their ecotoxicological mode of action, the IA reference model was followed to calculate the predicted responses of daphnids when exposed to both stressors simultaneously. Estimated mean values were obtained directly from equation 1, using the maximum response (i.e. 0) in the case of neonate production and intrinsic rate of population increase (r). Estimated effects by the reference IA model were compared to the average values observed and their respective 95% confidence intervals to infer significant antagonism or synergism for each mixture treatment. This approach was also applied to analyse feeding and respiration data to compare results in terms of the sensitivity of the different modelling approaches used.

Results

Mortality

The imidacloprid 48-h EC₅₀ (95% CI) for *D. magna* was 96.65 mg/L (95% CI: 87.83-105.60) with no predator cues and 90.68 mg/L (95% CI: 82.04-99.30) when simultaneously exposed to high concentration of predation cues.

Feeding and oxygen consumption

D. magna showed significant reduction in feeding ($F_{3, 59} = 414.97$, $p < 0.001$) and significantly higher respiration rates ($F_{2, 26} = 141.10$, $p < 0.001$) when exposed sublethal concentrations of imidacloprid. Furthermore, *D. magna* also showed significantly lower feeding rates ($F_{2, 59} = 53.41$, $p < 0.001$) and a significant increase in the oxygen consumption ($F_{2, 26} = 54.10$, $p < 0.001$) when exposed to predatory cues (Fig.1, 2). No statistically significant effects of the interaction between imidacloprid and predation cues were detected for feeding ($F_{6, 59} = 1.38$, $p = 0.243$) or for oxygen consumption ($F_{4, 26} = 0.91$, $p = 0.477$) by two-way analysis of variance.

In terms of predicted values using additive models for feeding, our data showed an accurate fit to the IA reference model ($SS = 2446.92$; $r^2 = 0.958$; $p < 0.001$). A significant dose level dependent deviation from the IA model was also observed ($SS = 2146.09$; $r^2 = 0.963$, χ^2 test, $p = 0.02$; $a = 5.72$; $b = 1.96$), where antagonism was shown for low doses of both stressors and a synergistic pattern for high doses of both stressors. This switching from antagonism to synergism occurs at the EC₅₀ level. Using the CA approach, there was also a good fit to the reference model ($SS = 3137.09$; $r^2 = 0.947$ $p < 0.001$) and the same dose level dependent deviation was observed ($SS = 2303.50$; $r^2 = 0.961$, χ^2 test, $p < 0.001$; $a = 3.40$; $b = 2.05$) with the switch from antagonism to synergism was also near the EC₅₀ value. The pattern of dose level dependent deviations from additivity is also observed (but not statistically significant) in the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each mixture treatment (Fig. 4 A). Analysis of variance also failed to detect significant interaction between stressors, thus indicating additivity.

Data from respiration measurements adequately fitted the IA reference model ($SS = 0.8719$; $r^2 = 0.931$; $p < 0.001$) with no significant deviation. On the other hand, and using the CA approach, a good fit to the reference model was observed ($SS = 1.19855$;

$p < 0.001$) but also with a significant deviation for synergism ($SS=0.7891$; $r^2=0.937$, χ^2 test, $p < 0.001$; $a=-5.43$). The synergistic deviations from additivity is also observed in the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each mixture treatment (Fig. 4 B) being only statistically significant at low doses of both stressors.

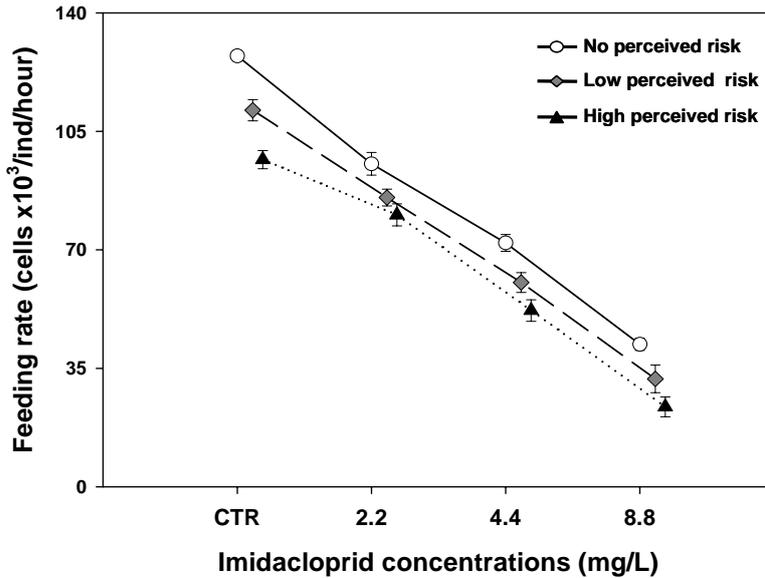


Fig 1 – Effects of Imidacloprid and perceived risk of predation on *D. magna* feeding rate (mean + SE).

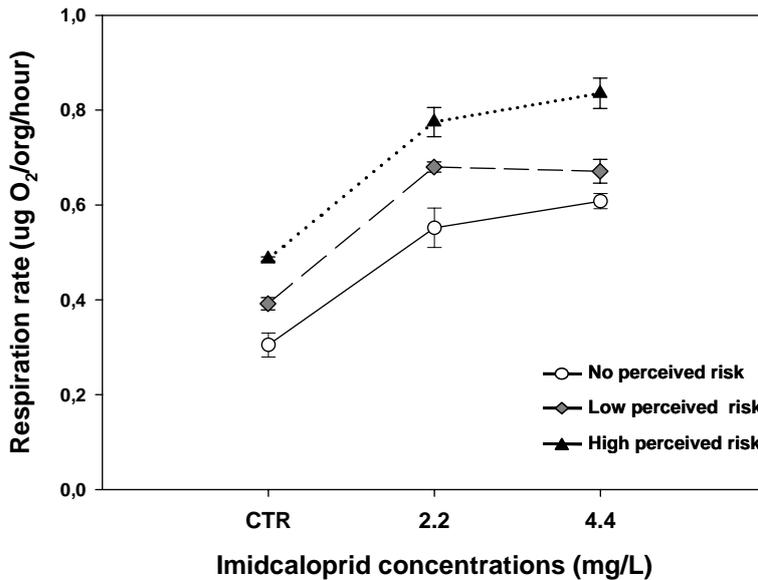


Fig 2 – Effects of Imidacloprid and perceived risk of predation on *D. magna* oxygen consumption (mean + SE).

Life history

In the 21d life table experiment, mortality did not exceed 10% except in treatments with 8.8 mg/L imidacloprid, where mortalities of 40-50% were observed (50% in the no predators' treatment and 40 % in the treatments with chemical cues). Furthermore there was an extremely low neonate production at this concentration (less than 5 neonates per treatment) which affects the jackknife procedure and the calculation of intrinsic rate of population increase. For this reason, and because we were mainly interested in sub-lethal effects, the 8.8 mg/L treatments were excluded from the statistical analysis of life history parameters.

The concentrations of imidacloprid at the end of day 2, i.e. before complete renewal of medium, were checked and the level corresponded to 71% - 65% of initial nominal concentrations. The analysis of imidacloprid concentrations on treatments with high levels of predation risk revealed no effect of chemical predatory cues on degradation of the pesticide.

D. magna exposed to chemical cues showed increased fecundity, reduction in size at maturity and in age at first reproduction, and an increase in the intrinsic rate of population increase (r) relatively to the control treatment (Fig.3 A-E, Table I).

Exposure to imidacloprid resulted in significant, contrasting effects with daphnids exposed to sublethal concentration of the pesticide showing delayed maturation, production of less neonates and thus significant reductions in intrinsic rate of natural increase, r , compared to control daphnids (Fig 3A,D,E, Table I). A significant reduction in size at first reproduction and number of broods were observed for daphnids exposed to higher concentrations of Imidacloprid (Fig 3 B, C, Table I).

There was a significant interaction between imidacloprid and predation cues on the life history parameters of *D. magna* (Fig 3A-E Table I). The imidacloprid inhibited the induced responses to predatory chemical cues in terms of age at first reproduction (one-way ANOVA; perceived predation risk effect in C0: $p < 0.001$, C1: $p = 0.056$, C2: $p = 0.501$; Figure 3D) and neonate production (one-way ANOVA; perceived risk effect in C0: $p < 0.001$, C1: $p < 0.001$, C2: $p = 0.009$, Figure 3A). Through these effects on reduction in offspring production and maturation time, imidacloprid also reduced the observed effects of predator cues on the intrinsic rate of natural increase (r) (one-way ANOVA; perceived risk effect in C0: $p < 0.001$, C1: $p = 0.006$, C2: $p = 0.227$; Figure 3E). This inhibition of

inducible responses on life history parameters was clearly more pronounced at higher concentrations of imidacloprid and also evident in size at maturity (one-way ANOVA; perceived risk effect in C0: $p < 0.001$, C1: $p = 0.042$, C2: $p = 0.661$, Figure 3B).

Thus, in the absence of imidacloprid, *D. magna* under low and high predation risk matured more rapidly and produced more neonates than daphnids not exposed to predatory cues. With simultaneous exposure to the pesticide this difference was reduced and daphnid populations exposed to imidacloprid under both low and high predation risk showed similar r -values to daphnid populations not exposed to predator cues. By comparing the experimental data with mean predicted additive values calculated using IA reference model in mixture treatments (Fig. 4 B, C), we can see that this inhibition of phenotypic responses can lead to synergistic effects in the high predation risk treatments. This is due to the contrasting effects of both stressors with imidacloprid inhibiting the increased fecundity and shorter maturation time of daphnids under high predation risk and thus greatly reducing intrinsic rate of population increase. The difference between predicted and observed data is not as clear for offspring production probably because the difference between neonate production in the different predation risk treatments although reduced, is still observed with exposure to imidacloprid.

Somatic growth

Daphnids exposed to imidacloprid were smaller than controls after 21 days ($F_{2, 88} = 2639.49$, $p < 0.001$). There was no significant effect of the chemical cues on the somatic growth of daphnids after 21 days ($F_{2, 88} = 1.61$, $p = 0.207$) nor of the interaction between imidacloprid and predation risk ($F_{4, 88} = 2.21$, $p = 0.075$) (Fig. 3F).

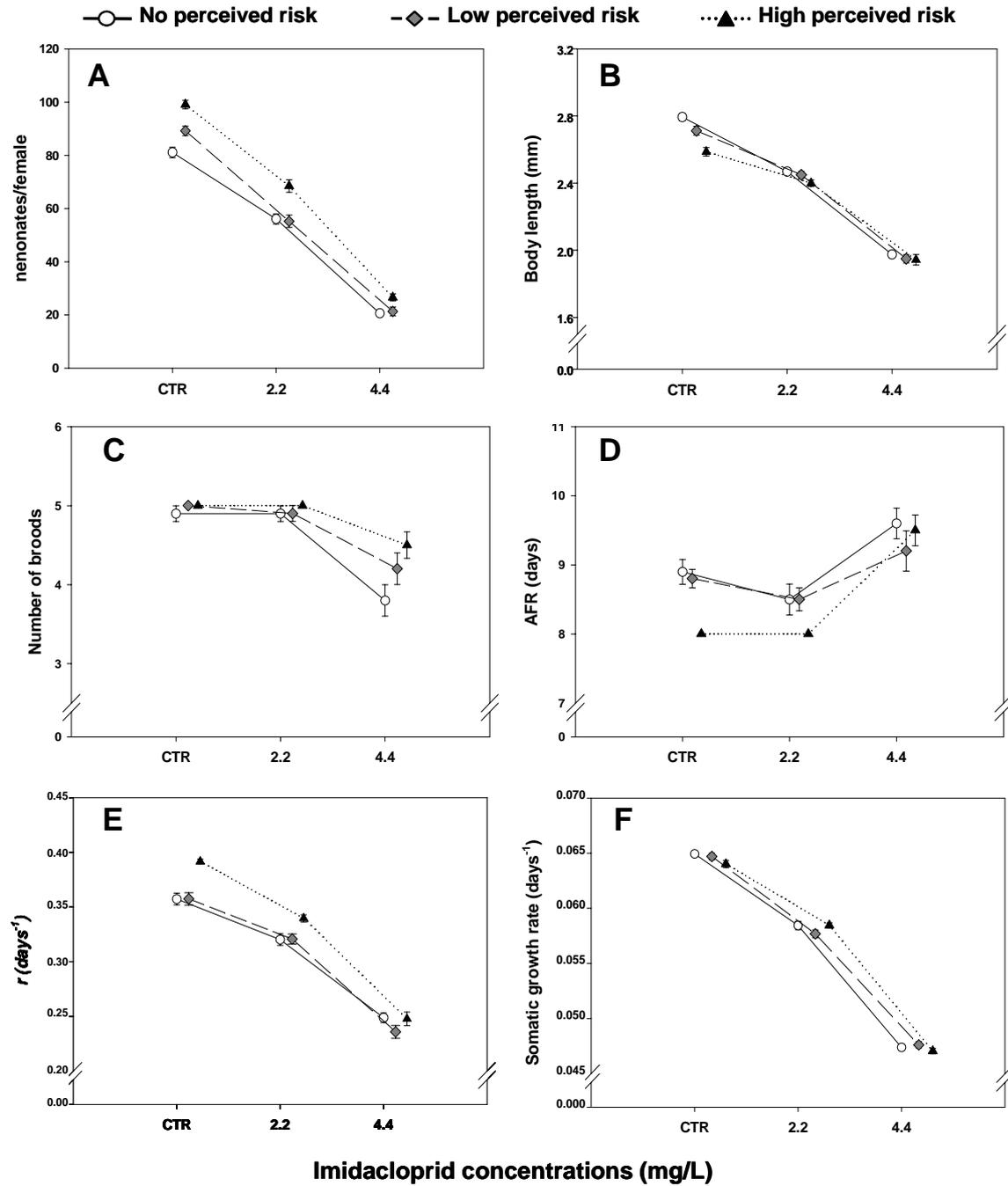


Fig 3 – Effects of Imidacloprid and different levels of perceived risk of predation on *D. magna* life history parameters and somatic growth over 21 days (mean + SE): A- Total number of neonates produced per female; B - Size at maturity; C – Number of broods; D - Age at first reproduction; E – Intrinsic rate of natural increase, r , and F - Somatic growth rate

Table I – Summary of results from MANOVA(A) and Univariate ANOVAs (B) testing the effects of Imidacloprid and predation cues on *D. magna* life history.

A			
MANOVA		Life history	
factor	Df	F	P
[IMI]	10, 154	107.67	<0.001
Predation risk	10, 154	14.51	<0.001
[IMI] x Predation risk	20, 256	4.51	<0.001

B											
ANOVA		Rate of pop. Increase (r)		Offspring		Age at first reproduction		Size at maturity		N° of broods	
factor	Df	F	P	F	P	F	P	F	P	F	P
[IMI]	2	501.37	<0.001	1020.35	<0.001	29.15	<0.001	799.38	<0.001	40.14	<0.001
Predation risk	2	16.76	<0.001	36.28	<0.001	5.63	0.005	14.86	<0.001	4.43	0.015
[IMI] x Predation risk	4	3.46	0.012	3.79	0.007	2.54	0.046	4.16	0.004	2.03	0.098

Table II - Interpretation of additional parameters substituted into the concentration addition (CA) and independent action (IA) reference models that define the functional form of the deviation pattern adapted from (Jonker *et al.* 2005)

Deviation Pattern	Parameter <i>a</i> (CA and IA)	Parameter <i>b</i> (CA)	Parameter <i>b</i> (IA)
synergism/antagonism (S/A)	a>0: antagonism a<0: synergism		
Dose-ratio dependent (DR)	a>0: antagonism except for those mixture ratios where negative <i>b</i> value indicate synergism	<i>b_i</i>>0: antagonism where the effect of the mixture is caused mainly by toxicant <i>i</i>	
	a<0: synergism except for those mixture ratios where positive <i>b</i> value indicate antagonism	<i>b_i</i><0: synergism where the effect of the mixture is caused mainly by toxicant <i>i</i>	
Dose-level dependent (DL)	a>0: antagonism low dose level and synergism high dose level	<i>b_{DL}</i>>1: change at lower EC50 level	<i>b_{DL}</i>>2: change at lower EC50 level
		<i>b_{DL}</i>=1: change at EC50 level	<i>b_{DL}</i>=2: change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	0<<i>b_{DL}</i><1: change at higher EC50 level	1<<i>b_{DL}</i><2: change at higher EC50 level
		<i>b_{DL}</i><1: No change but the magnitude of S/A is DL dependent	<i>b_{DL}</i><1: No change but the magnitude of S/A is effect level dependent

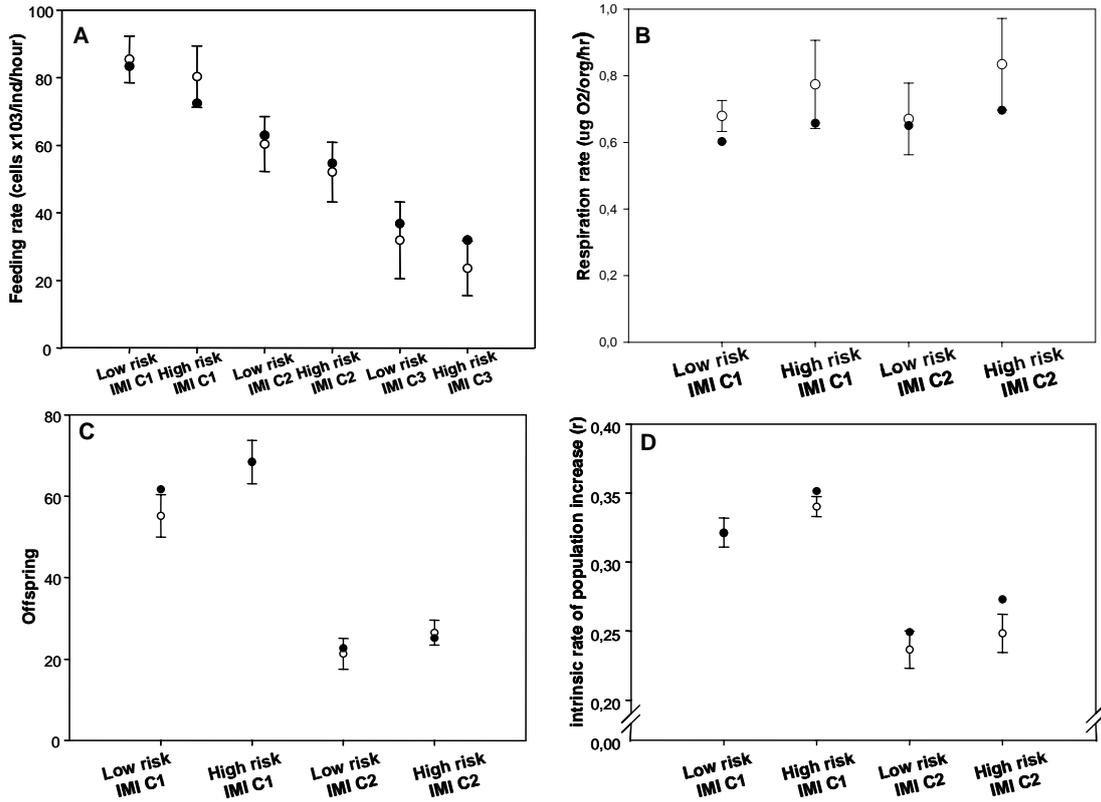


Fig 4 – Effects of combined exposures to Imidacloprid different levels of perceived risk of predation on *D. magna* feeding (A), respiration rate (B), offspring (C) and intrinsic rate of population increase, r , (D). Empty symbols denote observed responses (mean and 95% confidence intervals) and filled symbols represent effects predicted by independent action reference model.

Discussion

Imidacloprid is a relatively new pest control substance, which despite the favourable characteristics of low persistence and low toxicity towards vertebrates (Tomizawa & Casida 2005) is generating increasing concern regarding its potential impacts on natural ecosystems (Matsuda *et al.* 2001; Jemec *et al.* 2007). *Daphnia magna* has been shown to be less sensitive than other freshwater crustaceans and insects, being as tolerant as fish to imidacloprid (CCME 2007). Here, EC_{50} values of daphnids exposed to imidacloprid and predatory chemical cues were not significantly different compared to daphnids exposed to imidacloprid alone revealing that for short term exposures predation risk does not appear to increase the sensitivity of *D. magna* to imidacloprid exposure.

Short term exposure (24 hours) to sub-lethal concentrations of imidacloprid significantly reduced feeding and increased metabolic costs (i.e. increased oxygen consumption) in *D. magna*. Low doses of imidacloprid have been previously shown to induce several behavioural responses in aquatic insects such as reduced activity, uncontrolled muscular contractions, paralysis, all of which limit foraging activity of aquatic organisms and consequently impair feeding and growth (Alexander *et al.* 2007; Pestana *et al.* 2008a; Stoughton *et al.* 2008). Altered activity of daphnids treated with imidacloprid may affect feeding due to abnormal swimming behaviour, while also increasing metabolic costs due to extra activity and/or enhanced detoxification processes.

Exposure to increasing concentrations of predatory chemical cues also led to reduced feeding and increased oxygen consumption. This is in concordance with studies of behavioural and physiological responses of daphnids to fish infochemicals in which changes in swimming behaviour (Weber & Van Noordwijk 2002), avoidance (Pijanowska & Kowalczewski 1997; Roozen & Lurling 2001) and alertness (Pijanowska & Kowalczewski 1997; Brewer *et al.* 1999) while reducing the probability of encounters with fish predators (Lass & Spaak 2003a) can also reduce feeding (Rose *et al.* 2003; Beckerman *et al.* 2007) and place higher metabolic demands on daphnids reflecting the costs of vigilance (Beckerman *et al.* 2007). Furthermore, previous experiments with this clone have shown that *D. magna* exposed to a combination of kairomones and alarm substances experience stronger reductions of feeding rates and stronger effects in oxygen consumption than daphnids exposed to kairomones or alarm substances alone (Pestana *et al.* 2008b).

Combined exposure to imidacloprid and chemical cues revealed effects of both stressors on feeding and oxygen consumption of *D. magna* and that pesticide contamination can increase the costs of behavioural antipredator defences by further reducing energy intake and increasing energy expenditure of daphnids under fish predation pressure. Assessment of joint effects of these stressors on feeding and respiration showed significant deviations from additivity reference models contrasting with the results of analysis of variance which showed no significant interaction between the two stressors. The fact that models based on dose response curves detected deviation patterns from additivity that are not limited to just synergism or antagonism (such as dose level dependent deviation for feeding) is a clear sign that it is important to assess responses to more concentrations of each stressor and of the mixture to better calibrate

the models and to assess effects of a wider range of imidacloprid concentrations to daphnids under different levels of perceived predation risk.

Both reference models, IA and CA performed adequately in terms of prediction of feeding and respiratory responses under a combination of Imidacloprid and chemical cues simulating predation risk. Further studies are necessary but our results suggest that these approaches can be applied with respect to ecotoxicological modes of action. The assessment of effects of mixtures of different anthropogenic substances integrating different natural stressors such as predation can greatly improve the ecological relevance of pesticide risk assessments.

Chronic exposure to imidacloprid resulted in significant reductions in growth and fecundity with delayed maturation in *D. magna*. Consequently there was a significant reduction in r , the intrinsic rate of natural increase. The results of the chronic exposure to imidacloprid, including the mortality and reduced production of neonates observed in the 8.8 mg/L imidacloprid treatments are consistent with other studies (Jemec *et al.* 2007). However, the concentrations of imidacloprid that can be found in natural waters vary between 0.2 to 12 $\mu\text{g/L}$ (CCME 2007; Jemec *et al.* 2007) and thus are not likely to harm *D. magna* populations.

D. magna responses to trout kairomones and alarm cues from conspecifics were in agreement with many studies of phenotypic plasticity of daphnids in response to fish predation (Lass & Spaak 2003a). The reduction in age and size at maturity and increased fecundity observed here show that daphnids under the perceived risk of predation increase fitness through adaptive mechanisms that allow them to increase the probability of reproduction before being eaten by visually hunting, positive-size selective predators like fish and compensate for the density (i.e. mortality) effects of predation (Reede 1995, 1997; De Meester & Weider 1999; Spaak *et al.* 2000; Declerck & Weber 2003; Hulsmann *et al.* 2004; Gliwicz & Maszczyk 2007). Again, the pattern of *D. magna* life-history responses were also in concordance with previous experiments using the same predatory cues were a combination of kairomones from trout and alarm cues from conspecifics are necessary to elicit stronger life history responses of *D. magna* (Pestana *et al.* 2008b).

Simultaneous exposure to both stressors showed that the effects of predatory cues were stronger in daphnids under no pesticide exposure. This is clear in several life-history parameters such as production of neonates, age at first reproduction, size at maturity and consequently on intrinsic rate of natural increase, which show that

daphnids exposed to sub-lethal concentrations of imidacloprid lose the ability to recognize or to respond to the predatory cues. Thus, these two stressors caused contrasting effects in *D. magna* revealing that imidacloprid can inhibit the life-history responses induced by the risk of fish predation in *D. magna*.

The fact that the model approach suggested by Jonker (2005) is based on dose response curves was a limitation on our study due to the “stimulation” effects of predation risk in terms of daphnids reproductive output. It was therefore not considered for assessment of joint effects of these two stressors on the reproductive endpoints.

Results demonstrated also that imidacloprid and predatory cues elicit contrasting responses in terms of reproductive parameters (but not in terms of feeding or respiration). This is concordance with other studies where reproductive responses to predation are considered a physiological, inducible defence *per se* and not only a reflection of energy intake and expenditure (Beckerman *et al.* 2007). Bearing in mind the concept of ecotoxicological mode of action, the combined effects of neurotoxic pesticides and fish chemical cues on reproductive endpoints are probably correctly assessed by using the independent action reference models.

Significant interactions detected by analysis of variance showed that imidacloprid and fish predatory cues can have more than additive effects for many life-history parameters and the comparison between r mean values calculated based on IA approach with the experimental data clearly confirms this synergism for high predation risk treatments. Therefore we can conclude that daphnia populations are in fact more affected by pesticides such as imidacloprid when in the presence of predators. Furthermore it is also clear that an inhibition of phenotypic responses to predation caused by pesticide exposure will have serious consequences in terms of predation related mortality whenever predators show higher tolerance to the pesticide.

There is some evidence that pesticides acting on the nervous system of organisms can interfere with predator-prey interactions with additive or even synergistic effects. Chlorpyrifos has been shown to be more toxic to *Ceriodaphnia cf. dubia* under predation risk (Rose *et al.* 2001), with energy burdens imposed to organisms under predation risk due to reductions in feeding rates being suggested as responsible for the increased sensitivity (Rose *et al.* 2001; Rose *et al.* 2003). Carbaryl and invertebrate kairomones can act synergistically inducing reduced growth rate, mature size and clutch size in *Daphnia pulex* (Hanazato & Dodson 1995). The authors concluded that the presence of one stressor reduces tolerance to the other. There are also some examples of

inhibition of antipredator responses with pesticide exposure in zooplankton. Carbaryl inhibited the anti predatory morphology of *Bosmina fatalis* induced by invertebrate kairomones (Sakamoto et al. 2006) and low concentrations of endosulfan have been shown to inhibit the life-history antipredator responses of *Daphnia pulex* (Barry 2000). The interaction between pesticides and predation risk has also been observed in insects. Damselflies have also been shown to be more affected by pesticides such as endosulfan under predation risk (Campero et al. 2007). In the same way imidacloprid has been shown to act additively with predatory cues in several sub-lethal responses increasing the costs of antipredator responses and inhibiting antipredator behaviour of chironomids and caddisflies (Pestana et al. 2008a). At the same time behavioural responses of insects (Schulz & Dabrowski 2001) and daphnids (Dodson et al. 1995) to pesticide exposure have been shown to enhance mortality from fish predation. In our chronic experiment with *D. magna* we have also observed a spinning behaviour of organisms in the treatments with the highest concentrations of imidacloprid which can also further increase the risk of mortality from predation (Dodson et al. 1995).

The mechanism responsible for the interaction of pesticides and predation risk is not known. However, the neurotoxic pesticides acting by inhibition of acetylcholinesterase (e.g. chlorpyrifos, carbaryl), inhibition of neurotransmitter such as γ -aminobutyric acid, GABA, which down-regulates cholinergic nerve transmission (e.g. endosulfan) and on acetylcholine receptors (e.g. imidacloprid) although with slight different mechanisms can be considering similar acting since their neurotoxic effects are translated into an over-stimulation of cholinergic nerves which leads to uncontrolled nervous stimulation (Barry 1998; Walker et al. 2001). These pesticides can induce the stimulation of cholinergic nerves that also innervate kairomone receptors or nerves that innervate endocrine glands responsible for releasing hormones that are responsible for the induced responses to predation risk (Barry 1998) Thus, exposure to pesticides could disrupt the normal physiological and/or neuro-endocrine pathway of antipredator responses (Hanazato 1999; Barry 2002). We can hypothesize that this might also be the case for daphnids exposed to imidacloprid and predatory chemical cues since sub-lethal concentrations of imidacloprid, in the range of concentrations tested here, have been shown to reduce acetylcholinesterase (AChE) activity in *D. magna* (Jemec et al. 2007), and recent studies show that perceived predation risk can also reduce the activity of AChE in damselflies (Campero et al. 2007). Further research is necessary to explore the possibility of combined effects of predatory chemical cues and AChE- inhibiting

pesticides on AChE activity which can be responsible for additive or synergistic effects of pesticides on organisms under predation pressure. Nevertheless, imidacloprid concentrations tested here showed generalised toxicity with reductions of feeding and increased oxygen consumption, indicating that effects of pesticide on *D. magna* life-history responses to the risk of predation can be explained based on the energetic budget of organisms. The effects of pesticides can be stronger under predation risk because daphnids under fish predation risk allocate a greater proportion of their energy to reproduction (Stibor & Navarra 2000) even if they also reduce feeding rates and experience higher metabolic costs (Beckerman *et al.* 2007). Thus, daphnids facing the risk of predation would have less available energy for detoxification and repair processes. Due to these energy constraints, pesticide exposure (which also reduces energy intake and elevates metabolic costs) appears to increase the costs of phenotypic plasticity, reducing or inhibiting its expression (Hanazato & Dodson 1995; Hanazato 1999; Barry 2000; Sakamoto *et al.* 2006).

At the same time and because *D. magna* exhibits physiologically mediated life-history responses to size-selective predators like fish (Beckerman *et al.* 2007), these two mechanisms (i.e. effects on energy gain and neurophysiological interaction) might co-occur and jointly shape the effects of pesticides on responses of *D. magna* induced by the presence of fish. The strength of each mechanism may be dependent of pesticide concentration (and duration of exposure), with higher concentrations of pesticide causing important energy constraints and consequently overriding possible neurophysiological mechanisms of inducible defences.

Conclusion

Exposure to anthropogenic stressors, such as pesticides, can affect the ability of *Daphnia* populations to adapt to natural variations in their environment (Hanazato 2001). Our results show that imidacloprid and predation risk can reduce energy gain and that exposure to imidacloprid can enhance the risk of predation since it inhibits the phenotypic plasticity that *D. magna* exhibits under fish predation pressure. Daphnids exposed to imidacloprid reproduced later and at a smaller size than controls producing fewer neonates even in the presence of chemical cues from predators. Sublethal concentrations of pesticides can thus have significant detrimental consequences not only because of their toxic effects but also through effects on predator-prey interactions. If

we consider that behavioural, physiological, morphological and life-history plasticity can stabilize populations and community structure (Verschoor *et al.* 2004; Miner *et al.* 2005), then inductions of maladaptive responses in prey populations due to pesticide exposure can increase their risk of local extinction, thus reducing population viability.

We have demonstrated that a common approach, based on physiological mechanisms can be used to study the effects of different stressors such as predation and pesticides, alone and in combination, using standard ecotoxicological methodologies. In this way, we can further enhance the ecological relevance of ecological risk assessments through the concept of ecotoxicological modes of action of stressors (Barata & Baird 2000; Barata *et al.* 2007). More studies investigating combined effects of anthropogenic and natural stressors such as predation will be needed to gain an improved understanding of the indirect effects of toxic substances under natural conditions (Relyea & Hoverman 2006), thus permitting improved prediction of the effects of contamination on community structure and ecosystem function.

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Chapter V

Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk

Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk⁴.

Abstract

The influence of interactions between pesticide exposure and predation risk on the lethal and sublethal responses of two aquatic insects was investigated using the pesticide imidacloprid, and a combination of predator-release kairomones from trout and alarm substances from conspecifics. Laboratory experiments examined feeding and respiration rates of the caddisfly *S. vittatum* as well as the growth, emergence and respiration rates of the midge *C. riparius*, exposed to sublethal concentrations of imidacloprid. The results show significant effects of environmentally-relevant concentrations of imidacloprid on all endpoints studied. The joint effects of simultaneous exposure to both types of stressors were assessed using two different approaches: traditional analysis of variance and conceptual models normally used for the evaluation of contaminant mixture exposure. Additive effects of pesticide exposure and perceived risk of predation were observed for most endpoints tested. Exposure to imidacloprid also compromised antipredator behavioural responses of both insect species, with potential negative consequences in terms of mortality from predation in the field. *C. riparius* and *S. vittatum* showed slight differences in sensitivity to imidacloprid, and also to the levels of predation cues tested. These contrasting antipredator responses reflect differential adaptation to predation in terms of armouring and mobility between the two species.

Keywords: Insecticide, predator-prey interactions, multiple stressors, *Sericostoma vittatum*, *Chironomus riparius*, *Salmo trutta*.

⁴J.L.T. Pestana, S. Loureiro, D. J. Baird and A.M.V.M. Soares, *submitted to the journal Ecological Applications*.

Introduction

Macroinvertebrates that live in streams and rivers adjacent to agricultural areas are subjected to episodic and continuous inputs of pesticides which contribute to the contamination of inland waters and subsequent loss of biodiversity (Tilman *et al.* 2001). Under natural conditions, pesticides act in concert with other abiotic and biotic stressors to exert negative effects on aquatic ecosystems which are determined by stressor interactions. Nevertheless, stress ecology studies tend to focus on the effects of single factors, and often ignore the reality of stressor interactions (Relyea & Hoverman 2006). We need a better understanding of how multiple stressors interact with each other, to facilitate the assessment of possible additive or more-than-additive effects, and thus better inform risk assessment practices (Van Straalen 2003; Eggen *et al.* 2004; Relyea & Hoverman 2006). The effects of pesticides are typically assessed with classical laboratory ecotoxicological studies which are designed to generate information to be used to determine water quality criteria and safe concentrations of pollutants (Walker *et al.* 2001). The goal of increasing the ecological relevance in toxicity studies is being achieved with respect to several abiotic factors such as temperature, pH, water chemistry (Heugens *et al.* 2001), but ecotoxicologists tend to exclude from their investigations biotic stressors such as competition or predation. This will limit the extrapolation of laboratory results to effects on populations in the field because the effects of pesticides may be underestimated if possible interactions with natural stressors are not taken into consideration (Hanazato 2001; Relyea 2005a).

Predation, an important and pervasive form of biotic stress in natural ecosystems, is a major driver of population and community dynamics. In addition to the direct lethal effects arising from prey consumption, predators, simply by their presence within a system, can affect prey abundance, morphology, physiology, life history and behaviour (Lima & Dill 1990; Kats & Dill 1998; Lass & Spaak 2003a).

Prey organisms exhibit a variety of morphological, physiological, behavioural and life-history adaptations as defences against predation (Kats & Dill 1998). These anti-predator responses generally have fitness costs associated and therefore are only deployed when prey organisms perceive the risk of predation, and thus only when a clear benefit is gained from the expression of the defensive trait (DeWitt *et al.* 1998).

Recent studies have illustrated the proximate mechanisms involved in predator-prey interactions, focusing on mechanisms of assessing risk (Kats & Dill 1998). In aquatic

environments, chemical cues are important stimuli that mediate antipredator responses (Dicke & Grostal 2001), in combination with visual, mechanical and auditory cues. Infochemicals such as predator-release kairomones or alarm substances from disturbed or damaged conspecifics allow prey organisms to assess the level of risk and to deploy effective antipredator responses. Ecological theory predicts that increased predation risk which is associated with predator density or proximity will reduce prey activity, in order to reduce the probability of consumption by visual predators (Anholt & Werner 1998). This avoidance behaviour can lead to a decrease in foraging activity and feeding and thus, prey organisms' decisions reflect a trade-off between energy gain with consequences to growth and/or development rates, and mortality risk (Abrams & Rowe 1996; McPeck & Peckarsky 1998).

The combined effects of contaminants and predation stress have received little attention from ecologists and ecotoxicologists alike. Nevertheless, combined effects of pollutants and predators may be important if the uptake or effect of the pollutant is altered by the presence of predators, or if pollutants compromise normal anti-predator responses. Pesticide contamination has been shown to disturb predator-prey interactions (Preston *et al.* 1999; Lefcort *et al.* 2000; Schulz & Dabrowski 2001) and the detrimental effects of pesticides can even be influenced or modified solely by the perception of risk from predation, due to combined physiological or behavioural stresses (Rose *et al.* 2001; Relyea 2003b; Campero *et al.* 2007).

The purpose of this study was to examine the responses of the midge *Chironomus riparius* Meigen and the caddisfly *Sericostoma vittatum* Rambur, to imidacloprid, under different levels of predation risk simulated using a combination of chemical cues from brown trout and alarm substances from conspecifics.

Chironomids are an ecologically important group within freshwater ecosystems which often dominate the benthic communities of lotic and lentic environments in terms of numbers and biomass (Pery *et al.* 2002; Stief & de Beer 2002). Chironomids have been used as model organisms for sediment toxicity studies (Chappie & Burton 1997; Hirthe *et al.* 2001; OECD 2001; Soares *et al.* 2005; Faria *et al.* 2006; Stoughton *et al.* 2008). As a major component of fish diets, they have been also used to investigate predator impacts on invertebrate communities and changes in activity (Holker & Stief 2005), growth and development (Ball & Baker 1996; Noonburg & Nisbet 2005) have been shown for chironomids in response to nonlethal cues indicating the presence of predators.

The caddisfly *Sericostoma vittatum* Rambur, (Trichoptera: Sericostomatidae) is an endemic species of the Iberian Peninsula, playing a key role in the fragmentation of allochthonous organic matter of streams in central Portugal (Feio & Graça 2000; Gonzalez & Graça 2003). Caddisflies have been previously used in ecotoxicological studies (Schulz & Liess 2000), and responses induced by chemical cues from predators have also been demonstrated (Kuhara *et al.* 2001; Wissinger *et al.* 2006).

In this study brown trout, *Salmo trutta* (Linnaeus, 1758) was chosen as a model vertebrate predator. Brown trout are native to Europe, and prefer cold, well-oxygenated upland waters. They are mainly diurnal, feeding on aquatic and terrestrial insects, crustaceans and small fish. Chironomids and caddisflies are two of the most important items in the diet of brown trout (Penczak & Formigo 2000) and several studies have previously demonstrated antipredator responses induced by chemical cues from trout in several different insect species (Huryn & Chivers 1999; Dahl & Peckarsky 2002; Peckarsky *et al.* 2002).

Imidacloprid was chosen as a model pesticide because it is a relatively new pest control substance, which is generating increasing concern on its impacts on natural ecosystems (Matsuda *et al.* 2001; Jemec *et al.* 2007). Imidacloprid is a systemic insecticide, belonging to a class of chloronicotinyl insecticides, acting on the nicotinic acetylcholine receptors (nAChRs) which are common to many invertebrate taxa, and insects in particular (Tomizawa & Casida 2003). Due to its relatively high solubility (~510 mg/L) and mobility in soils has the potential to enter streams by dissolved runoff and leach to ground water (Gupta *et al.* 2002). Imidacloprid has been found in inland waters at concentrations ranging from 0.2 to 12 µg/L (CCME 2007; Jemec *et al.* 2007). Our main objective was to investigate possible interactions between these two stressors (imidacloprid and perceived predation risk), employing standard tests currently used in pesticide hazard assessments in order to improve our knowledge of the potential effects of pesticide exposure on macroinvertebrate populations under field conditions, where predators are generally present.

Material and Methods

Animals

C. riparius larvae were obtained from laboratory cultures at the University of Aveiro, Portugal which had been maintained for more than five years in standard

conditions, at 20°C, in a light-dark cycle of 16-8 h, in hard water ASTM and fed twice a week with macerated fish food, Tetramin®. *S. vittatum* larvae were collected from Ribeira S. João, Serra da Lousã, Central Portugal (40°06'N, 8°14'W), using a hand net. Organisms were acclimated for two weeks to laboratory conditions: 20°C, light-dark cycle of 14:10h, in aerated Artificial Pond Water (APW) (Naylor et al. 1989) and were fed *ad libitum* with unconditioned alder (*Alnus glutinosa*) leaves.

Young brown trout (*Salmo trutta* L.) were obtained from a fish farm and transferred to laboratory where they were kept at 17 ±1°C in 60L plastic tanks with aerated APW and fed every day with commercial fish food. After one month we selected healthy fish to be used in the preparation of the fish chemical cues.

Preparation of “predation cues”

Ten young brown trout (12-15 cm) were held in 10 L aerated APW for 24 hrs. After that period, the water, containing trout exudates, was filtered (0.45 µm Whatman acetate cellulose filter), frozen at -20°C and thawed as necessary. Fish were not fed for 3 days before this 24 hour period to reduce the level of ammonia and faeces in the exudates water. For the production of alarm substances we macerated 5 *S. vittatum* larvae (6-8 mg dry weight) or 50 *C. riparius* 6 day old larvae in 100 ml of APW. These solutions were filtered (0.45 µm Whatman acetate cellulose filter) and frozen at -20°C.

The stock solutions of chemical cues were used to prepare the treatments described below as “Low predation risk” and “High predation risk”. The concentrations of fish used in treatments were comparable to low and high densities of Brown trout found in a long term study in the British Lake District (Elliot, 1994). Likewise, the concentrations of alarm substances, although somewhat arbitrary, were chosen based on the number of prey (chironomids and caddisflies) actively consumed by a single trout in 15 minutes in our tanks in the laboratory:

for *S. vittatum* :

- No risk = APW with no cues added
- Low predation risk = 0.02 fish L⁻¹ + 0.08 macerated caddis larvae L⁻¹
- High predation risk = 0.1 fish L⁻¹ + 0.4 macerated caddis larvae L⁻¹

for *C. riparius*:

- No risk = APW with no cues added
- Low predation risk = 0.02 fish L⁻¹ + 0.4 macerated chironomid larvae L⁻¹
- High predation risk = 0.1 fish L⁻¹ + 2 macerated chironomid larvae L⁻¹

Test chemical

Confidor® 200 SL was purchased from Bayer CropScience AG (Monheim, Germany) and was used to prepare the appropriate stock solutions of imidacloprid with distilled water. The concentrations determined by HPLC-UV of the stock solutions were 1666 µg L⁻¹ for *C. riparius* tests and 7840 µg L⁻¹ for the experiments with *S. vittatum*. Stock solutions were stored at 4 °C protected from light. Tests solutions were prepared by adding an appropriate amount of stock solution in APW. The concentrations presented in graphs are nominal concentrations corrected by the measured concentrations of these stock solutions.

Acute toxicity experiments

Acute lethality was estimated to gauge the inherent sensitivity of each species to the pesticide, and also to establish a range of sub-lethal concentrations to be used in the feeding and respiration experiments. Test solutions of Imidacloprid (Confidor®) were prepared in APW. There were ten replicates with one organism each per treatment in of *S.vittatum* acute tests and 5 replicates with 25 larvae (six days old) for *C. riparius*. Organisms were exposed in glass vials containing 150 ml of pesticide solutions and no food. After 96 h exposure, mortality was determined by mechanical stimulation, with animals that failed to show any response being considered as dead. The mortality assessments for both species were also conducted in the presence of predator exudates (high predation risk only), to find out if the predator's chemical cues altered the lethal sensitivity of *S. vittatum* and *C. riparius* to Imidacloprid.

***S. vittatum* feeding experiments**

An experimental design for the feeding experiments was adapted from studies with *Gammarus pulex* (Naylor et al. 1989). We used a full factorial design where pesticide exposure was tested in combination different levels of predation risk. Thus, for every predation risk level (no risk, low and high) we tested three concentration of pesticide: 1.9; 3.9; and 7.8 µg/L of Imidacloprid plus the control treatment.

We used ten replicates with one animal per replicate in all experiments, which were allocated to individual glass vessels, containing a 1cm layer of inorganic fine sediment (<1 mm), 150 ml of experimental medium and food in the form of 5 alder leaf discs. Alder leaf discs used in feeding experiments were autoclaved and then dried at 60°C for

4 days and weighed. They were soaked in APW 96h prior to use in the feeding trials. After 6 days animals and the remaining food were removed, dried at 60°C for 4 days and reweighed. Feeding rate was calculated as the difference between the initial and final leaf disc dry mass (mg) and divided by the dry mass of organisms (mg) and elapsed time (days). Five control cages per treatment with leaf discs but no animals were used to correct for weight change due to factors other than feeding. Every 48h, 100mL of the solution (Imidacloprid and chemical cues) was renewed. During the experiment all replicates were examined daily. All tests were conducted at 20 ± 1 °C with a photoperiod of 14 h light: 10 h dark.

***C. riparius* growth experiment**

We performed our experiment as a sediment-water chironomid toxicity test according to the OECD guideline (OECD 2001). The experiment was performed with 200 ml glass vials (10 replicates per treatment) with five larvae (three days old) per replicate. Each replicate contained a 1cm layer of inorganic fine sediment (<1mm), and 150 ml of experimental medium. Food (Tetramin®) was provided every other day at a ration of 0.5 mg/larvae/day). Survival, head capsule width, total length and time to emergence were the response parameters measured. Every 48h, 100mL of the test solution (imidacloprid and chemical cues) was renewed. 0.4, 1.2 and 3.7 µg/L of Imidacloprid were used in combination with different predator treatments: No risk (i.e. no chemical cues added), low and high predation risk. 5 replicates (= 25 organisms) were used to measure growth and 5 replicates to measure emergence. During the experiment all replicates were examined daily. All tests were conducted at 20 ± 1 °C with a photoperiod of 14 h light: 10 h dark. Growth was estimated by measuring the total length and head capsule width of each larva at day 10 with a stereo microscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer.

Measurement of oxygen consumption

Oxygen consumption was determined by simple static respirometry, using larvae held for 24 hrs in 50-ml gastight syringes (Hamilton, USA). To measure oxygen consumption in *C. riparius*, we employed three syringes per treatment, each holding five 6-day old larvae; for *S. vittatum* we used five replicates each holding three organisms of approximately similar size. Syringes were filled with the appropriate test

solutions and organisms, the air remaining was expelled from each syringe and they were left in the dark in a water bath (20°C). After 2 hours, initial oxygen concentrations were measured with an oxygen meter ((model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow). After 22 hrs, the final oxygen concentrations were measured in the same way. In the case of *S. vittatum*, larvae were dried (with no case) for 4 days at 60°C and weighed. Oxygen consumption was determined by the differences in the oxygen content of water before ($T_0= 2h$) and after ($T_{final}= 24h$) the exposure period, and the respiration rate was expressed as μg oxygen consumed per mg of organism per hour per ml. For *C. riparius*, respirometry we followed the same protocol but the respiration rate was expressed as μg oxygen consumed per organism per hour per ml. For each treatment we used three blank controls (syringes with no organisms) to correct for the ambient oxygen depletions due to factors other than organism respiration (e.g. from background microbial oxygen consumption). These results were used as correction factors in appropriate treatments. We used a full factorial design where pesticide exposure was tested in combination with all the different levels of predation risk. Thus, for every predation risk level (no risk, low and high) we tested three concentrations of imidacloprid: 1.9; 3.9; and 7.8 $\mu\text{g/L}$ plus the control treatment for *S. vittatum* and 0.4; 1.2; and 3.7 $\mu\text{g/L}$ plus the control treatment for *C. riparius*. Due to the limited number of syringes available, these experiments were performed in two runs over two consecutive days for *C. riparius* and three runs over three consecutive days for *S. vittatum* in a randomised block design.

Statistical analysis

EC50 values for mortality (measured as immobilization) were calculated using the probit method (Minitab 2000). For all other experiments, two-way ANOVAs were performed using Imidacloprid concentrations and chemical cues as treatments. For respiration rates, day of measurement was blocked as a random factor. Whenever significant differences were observed Dunnett post hoc test was used for multiple comparisons to determine which treatments were significantly different from the control. Data from *S. vittatum* feeding experiments were $\log(x+1)$ transformed to stabilise variances across treatments (Zar 1996). All statistical analyses were performed using the Minitab 13.0 statistical package (Minitab 2000).

To address the effects of the combined stressors, the observed effect on feeding, of *S. vittatum*, and growth (length and head width) and respiration of *C. riparius* were

compared to the expected effects of stressors combinations calculated from effects of single compound exposures. This procedure is usually based on already described conceptual models: concentration addition (CA) and independent action (IA) (e.g. Backhaus *et al.* 2003; Altenburger *et al.* 2004; e.g. Jonker *et al.* 2004; Jonker *et al.* 2005). Although these two conceptual models take into consideration the information available on chemicals' mode of action, it has been suggested to consider the ecotoxicological mode of action of chemicals when considering effects at the whole organism level such as physiological and life historical responses (Barata *et al.* 2007).

Although the mechanism of action of predatory cues is unknown; imidacloprid and predation risk can impair food acquisition and affect the respiration rates due to effects on behaviour of insects. Although we can consider that they share a common ecotoxicological mode of action (sensu Barata & Baird 2000) both conceptual models were applied to our data.

For the IA conceptual model the fit to our data was made using equation 1:

$$Y = \mu_{\max} \prod_{i=1}^n q_i(C_i) \quad (\text{equation 1})$$

where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, $q_i(C_i)$ the probability of non-response, μ_{\max} the control response for the selected endpoint and \prod the multiplication function.

For the CA model fit, equation 2 was applied, where C_i is the dose used for stressor i in the mixture and EC_{x_i} is the effect dose of stressor i that produces the same effect (x%) as the whole mixture.

$$\sum_{i=1}^n C_i / EC_i = 1 \quad (\text{equation 2})$$

The procedure analysis suggested by Jonker (2005) was used here for the analysis of feeding of *S. vittatum* as well as oxygen consumption and growth (head capsule width) of *C. riparius* since it permitted significance testing of model fit for both the independent action model and the concentration addition model and also because the analysis takes into account different nonlinear dose–response characteristics of stressors (Jonker *et al.* 2005). Deviations from reference models such as synergism/antagonism,

dose-ratio and dose level were assessed by additional parameters used in the mathematical models that describe both CA and IA reference models and are tested within a nested framework (see (Jonker *et al.* 2005). The models are fitted to the data using a maximum-likelihood method. The best fit is chosen using a Chi-square test which minimizes the objective function based on the log likelihood. The biological interpretation of these additional deviation parameters are described in Table III. Here, average data from *S. vittatum* feeding and *C. riparius* growth experiments was used as a preliminary approach (Jonker *et al.* 2005) because the raw data set showed a poor fit to the models. The complete data set from *C. riparius* respiration experiments was used in the analysis of joint effects of the stressors tested.

A different approach using the IA reference model was followed to calculate the predicted responses of both insect species when exposed to both stressors simultaneously. Estimated mean values were obtained directly from equation 1, using the maximum response from the data set. Additive effects estimated by the reference IA model were compared to the average values observed and their respective 95% confidence intervals to infer significant antagonism or synergism for each mixture treatment. This approach was applied to compare results in terms of the sensitivity of the different modelling approaches and was also used to calculate the predicted responses of the mixture on the respiration rates of *S. vittatum*. For this latter parameter, the approach described by Jonker et al (2005) was not applicable, as it relies on dose response curves which cannot be estimated if the stressors in the mixture cause contrasting effects (stimulation *vs.* inhibition), as was observed for *S. vittatum* respiration.

Results

C. riparius

The imidacloprid 96-hr EC50 (95% CI) for *C. riparius* was 12.94 µg/L (9.74 – 18.22) with no predator cues and 14.06 µg/L (10.74 – 20.18) when simultaneously exposed to high concentration of predation cues.

In the 10-day growth experiment, mortality was below 10% except in treatments with highest concentrations of imidacloprid, where mortalities of 80-100% were observed. These treatments were excluded from analysis of growth, behaviour and emergence. The concentrations of imidacloprid measured at the end of day 2, i.e. before

partial renewal of medium, were 96% of initial concentrations for the lower concentration, 64% for the medium concentration and 40% for the highest concentration of initial concentrations. The degradation of pesticide increased along the experimental period probably as a result of bacterial growth in vials due to continuous food addition which can greatly increase the degradation rate of imidacloprid (CCME 2007). The analysis of imidacloprid concentrations on treatments with high levels of predation risk revealed no effect of chemical predatory cues on degradation of the pesticide.

There was a reduction in *C. riparius* growth when exposed to sub-lethal concentrations of imidacloprid (Table I, Fig.1 and 2). NOECs and LOECs for imidacloprid were 0.4 µg/L and 1.2 µg/L respectively. Growth of *C. riparius* larvae was also significantly affected by the presence of high levels of predator cue, with a decrease in the total length and head capsule width being observed. Emergence results revealed a significant reduction, relative to controls, in emergence ratio for larvae exposed to 1.2 µg/L imidacloprid (Table I, Fig. 3), and a significant delay in time-to-emergence when larvae were exposed to 0.4 µg/L and to high levels of predation cue (Table I, Fig. 4).

In the respirometry experiments, mortality was always below 10%. *C. riparius* showed a significant increase in their respiration rates when exposed to sub-lethal concentrations of imidacloprid (Table I, Fig. 5), with LOEC values for Imidacloprid of 1.2 µg/L. Predation cues also significantly increased oxygen consumption in *C. riparius*. No significant effects were observed for day of measurement (ANOVA $F_{1,38}=0.72$, $p=0.405$). As shown in Fig. 4, after six days of exposure to imidacloprid, *C. riparius* burrowing behaviour was also affected. A higher percentage of larvae were found on top of sediment (or in the water column) as a result of exposure to 0.4 µg/L of imidacloprid. No statistical differences in the burrowing behaviour of *C. riparius* larvae were noted under exposure to varying concentrations of predation cues (Table I, Fig 6).

Concerning the prediction of joint effects of both stressors no statistically significant interaction of imidacloprid and predation cues were detected by analysis of variance for any of the parameters tested for *C. riparius* thus indicating additivity of effects (Table I). The application of conceptual models held similar results. However data from respiration measurements did not show a good fit to the CA reference model (SS=47.9215; $r^2=0.420$; $p=0.001$) but a significant dose ratio dependent deviation was found (SS=18.8944; $r^2=0.772$; $p<0.001$ $a=0.821$; $b=-5,695$) where synergism is observed where the toxicity of the mixture is caused mainly by imidacloprid. The shift

between synergism to antagonism occurs when $[cues]= 0.2 [IMI]$ meaning that for the concentrations ratios used here, antagonism is only observed in the treatments with the lowest imidacloprid concentration and the highest predation risk. Using the IA approach data also showed a weak fit to the reference model ($SS=43.9189$; $r^2=0.469$; $p<0.001$) also with a significant dose ratio dependent deviation ($SS=19.1956$; $r^2=0.768$, χ^2 test, $p<0.001$; $a=1.769$; $b=-1,294$) where synergism is observed where the toxicity of the mixture is caused mainly by imidacloprid. The ratio at which synergism shifted to antagonism was not possible to calculate meaning that the CA approach is probably more suitable for this type of data.

For *C. riparius* respiration data, the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each combination treatment, significant deviation from additivity (synergism) could only be observed for the treatment with high concentrations of both stressors (Fig. 9 C).

Concerning the prediction of joint effects of both stressors on *C. riparius* growth, average data (head capsule width measurements) adequately fitted the IA reference model ($SS<0.0001$; $r^2=0.966$; $p<0.05$) and also the CA approach ($SS<0.0001$; $r^2=0.951$, $p<0.05$) with no significant deviations found. Additive effects of imidacloprid and perceived predation risk on head capsule width of *C. riparius* were also observed in the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each mixture treatment (Fig. 9 D). These results are also in concordance with the results from analysis of variance where no significant interaction between stressors was found (Table I).

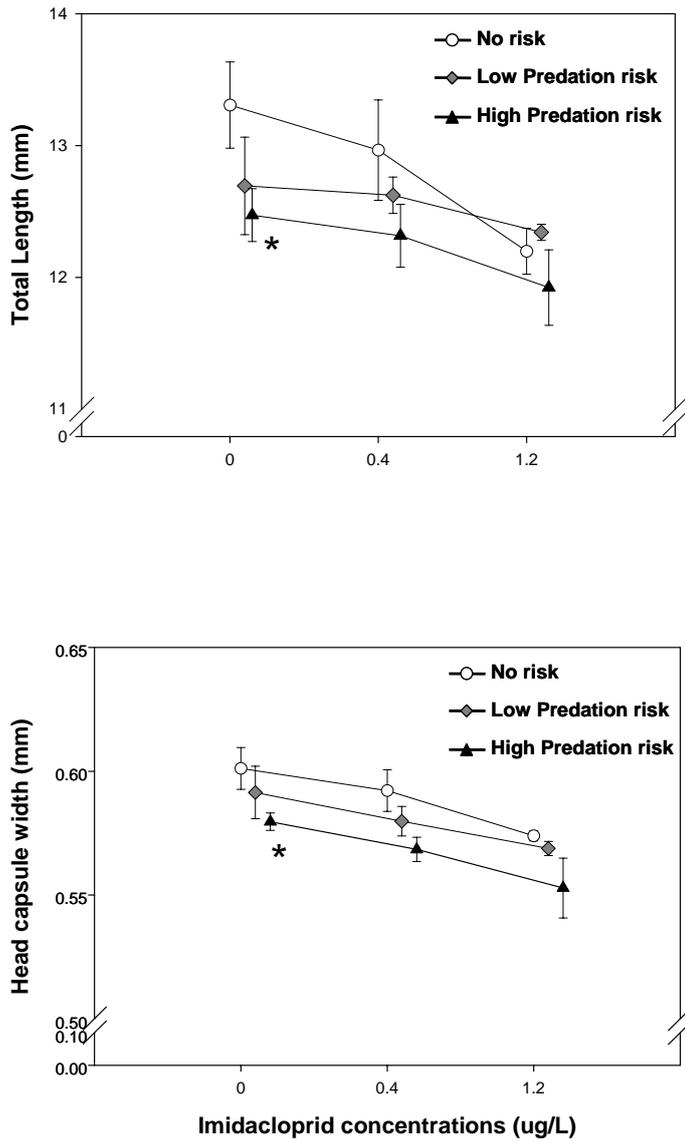


Fig 1, 2 - *C. riparius* growth measurements: total length and head capsule width (mean \pm SE) at day 10. Asterisks denote predation risk treatments that are significantly different ($p < 0.05$) compared to the control treatment (no predation).

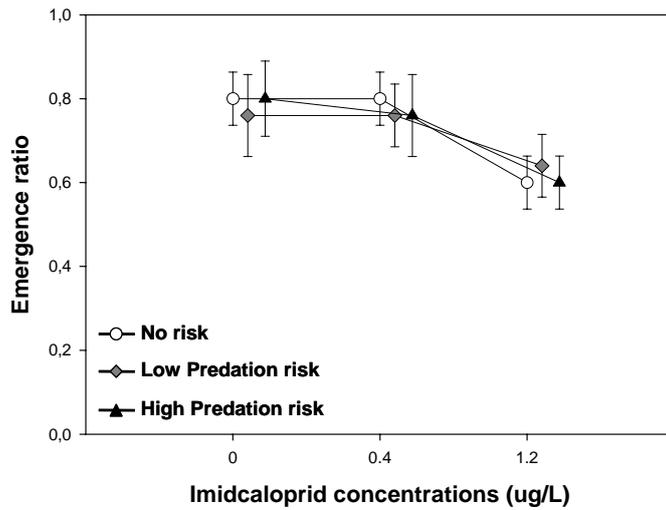


Fig 3 - *C. riparius* mean emergence ratio

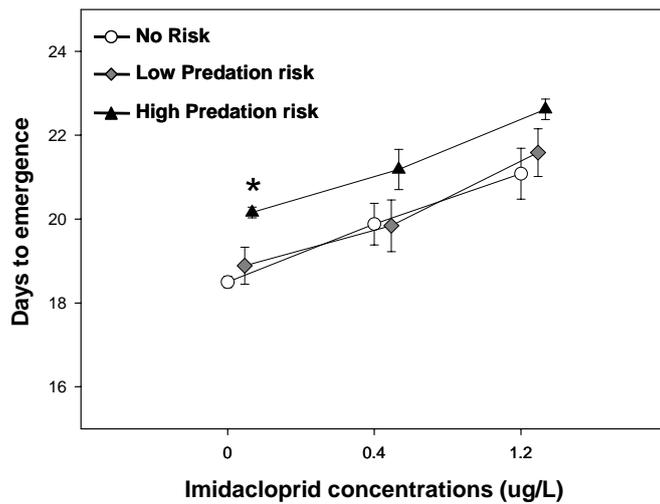


Fig 4 - *C. riparius* mean development time (1/development rate) (mean \pm SE). Asterisks denote predation risk treatments that are significantly different ($p < 0.05$) compared to the control treatment (no predation).

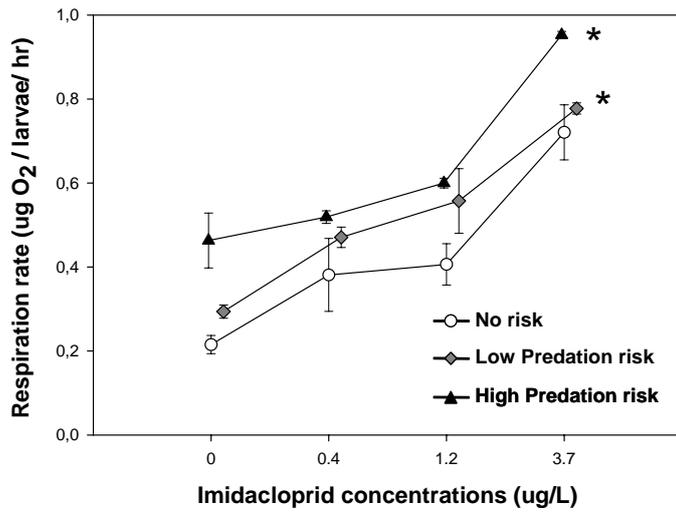


Fig 5 - *C. riparius* respiration rate (mean \pm SE). Asterisks denote predation risk treatments that are significantly different ($p < 0.05$) compared to the control treatment (no predation).

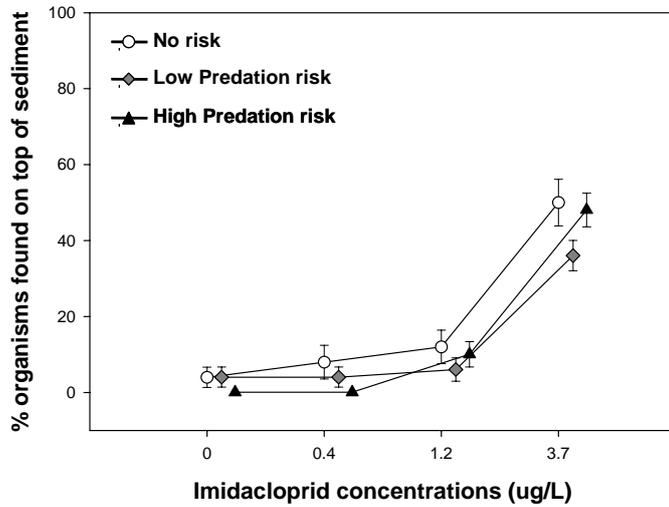


Fig 6 - *C. riparius* burrowing behaviour. The observations of number of larvae visible on top of sediment (mean \pm SE), were taken at day 6 for a 15 min period for each treatment and dead organisms were excluded from the analysis.

Table I - Two-Way ANOVA results for *C. riparius* experiments.

Factor	<i>df</i>	<i>F</i>	<i>p-value</i>	LOEC for IMI
<i>C. riparius</i>				
Total length				
[IMI]	2	5.20	0.010	1.2 µg/L
Predation cues	2	3.77	0.033	
[IMI] x Predation cues	4	0.58	0.682	
Head Capsule width				
[IMI]	2	9.19	0.001	1.2 µg/L
Predation cues	2	6.87	0.003	
[IMI] x Predation cues	4	0.07	0.991	
Burrowing behaviour				
[IMI]	2	92.22	<0.001	3.7 µg/L
Predation cues	2	2.90	0.059	
[IMI] x Predation cues	4	1.31	0.257	
Development rate				
[IMI]	2	22.86	<0.001	0.4 µg/L
Predation cues	2	9.03	0.001	
[IMI] x Predation cues	4	0.19	0.941	
Emergence ratio				
[IMI]	2	4.62	0.016	1.2 µg/L
Predation cues	2	0.03	0.971	
[IMI] x Predation cues	4	0.12	0.975	
Respiration				
[IMI]	3	61.79	<0.001	0.4 µg/L
Predation cues	2	9.62	0.001	
[IMI] x Predation cues	6	0.72	0.677	

S. vittatum

The imidacloprid 96-hr EC50 (95% CI) for *S. vittatum* was 47.22 µg/L (34.17 - 70.74) with no predator cues and 35.86 µg/L (25.47 - 52.15) when simultaneously exposed to high concentration of predation cues.

In the feeding and respiration experiments, observed mortality was always below 10% with the exception of the feeding experiment treatment of 1.9 µg/L + high level of predation cues, in which mortality was 20%. The concentrations of imidacloprid at the end of day 2, i.e. before partial renewal of medium, were checked and the level corresponded to 66% - 63% of initial concentrations. The same relative concentrations were measured at the end of the experimental period. The analysis of imidacloprid concentrations on treatments with high levels of predation risk revealed no effect of chemical predatory cues on degradation of the pesticide.

The feeding rate of *S. vittatum* was significantly reduced under exposure to sub-lethal concentrations of imidacloprid (Table II Fig. 7). Feeding-related NOEC and LOEC values for imidacloprid were 1.9 µg/L and 3.9 µg/L respectively.

S. vittatum showed reductions in oxygen consumption when exposed to sub-lethal concentrations of imidacloprid, but in contrast, significant increases in respiration rates were observed under exposure to predation cues (Table II Fig. 8) NOEC and LOEC values for imidacloprid were 3.9µg/L and 7.8 µg/L respectively. No significant effects were observed for day of measurement (ANOVA $F_{1,64}=0.45$, $p= 0.640$).

Sub-lethal concentrations of the insecticide also affected the burrowing behaviour of *S. vittatum* with a significantly higher percentage of larvae found on top of the sediment as a result of exposure to the highest concentration of imidacloprid (Table II, Fig 9). No statistical differences were observed in the burrowing behaviour of *S. vittatum* larvae under exposure to different levels of predation cues. Nevertheless, results indicated that caddis larvae exposed to predation cues appeared to remain hidden in the sediment for longer periods of time since more larvae were observed on top of the sediment in the treatments where no cues were added

Concerning the prediction of joint effects of both stressors, average data from feeding experiment with *S. vittatum* adequately fitted the IA reference model (SS=0.0093; $r^2=0.949$; $p<0.001$) and also the CA approach (SS=0.0094; $r^2=0.948$, $p<0.001$) with no significant deviations found. Additive effects of imidacloprid and perceived predation risk on feeding rates of *S. vittatum* were also observed in the comparison between the observed data and the mean estimated responses calculated

directly using the IA reference model for each mixture treatment (Fig. 9 A). These results are also in concordance with the results from analysis of variance where no significant interaction between stressors was found (Table II).

For *S. vittatum* respiration data the analysis of variance couldn't detect a significant interaction between stressors (Table II), but the average values observed were higher than the predicted responses calculated directly using the IA in all treatments. A significant deviation from additivity was found in the treatments with the highest concentrations of both stressors in the comparison between the observed data and the mean (Fig. 9 B).

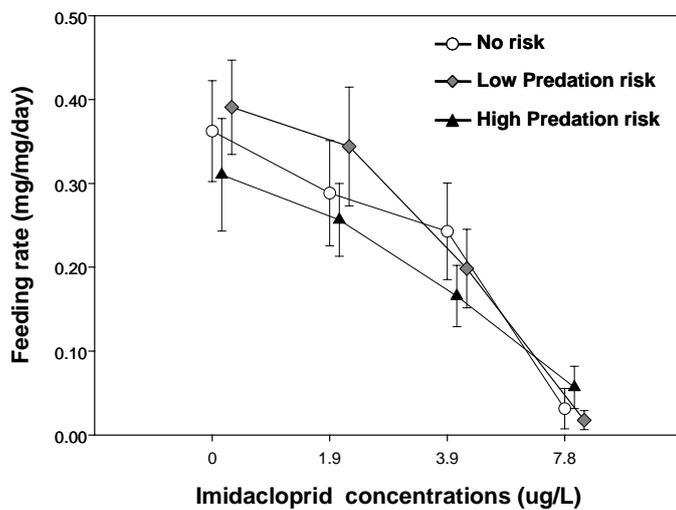


Fig 7 - *S. vittatum* feeding rate (mean \pm SE).

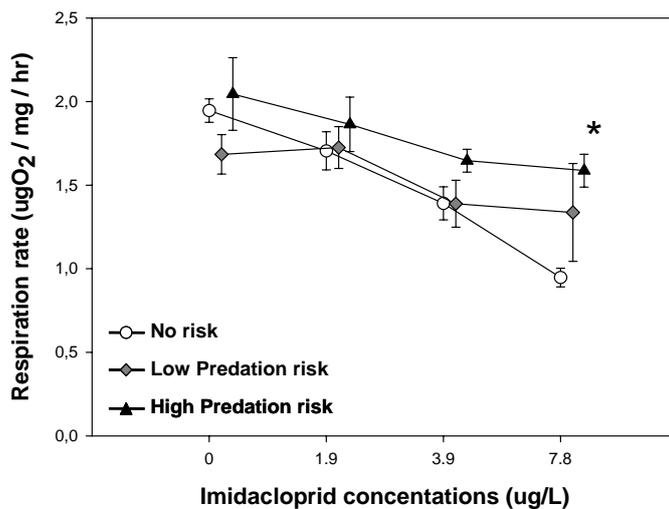


Fig 8 - *S. vittatum* respiration rate (mean \pm SE). Asterisks denote predation risk treatments that are significantly different ($p < 0.05$), compared to the control treatment (no predation).

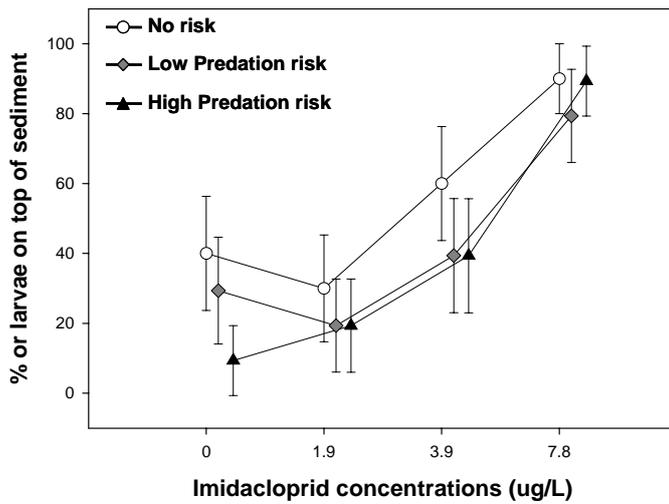


Fig 9 – *S. vittatum* burrowing behaviour. The observations of number of larvae visible on top of sediment (mean ± SE), were taken at day 3 for a 15 min period for each treatment. Dead organisms were excluded from the analysis.

Table II - Two-Way ANOVA results for the experiments with *S. vittatum*

Factor	<i>df</i>	<i>F</i>	<i>p</i>	LOEC
<i>S. vittatum</i>				
Feeding				
[IMI]	3	22.90	<0.001	3.9 µg/L
Predation cues	2	0.70	0.501	
[IMI] x Predation cues	6	0.64	0.701	
Respiration				
[IMI]	3	10.99	<0.001	3.9 µg/L
Predation cues	2	4.26	0.020	
[IMI] x Predation cues	6	1.16	0.342	
Burrowing behaviour				
[IMI]	3	11.89	<0.001	7.8 µg/L
Predation cues	2	1.02	0.363	
[IMI] x Predation cues	6	0.15	0.989	

Table III - Interpretation of additional parameters substituted into the concentration addition (CA) and independent action (IA) reference models that define the functional form of the deviation pattern adapted from (Jonker *et al.* 2005)

Deviation Pattern	Parameter <i>a</i> (CA and IA)	Parameter <i>b</i> (CA)	Parameter <i>b</i> (IA)
synergism/antagonism (S/A)	a>0: antagonism a<0: synergism		
Dose-ratio dependent (DR)	a>0: antagonism except for those mixture ratios where negative <i>b</i> value indicate synergism	b_i>0: antagonism where the effect of the mixture is caused mainly by toxicant <i>i</i>	
	a<0: synergism except for those mixture ratios where positive <i>b</i> value indicate antagonism	b_i<0: synergism where the effect of the mixture is caused mainly by toxicant <i>i</i>	
Dose-level dependent (DL)	a>0: antagonism low dose level and synergism high dose level	b_{DL}>1: change at lower EC50 level	b_{DL}>2: change at lower EC50 level
		b_{DL}=1: change at EC50 level	b_{DL}=2: change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	0<b_{DL}<1: change at higher EC50 level	1<b_{DL}<2: change at higher EC50 level
		b_{DL}<1: No change but the magnitude of S/A is DL dependent	b_{DL}<1: No change but the magnitude of S/A is effect level dependent

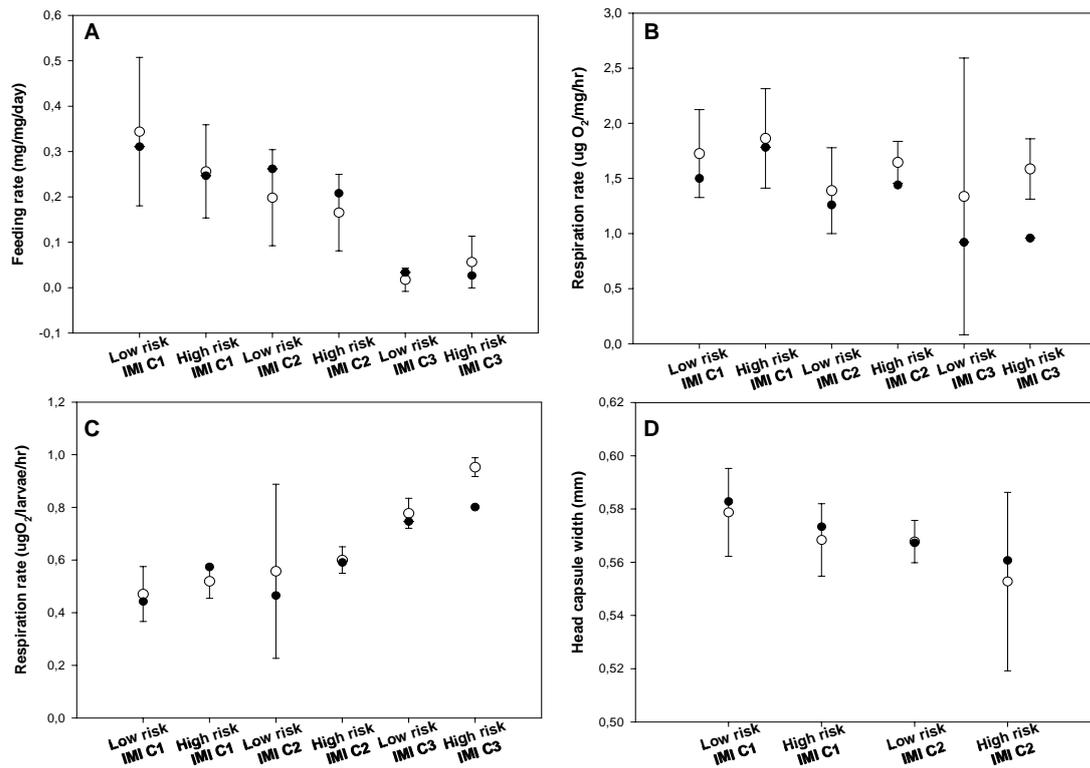


Fig 10 – Effects of combined exposures to Imidacloprid different levels of perceived risk of predation on *S. vittatum* respiration rate (A) and feeding (B), and on *C. riparius* respiration rate (C) and growth (head width) (D). Empty symbols denote observed responses (mean and 95% confidence intervals) and filled symbols represent effects predicted by independent action reference model.

Discussion

Since inland waters commonly exhibit at least some degree of pesticide contamination, and since predation has a major impact on prey communities, populations and individual traits, it is prudent to study the effects of pesticides and predation to aquatic biota and the consequences of their interaction. Assessing the effects of pesticides in organisms facing different levels of predation risk is important to reduce the uncertainty surrounding pesticide effects on natural populations and communities and thus facilitate the extrapolation of laboratory ecotoxicological test results to natural ecosystems (Sih *et al.* 2004; Relyea *et al.* 2005).

Ecological theory predicts that when sensing situations of predation risk, aquatic insects engage in behavioural and physiological based adaptive responses that are translated in a trade-off between the fitness benefits of energy gain and the risks of being eaten by predators (Benard 2004). When in conditions of perceived high predation risk, prey organisms can decrease activity, reducing their risk of encountering predators, yet also reducing energy returns from foraging (Peckarsky *et al.* 1993; Rose *et al.* 2003; Holker & Stief 2005; Gyssels & Stoks 2006; Beckerman *et al.* 2007). The presence of predators has also been shown to place higher metabolic demands on prey organisms, manifested in elevated rates of oxygen consumption, reflecting an increased cost of vigilance under predation risk (Bengtsson 1982; Beckerman *et al.* 2007). Thus, in the presence of predators like fish, the energy budget of aquatic insect larvae can be reduced, leading to delayed maturity and/or smaller size at maturity (Ball & Baker 1996; Peckarsky *et al.* 2002; Dahl & Peckarsky 2003a; Benard 2004; Holker & Stief 2005).

The energy budget of organisms has also been used in ecotoxicology to assess the sublethal effects of different types of contaminants (Maltby *et al.* 1990; Lam 1996b; Maltby 1999; Verslycke *et al.* 2004). Low levels of contaminants have been previously shown to cause alterations in feeding and respiration rates of many species with potential consequences for growth, reproduction and survival (Widdows 1985; Kedwards *et al.* 1996; McLoughlin *et al.* 2000; Soucek 2006). In turn, such effects can propagate through higher levels of organisation, to populations, communities and ecosystems (Allen *et al.* 1995; Forrow & Maltby 2000; Slijkerman *et al.* 2004).

Imidacloprid is a systemic insecticide that has been previously shown to have potentially harmful effects on aquatic non-target insects (Alexander *et al.* 2007; Stoughton *et al.* 2008). Our results support these previous studies, showing that imidacloprid is acutely toxic to *C. riparius* and *S. vittatum* at low concentrations. The EC₅₀ (immobilization) for imidacloprid found for both species was within the range of acute effect concentrations observed for other insect species (Song *et al.* 1997; Alexander *et al.* 2007; Stoughton *et al.* 2008) and near concentrations measured in natural systems in the case of *C. riparius*. *S. vittatum* showed a relatively higher tolerance to imidacloprid than *C. riparius*.

Low doses of imidacloprid have been shown to induce several behavioural responses such as reduced activity and uncontrolled muscular contractions, which can limit foraging

activity of aquatic insects and consequently impair feeding and growth (Alexander et al. 2007). This was also the case for the species tested here. Growth, development rates and emergence ratio of *C. riparius* were significantly reduced by exposure to low concentrations of imidacloprid. *S. vittatum* feeding rates were also significantly reduced with exposure to sub lethal concentrations of imidacloprid.

The respiration of insect larvae was also affected by imidacloprid, and we observed increased respiratory rates in *C. riparius*, likely a reflection of uncontrolled muscular activity, which increases energy costs. Curiously, imidacloprid exposure induced a reduction in the respiratory rates of *S. vittatum*. We hypothesize that the different responses in terms of respiration patterns were due to different behavioural responses of *C. riparius* and *S. vittatum* larvae when exposed to imidacloprid in the respirometers (gastight syringes). It is important to reinforce also that respiratory experiments were done with no food, no sediment and for only a 24 hour exposure period. In response to the lack of sediment, larvae of each species respond differently: *S. vittatum* larvae showed low locomotor activity, whereas *C. riparius* larvae increased activity by continuous swimming in the water column. Furthermore, in these respirometry experiments, we exposed *S. vittatum* larvae with their cases but chironomids were exposed without their tubes. Cased caddisflies use body undulations to pump water through their cases to promote efficient ventilation by restricting and directing water flow (Merritt & Cummins 1996). Since we failed to observe uncontrolled muscular contractions in *S. vittatum* exposed to the imidacloprid concentrations tested, we conclude that reduced respiration rates with increasing insecticide concentration might be due to a disruption of these ventilatory movements together with decreased locomotor activity. Nevertheless, the measured respiration rates in the control treatment are in the range of the observed before for this species in laboratory flow-through respirometry experiments (Feio & Graça 2000). *C. riparius* larvae on the other hand, having no sediment to burrow into, swam continuously, which, together with continuous stimulation of the nervous systems (mild tremors) caused by exposure to imidacloprid, led to higher respiration rates reflecting increased metabolic costs. Thus, differing behavioural responses of the two species in response to different concentrations of imidacloprid and the lack of sediment substrate might explain the apparent contradictory effects of imidacloprid on respiration of *C. riparius* and *S. vittatum*.

C. riparius responded to predation cues in a concentration dependent manner, i.e., responses were stronger when higher concentration of chemical cues from predators and macerated conspecifics were present. This implies that *C. riparius* could detect cue concentrations, leading to perceived differences in levels of predation risk. This is in accordance with empirical results showing that prey species adjust the level of responses to the perceived level of predation risk (Reede 1995; McIntosh & Peckarsky 2004; Holker & Stief 2005; Mirza *et al.* 2006). The combination of chemical cues from predators, or kairomones, with alarm substances from alarmed, injured or dead conspecifics has also been shown to produce a stronger response and to be a more reliable cue in terms of assessment of threat and level of risk to different prey species (Wisenden *et al.* 1997; Slusarczyk 1999; Korpi & Wisenden 2001; Wisenden & Millard 2001; Stabell *et al.* 2003; Schoeppner & Relyea 2005; Laforsch *et al.* 2006; Beketov & Liess 2007). Other experiments in our laboratory have shown that these and other invertebrate species show similar, although weaker, responses to fish kairomones alone. We have decided to use a combination of the two cues as this is a more realistic scenario under which to investigate responses to predation risk instead of responses to fish kairomones alone.

C. riparius were significantly smaller, and emergence was delayed after 10 days of exposure to high levels of predation cues. As previously shown in chironomids, reductions in activity and the consequent decrease in time spent foraging can decrease growth and development rates under high levels of predation risk (Noonburg & Nisbet 2005). Our results support the view that elevated respiration rates arising from increased awareness of risky situations is a sensitive response to the presence of predators. The trade-off between energy gain and mortality risk is thus apparent in *C. riparius* under risk of predation simulated by exposure to non-lethal cues, kairomones from trout combined with alarm substances from conspecifics.

S. vittatum feeding rates observed under different levels of predation risk were not statistically different from the control (no chemical cues added), although a non-significant decrease in feeding rates was noted when larvae were exposed to a high concentration of chemical cues. Lower feeding rates observed in organisms exposed to treatments with high concentration of predatory cues were possibly a consequence of reduced activity, also observed as a response to chemical cues from fish in other cased caddisfly species (Kuhara *et al.* 2001). The lack of statistically significant results here could indicate a lower

responsiveness to presence of predators in this species. A lack of responses to nonlethal cues from fish has been observed before for caddisflies species (Kohler & McPeck 1989; Lefcort *et al.* 2000), and could be a consequence of their defensive armouring. The mineral cases of cased caddisflies constitute an effective antipredator defence, and can be considered as armour that not only camouflages larvae but also increases the handling time for predators (Kuhara *et al.* 1999; Boyero *et al.* 2006). Furthermore, they show low locomotor activity and are normally found hidden under the sediment or organic material, and are usually more active at night, all of which can be considered behavioural adaptations to avoid predators. This lifestyle may explain the weaker feeding response of caddisflies when under fish predation risk (Kuhara *et al.* 2001).

In our experiments with *S. vittatum* respiration was a more sensitive measure of effects of the predatory chemical cues than feeding. Respiration rates of *S. vittatum* larvae increased under high levels of perceived predation threat and as for chironomids, we hypothesise that this response is due to the increased alertness exhibited by organisms under risky situations (Woodley & Peterson 2003; Beckerman *et al.* 2007). Higher nocturnal respiration rates of *S. vittatum* were previously shown to be as consequence of higher activity shown by larvae at dark possibly to avoid competitors and predators (Feio & Graça 2000). Here, the respiratory experiments were performed with no light so it remains unclear if *S. vittatum* shows stronger behavioural responses to chemical cues from predators during the day.

Although we did not measure growth or emergence in *S. vittatum*, it is likely that respiration (as an indication of metabolism) and feeding results could be used cautiously to infer potential growth impairment in *S. vittatum*, keeping in mind that other factors such as assimilation efficiency could complicate this interpretation (Stoks 2001).

We failed to observe any combined effect of imidacloprid and predation cues on the acute lethal responses of either species. However, in the sublethal exposures, the highest imidacloprid concentrations tested increased the metabolic costs of antipredator responses of *C. riparius*, and inhibited the feeding responses to perceived predation risk observed in *S. vittatum*. For *S. vittatum*, the lack of statistically significant results, in the case of feeding response to predation cues, and contrasting responses to different stressors (in the case of respiration), complicate our interpretation of the interaction between these major stressor classes.

The assessment of joint effects of these stressors with conceptual models generally agreed with results of analysis of variance which showed no significant interaction between the two stressors. Although only additive effects were observed for growth and development rates of chironomids, it is possible that effects of imidacloprid under predation pressure could be stronger in terms of reproductive fitness of insects through reductions of size at emergence that although not measured here, have been observed in aquatic species in response to perceived risk of predation (Peckarsky *et al.* 2001) and also to sub-lethal concentrations of imidacloprid (Alexander *et al.* 2008). Despite this, the results presented above provide clear evidence that pesticide contamination can have enhanced negative effects on aquatic insects under the perceived presence of predators. Moreover it was demonstrated how simple additive models can be employed to predict the joint effects of two different stressors. The fact that the models used here, based on dose response curves, detected deviation patterns from additivity that are not limited to just synergism or antagonism (such as dose ratio dependent deviation for *C. riparius* respiration) is an indication that the effects of pesticides under different levels of perceived risk of predation can change considerably according to the level of both stressors and also their ratio in the mixture. A better calibration of models is also necessary with the assessments of responses to more concentrations of each stressor and of their combinations.

With the ecotoxicological mode of action in mind, our results suggest that concentration addition and independent action conceptual approaches can be applied to different parameters measured at the organism level to predict the responses to combinations of pesticides and biotic natural stressors. In insects with a complex life cycle and that do not feed as adults the effects of perceived predation risk on growth are thought to be driven by a reduction in activity with consequently reductions on food intake (Ball & Baker 1996) Here, the *C. riparius* data show this behavioural mediated life-history changes with an increase in predation risk resulting in slower growth and also slower development rates. Because the toxicity of imidacloprid is also manifested through feeding impairments, these two stressors share a common ecotoxicological mode of action being responses mediated through behaviour (Ball & Baker 1996). However other insects species have shown to increase development rates at the cost of size at maturity in response to fish predation risk (Dahl & Peckarsky 2003b) suggesting a possible physiological basis for the

antipredator response. Because sublethal concentrations of pesticides have also been shown to affect the size of emergent insects (Alexander *et al.* 2008), it is important to expand the mechanistic study of effects of combined exposure to pesticides and predation risk by measuring different endpoints (digestive physiology, energetic reserves, size at maturity, fecundity) over a wider range of pesticide concentrations.

To better understand and evaluate the effects of predation risk on insect population dynamics it crucial to consider other environmental variables such as food deficiency or time stress that can change the magnitude of predator sub-lethal effects (Stoks *et al.* 2005b; Beketov & Liess 2007)

Our results further suggest the possibility for additional indirect effects of pesticides on susceptibility to predation: since imidacloprid significantly impairs the burrowing behaviour of larvae of both species, this in itself could further increase the risk of mortality from predation. Effects of pesticides on the drifting behaviour of insects have been shown to enhance mortality from fish predation (Schulz & Dabrowski 2001). Here we have only examined the responses of insects to nonlethal cues, simulating the presence of predators. Nevertheless we hypothesise that in natural ecosystems, exposure to low 'environmentally relevant' concentrations of imidacloprid, in addition to its direct effects, could indirectly increase mortality of chironomids and caddisflies due to fish predation.

Conclusion

Predator-avoidance behaviour by aquatic insect larvae can result in reductions in time spent feeding, with consequences for rates of growth and development (McPeck & Peckarsky 1998). Behaviour that results in reduced growth and/or changes in development time may be particularly important for insects with short adult stages such as *C. riparius* and *S. vittatum* because fecundity is determined by the size of larva upon metamorphosis (Peckarsky *et al.* 2001). If we consider that many of these organisms under predation pressure can also be exposed to pesticides and that sublethal concentrations of pesticides can also affect energy intake, it is clear that additive reductions in growth rate and/or size upon emergence could have significant consequences for reproductive success and thus influence population dynamics and viability. Nonlethal effects of predator-avoidance behaviour and pesticide contamination will also have consequences at ecosystem level,

since any reduction in secondary production by prey populations will influence food availability to predators.

In this study we have assessed the effects of sublethal concentrations of imidacloprid on two insect species under different levels of predation risk. The experimental designs employed closely matched methods commonly used in ecotoxicological hazard assessment and clearly demonstrated responses of *C. riparius* and *S. vittatum* to predation cues both in the presence and absence of pesticide stress. We have further demonstrated that predation cues can be additively combined with another stressor, which can be considered 'similarly-acting' in an ecological sense. Previous studies (Barata & Baird 2000; Barata *et al.* 2007) have indicated the need for a paradigm shift in ecological risk assessment, moving away from narrow, toxicological definitions of stressor modes of action towards broader, more encompassing definitions of ecologically relevant stressor effects. The results discussed above further advances this approach by demonstrating that 'natural stressors' such as predation can be studied employing similar experimental approaches. Moreover, treating natural and anthropogenic stressors within the same framework can yield compatible data for modelling, allowing improved interpretation of ecological effects within a broader ecosystem context.

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Chapter VI

Concluding remarks and
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Concluding remarks and perspectives for future research

The continued use of pesticides in modern agriculture has maintained concern about their impacts on non-target species, on natural communities and on ecosystem processes. The traditional toxicological approach of examining a few species of model organisms under highly controlled laboratory conditions is an efficient and necessary process for assessing the thousands of existing registered chemicals. However, the effects of pesticides under more natural conditions can be very different due to both density- and trait-mediated indirect effects that are often absent in single species experiments performed under laboratory controlled conditions (Heugens *et al.* 2001; Van Straalen 2003; Eggen *et al.* 2004; Rohr *et al.* 2004; Relyea *et al.* 2005). Studying natural and anthropogenic stressors and their combined effects under the same ecological framework with similar experimental approaches is of great value in ecological risk assessment (Relyea & Hoverman 2006; Rohr *et al.* 2006). Such a framework can support a mechanistic understanding of the conditions under which density and trait mediated effects of pesticides affect species interactions, communities and ecosystems. By understanding how ecologically relevant concentrations of pesticides under different environmental situations affect species' traits, we will improve our ability to predict if and how these trait changes should affect the community as a whole (Baird *et al.* 2008). Since freshwater insects are subjected to time-varying levels of predation, and similarly episodic levels of pesticide exposure (Sih *et al.* 2000; Hanazato 2001; Sih & McCarthy 2002; Sih *et al.* 2004) it is prudent to study the effects of pesticides combined with different levels of predation risk minimizing uncertainties from extrapolating laboratory results to natural systems where predators are commonly present (Hanazato 1999; Fleeger *et al.* 2003; Rohr & Crumrine 2005).

The main objective of this dissertation was to improve understanding on the potential effects of pesticide exposure on macroinvertebrate populations under different levels of predatory stress. Imidacloprid, a widely used neurotoxic insecticide and predation risk were used as stressors. Perceived predation risk was simulated with chemical cues from trout and alarm cues from macerated conspecifics that are known to induce behavioural, physiological and life-history responses in a variety of species.

The effects of imidacloprid in relevant exposure conditions were studied with the use of freshwater field mesocosms. Exposure to pulses of imidacloprid caused important

alterations on structural and functional community endpoints which related well to single species toxicity tests using a pteronarcid stonefly species.

A mechanistic approach was then employed, to identify variables at the organism level that may shape the interaction between predation stress and imidacloprid toxicity in single species laboratory standard tests currently used in pesticide hazard assessment. Exposure to sub-lethal concentrations of imidacloprid caused reduced energy intake, increasing oxygen consumption rates, reduced growth and delayed maturity in *C. riparius* and *D. magna*. Same responses were observed for *S. vittatum* and *P. comstocki* but imidacloprid reduced oxygen consumption rates in these two test organisms. In *D. magna*, reduced fecundity and a smaller size at maturity were also observed. Perceived predation risk also reduced energy intake and increased oxygen consumption rates of test species. Specific responses of *D. magna* to size selective predators were also observed with an increase in development rates and fecundity with smaller size at maturity being observed in organisms under perceived predation risk.

We provided evidence which show that sublethal concentrations of pesticides can have significant detrimental consequences not only because of their toxic effects but also through effects on predator-prey interactions. By improving our understanding of the behavioural and physiological mechanisms underlying this interaction we showed that imidacloprid can increase the metabolic costs of induced antipredator defences or inhibit induced responses to predation risk. The joint effects of these stressors were assessed with predictive conceptual models currently used to assess combined effects of different toxicants and with analysis of variance. Deviations from additivity were observed for some endpoints measured in daphnids and insects demonstrating that the effects of imidacloprid on different test species vary depending on the perceived predation risk and that prey organisms under predatory stress will be more affected by pesticides. The results further suggest the possibility for additional indirect effects of pesticides on the susceptibility to predation through effects on prey behaviour that can increase the risk of mortality from predation.

The results presented in this dissertation demonstrate that 'natural stressors' such as perceived predation risk, pesticides and their combination can be studied employing similar experimental approaches that in turn can yield compatible data for modelling allowing improved interpretation of ecological effects within a broader ecosystem context.

Although the concentrations of both stressors elicited a response in all tested species, experiments to assess the combined effects of pesticides and predation risk require further refinement. First, understanding the ecological role of chemical cues in predator-prey interactions (i.e. kairomones and alarm cues) would greatly benefit from the determination of the chemical identity of the compounds involved in inducing the defences. This would facilitate the use of ecologically realistic concentrations of those chemical cues in future experiments, in terms of perceived predation risk (Burks & Lodge 2002; Pohnert *et al.* 2007). Furthermore, once we know what infochemicals a given species use, we can look for differences in sensitivities to those chemicals among populations and species to address the questions about how differences in detection and response to predation cues may be related to local differences in predation risk.

Moreover, this study shows that organisms process information present in alarm cues from macerated conspecifics and in predator kairomones to assess the level of predation risk, adjusting the magnitude of their responses to the different levels of perceived threat and that the magnitude of the prey's response to chemical cues is concentration dependent.

We also provide evidence that a combination of prey-specific alarm cues and predator-specific substances is necessary to trigger the full deployment of antipredator responses in daphnids although more reversible and immediate responses, with lower costs, can be triggered by alarm cues alone. This is in agreement with literature which state that costly defences such as morphological or life-history responses probably require more reliable cues for their induction, explaining why they are less frequently observed as responses to single cues (Huryn & Chivers 1999; Slusarczyk 1999; Schoepner & Relyea 2005; Brodin *et al.* 2006; Beketov & Liess 2007). In the experiments reported here, it was necessary to maintain the same concentration of kairomone and alarm cues across treatments, and thus we did not feed trout with prey when preparing the kairomone stock solutions. Water conditioned by fish fed with conspecifics could induce even stronger responses, compared to a mixture of macerated conspecifics and kairomones, due to the activation of alarm cues by digestive enzymes or digested prey tissues (Stabell *et al.* 2003; Jacobsen & Stabell 2004), thus enhancing the antipredator responses and possibly changing prey responses to pesticides under predation risk.

Although the range of concentration tested was limited, the dose response relationships should be determined using more concentrations of predatory cues, of the pesticide and of

their combinations. This will contribute to a better calibration of the models used, which rely on dose response curves. Moreover, deviations from additivity that were not limited to antagonism or synergism were detected by models, suggesting that the effects of pesticides under different levels of predation risk depend on the level of each stressor and also on the ratio of the mixture. This is especially important since other studies have pointed out that synergism between pesticide stress and predatory cues may only be present at intermediate relatively low concentrations of pesticides which may not have been tested here in the case of the experiments with insects (Relyea 2004b, 2005b; Campero *et al.* 2007).

The fact that only additive effects of combined exposure of imidacloprid and predation risk were found in experiments with insects, may be related to the endpoints tested. By not conducting longer term experiments, costs of induced phenotypic responses to predation risk in terms of reproductive fitness such as size or mass at emergence and reduced fecundity, might have been neglected. In fact, some authors show that insects can increase development rates at the cost of size at maturity in response to fish predation risk (Peckarsky *et al.* 2001; Dahl & Peckarsky 2003b) suggesting a possible physiological basis for the antipredator response. Furthermore effects of the interaction of pesticide stress and predation risk in insects have been shown to be not only behavioural mediated by food intake but also by physiological mechanisms such as food assimilation and conversion (Campero *et al.* 2007), reduced allocation of acquired resources to growth (Stoks *et al.* 2005b) or reductions of energy storage levels (Stoks *et al.* 2005a) all of which we did not measure in the experiments with insects. Because sublethal concentrations of pesticides have also been shown to affect the size of emergent insects (Alexander *et al.* 2008), it will be important to expand the mechanistic study of effects of combined exposure to pesticides and predation risk by measuring different responses (digestive physiology, energetic reserves, size at maturity, fecundity) which will ultimately determine the overall effects on reproductive fitness (Pauwels *et al.* 2005; Stoks *et al.* 2005b; Beckerman *et al.* 2007; Campero *et al.* 2007).

Although our studies reported phenotypic plastic responses in different organisms, and extrapolated from the results, there was no further consideration of the adaptive value of plasticity. If plasticity really is adaptive, then it should be favoured not only because of fitness trade-offs associated with induced responses, but also because different selective pressures on the traits exhibiting plasticity should be evident (Tollrian & Harvell 1999). It

is thus essential to measure the benefits of induced responses (reduction in predation rates) and their fitness costs (preferably over a longer time period and under more natural conditions) and also to test if selection truly favours plasticity, in order to understand the adaptive value of phenotypic plasticity (DeWitt *et al.* 1998; Stibor & Navarra 2000; Relyea 2002).

Similarly, when considering sub-lethal effects of pesticides and the effects of pesticides on predator prey interactions, the “non-adaptive” value of prey responses simultaneously exposed to pesticides and predation risk should be also assessed. One cannot completely address the question of negative effects of pesticides on predator prey interactions without testing the fitness costs and divergent selective pressures on the plastic traits. However, most studies have been using simple experimental designs to address the adaptive value of inducible defences to the presence/absence of a single type of predator. This approach although straightforward in terms of interpretation can mask and underestimate potential costs that inducible defences may have in the multitude of other environments that prey encounter.

The increasing number of studies dealing with induce antipredator defences improved our understanding on several physiological and behavioural aspects that mediate the interactions between pesticide exposure and predation risk at the organism level. Nevertheless, higher-tier experiments are needed to verify if these observed responses have effects in the population, and potentially play a role in structuring populations and communities. A more comprehensive approach including relevant selective environments for prey and predator(s) will contribute substantially to our understanding of the ecology and evolution of phenotypic plasticity while improving our predictive ability to predict effects of pesticides in complex natural ecosystems.

Therefore we need to identify other environmental factors that are relevant for a particular organism, determining how these factors may affect phenotypic responses and responses to pesticide exposure, and to assess the fitness consequences of those responses. For example, natural systems typically contain diverse predator assemblages that include predators with different foraging techniques, prey size selection, and population densities that can modify the magnitude and direction of induced responses, and thus alter the effects of pesticides (Relyea *et al.* 2005). It is also important to consider the relative tolerance of predators and prey in any particular system and therefore density and trait

mediated effects should be considered with exposure of pesticides to both prey and predator since they vary according to their relative tolerance to the pesticide (Mills & Semlitsch 2004). Organisms with induced antipredator responses may also have lower fitness (i.e. slower growth, increased susceptibility, increased costs or constrains) in the presence of other biotic and abiotic stressors such as competitors (Mills & Semlitsch 2004; Bolnick & Preisser 2005), parasites or food shortage (Anholt & Werner 1998; Stoks 2001; Beketov & Liess 2007; Gliwicz & Maszczyk 2007), or seasonal time stress (for organisms with complex life cycles) (Benard 2004; Stoks *et al.* 2006; Relyea 2007) that can also change the way prey balance the growth-predation risk trade off due to physiological and behavioural constrains. Since the toxicity of pesticides can also be affected by these environmental variables, it is important to study the effects of pesticides under more natural conditions to correctly assess the fitness costs of the combined exposure and the overall impacts of pesticides on populations and natural communities.

From the ecological community perspective, increased research on phenotypic plasticity has altered the view that density-mediated effects of predation are the driving forces behind indirect interactions. Indeed, when a species alters its behaviour, physiology, morphology or life history according to different environments (predation, competition, abiotic cues) it can significantly affect interactions with other members of the community. It has been suggested that trait-mediated indirect interactions can vary in magnitude but can be just as strong as density mediated indirect interactions (Peacor & Werner 2001; Miner *et al.* 2005; Preisser *et al.* 2005). More input is needed to verify the relative importance of trait and density mediated indirect effects by testing not only non-lethal predators but also reducing prey density to simulate thinning by predators. Moreover, for trait mediated effects to affect community structure, they must alter the population parameters (e.g. r) of the species within the community. Therefore, there is a need to conduct multigenerational, long-term studies that examine population parameters. Due to practical reasons such as long generation times of model species or and the difficulty to manipulate predators over long time scales, assessing the long term importance of density and trait mediated indirect effects of predation or pesticide stress has been a challenging task. Methodologies used in this work can facilitate in part these logistical aspects.

To conclude, while we continue to gain a basic understanding of the indirect effects of pesticides on predator-prey interactions, future studies that incorporate field-relevant

scenarios such as different predator and prey species, relevant pesticide concentrations tested in complex environments varying in the level of resources and including other stressors like competition or time stress, preferably accounting for long-term effects, would be extremely valuable. By incorporating more ecological complexity into our experiments (e.g. multispecies and multitrophic designs) we will better understand the ecology and evolution of predator induced plasticity and better predict the indirect effects of pesticides on natural communities and ecosystem processes.

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