The Role of Crustaceans within an Integrated Mosquito Control Management

by

Iris Angela Kröger
from Munich

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Fachbereich 7: Natur- und Umweltwissenschaften
Universität Koblenz-Landau

Thesis examiners:
PD Dr. Matthias Liess, Helmholtz Centre of Environmental Research, Leipzig
Jun.-Prof. Dr. Ralf Schäfer, University Koblenz-Landau

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Summary

Mosquito control is an important issue for human society, as many mosquito species are vectors for diseases (e.g., malaria, dengue, west-nile). Common mosquito control strategies are mainly based on the use of insecticides, which often have side- effects on non-target species. Increasing environmental awareness as well as the development of insecticide resistance caused a turn in mosquito control strategies towards biological control and integrated mosquito management.

The detection of the biological insecticide Bti (*Bacillus thuringiensis* var. *israelensis*) was an important step towards an environmentally friendly control strategy. Bti acts specific on mosquito larvae (Diptera: Culicidae); however, its effectiveness is limited in time due to natural degradation. The aim of the following investigations was to prolong the impact of Bti by using natural antagonists of mosquito larvae. Antagonistic crustaceans like filter feeding Cladocera or predatory Copepods are very common in nearly all kind of water bodies. Their negative impact on mosquito larval populations has been demonstrated under lab conditions and within two-species set-ups. Most investigations, however, were focused on predatory Copepods (e.g. Mesocyclops). The role of competing crustaceans (e.g. Cladocera) and mixed communities consisting of both predatory and competing crustaceans for mosquito larval control has been poorly investigated. The present thesis was conducted to investigate this topic. In particular three questions need to be answered:

1) Are crustaceans **relevant** for mosquito larval control under field conditions in terms of spatial and temporal niche overlap? Which crustacean taxa are most suitable for biological control of which mosquito species?
2) Which **mechanisms** drive the negative impact of crustaceans on mosquito larvae? Does species diversity of mixed crustacean communities influence the impact on mosquito larval populations? Which stages of mosquito development are affected by crustaceans (e.g. oviposition, larval development)? Do crustaceans alter the impact of Bti treatment on mosquito larvae?
3) How strong is the **effect** of crustaceans on mosquito larval populations either alone or in combination with insecticide application?

At a first step field observations were done, to investigate spatial and temporal habitat overlap between different mosquito species and different crustacean taxa. Synchron development was reported for larvae of *Ae.vexans* and Cyclopoida, which colonized newly established ponds most rapidly. Larvae of *Cx.pipiens* coocurred with Cladocera, but the latter established delayed in time; hence the impact of Cladocera on larvae of *Cx.pipiens* was restricted to the later part of the hydroperiod (≥ 3 weeks). Habitat structure influenced time of species occurrence with ponds at reed-covered wetlands favouring crustacean development, while ponds at grassland habitats favoured colonization by mosquito larvae. Established populations of crustaceans prevented colonization of mosquito larvae until the end of hydroperiod

The mechanisms driving the negative effect of crustaceans on mosquito larvae were investigated within an experiment under artificial conditions. Crustacean communities were found to reduce both oviposition and larval development of *Cx.pipiens*. Crustacean communities of high taxa diversity, including both predatory and competing crustaceans, were more effective compared with crustacean communities dominated by single taxa. Presence of crustacean communities characterised by high taxa diversity increased the sensitivity of *Cx.pipiens* larvae towards Bti and prolonged the time of recolonization. However establishment of diverse crustacean communities took about 2 weeks.

In a final step the combined approach, using Bti and crustaceans, was evaluated under field conditions. The joint application of Bti and crustaceans was found to reduce mosquito larval populations over the whole observation period, while single application of Bti caused only short-term reduction of mosquito larvae. Single application of crustaceans had no significant effect, because high abundances of prior established mosquito larvae impeded propagation of crustaceans. At combined treatment, mosquito larvae were reduced by Bti application and hence crustaceans were able to proliferate without disturbance by interspecific competition.

In conclusion, natural competitors were found to have a strong negative impact on mosquito larval populations. However, a time span of about 2 weeks has to be bridged, before crustacean communities reached a level sufficient for mosquito control. Results of a combined approach, complementing the short-term effect of the biological insecticide Bti with the long-term effect of crustaceans, were promising. Using natural competitors within an integrated control strategy could be an important tool for an effective, environmentally friendly and sustainable mosquito management.

Zusammenfassung

Die Kontrolle von Stechmücken ist in vielen Ländern der Erde ein wichtiges Thema, da einige Mückenarten gefährliche Krankheiten übertragen können (z.B. Malaria, Dengue). Herkömmliche Kontrollmethoden beinhalten hauptsächlich den Einsatz von chemischen Insektiziden, die jedoch nicht nur Mücken sondern auch Nicht-Ziel Organismen schädigen. Intensiver Einsatz von Insektiziden führt zudem schnell zu Resistenzbildungen in den Mückenpopulationen. Daher richtet sich der Fokus mehr und mehr auf die Entwicklung biologischer Kontrollmethoden, um Mückenmanagement nachhaltig und umweltfreundlich zu gestalten.

Ein erster Schritt in diese Richtung war die Entwicklung des biologischen Insektizids Bti (*Bacillus thuringiensis* var. *israelensis*). Dieses Insektizid wirkt spezifisch auf Mückenlarven (Culicidae). Allerdings ist seine Wirkungsdauer begrenzt, da Bti innerhalb kurzer Zeit biologisch abgebaut wird. Ziel der folgenden Untersuchungen ist es, die Wirkungsweise von Bti zu verbessern. Dabei sollen natürliche Gegenspieler der Mückenlarven zum Einsatz kommen. Räuberische und konkurrierende Kleinkrebse (Crustacea) kommen natürlicherweise in fast allen aquatischen Biotopen vor. Bislang wurde aber hauptsächlich die Wirkung räuberische Kleinkrebse (z.B. Mesocyclops) auf Mückenlarven untersucht. Wie wichtig Nahrungskonkurrenten (z.B. Cladocera) und gemische Artgemeinschaften (bestehend aus Räubern und Konkurrenten) für die Mückenkontrolle sind, ist weitgehend unbekannt. Dies soll in der hier vorgestellen Arbeit untersucht werden. Dabei sind 3 Fragestellungen von besonderem Interesse:

1) Sind Kleinkrebse unter natürlichen Bedingungen überhaupt **relevante** Gegenspieler der Mückenlarven? Besteht eine zeitliche und räumliche Überlappung in der Entwicklung dieser beiden Gruppen und welche Mückenarten werden von welchen Kleinkrebs-Klassen beeinflusst?
2) Auf welchen **Mechanismen** beruht die Interaktionen zwischen Kleinkrebsen und Mückenlarven? Spielt neben der Kleinkrebs-Dichte auch die Artenvielfalt eine Rolle? Welche Stadien der Mückenentwicklung (Eiablage, Larvenentwicklung) werden durch Kleinkrebse beeinflusst? Verändert die Anwesenheit von Kleinkrebsen den Effekt von Bti auf Mückenlarven?
3) Wie stark ist der **Effekt** von Kleinkrebsen auf Mückenlarven im Vergleich zum Insektizid Bti? Wird die Wirkung verstärkt, wenn man Kleinkrebse und Bti kombiniert?

In einem ersten Schritt wurden Feldbeopbachtungen durchgeführt um die räumliche und zeitliche Einnischung von Kleinkrebsen und Mückenlarven zu erforschen. Larven der Mückenart *Ae.vexans* etwickelten sich synchron mit der Kleinkrebsordnung Cyclopoida innerhalb einer Woche nach Entstehung eines Gewässers. Die Abundanzen von *Ae.vexans* und Cyclopoida korrelierten negativ miteinander. Larven der Mückenart *Cx.pipiens* besiedeln dieselben Gewässer wie Cladocera, wobei letztere zeitlich verzögert auftraten. Der negative Effekt von Cladocera auf *Cx.pipiens* Larven war daher erst nach ca. 3 Wochen sichtbar. Das zeitliche Auftreten der Arten wurde durch die Biotopstruktur beeinflusst. Schilfbiotope beschleunigten die Entwicklung von Kleinkrebsen, wohingegen Gewässer auf Wiesenflächen schneller von Mückelarven besiedelt wurden. Sobald sich aber Populationen von Kleinkrebsen etabliert hatten, wurde Mückenlarven nachhaltig aus den Gewässern verdrängt.

In einem zweiten Schritt wurde untersucht, auf welche Weise Mückenlarven von Kleinkrebsen beeinflusts werden. In einem Mikrokosmen-Experiment zeigte sich, dass Kleinkrebse sowohl die Eiablage der Mückenart *Cx.pipiens* vermindern als auch die Entwicklung der Mückenlarven beeinträchtigen. Artgemeinschaften mit hoher

Artdiversität, bestehend aus verschiedenen räuberischen und filtrierenden Kleinkrebsen, reduzierten Mückenpopulationen effektiver als Artgemeinschaften mit geringer Artdiversität. Die Anwesenheit diverse Krebsgemeinschaften bewirkte zudem, dass Bti bereits in geringerer Dosierung einen Effekt erzielte und *Cx.pipiens* Larven längere Zeit benötigten, um ein Gewässer nach der Behandlung durch Bti wieder zu besiedeln. Allerdings benötigten Kleinkrebse ca. 2 Wochen um eine diverse Artgemeinschaft zu etablieren.

In einem letzen Schritt wurde der kombinierte Einsatz von Bti und Kleinkrebsen under Freilandbedingungen erprobt. Die Kombination aus Bti und Kleinkrebsen reduzierte die Anzahl der Mückenlarven (*Cx.pipiens*) über den gesamten Beobachtungszeitraum hinweg. Die alleinige Ausbringung von Bti bewirkte nur eine kurzfristige Reduktion der Mückenlarven, während die alleinige Ausbringung von Kleinkrebsen keinen Effekt erzielte. Die Anwesenheit der Mückelarven verhinderte eine ausreichende Vermehrung der Kleinkrebse, während im kombinierten Ansatz die Mückenlarven kurzzeitig durch das Bti eliminiert wurden, was die Entwicklung der eingesetzten Kleinkrebse begünstigte.

Zusammenfassend konnte gezeigt werden, dass sowohl räuberische als auch filtrierende Kleinkrebse einen starken negativen Effekt auf Mückenlarven ausüben. Allerdings muss ein Zeitraum von ca. 2 Wochen überbrückt werden, bis sich eine ausreichend starke Artgemeinschaft aus Kleinkrebsen etabliert hat. Der Einsatz von Kleinkrebsen in Kombination mit dem Insektizid Bti erweist sich als vielversprechend und könnte in Zukunft dazu beitragen, Mücken effektiv und nachhaltig zu kontrollieren.

1 Introduction

1.1 Problem of mosquitoes as vector for diseases

Mosquitoes are an important part of wetland ecosystems, serving as prey for many other species, like insects, amphibians, fish and birds. However, from the human point of view, mosquitoes are mainly noticed as nuisance and, even worse, as vector for diseases. Indeed, several 100.000 deaths, caused by mosquito-born diseases (e.g., malaria, dengue, yellow-fever) are reported each year by the WHO. Most affected are people of developing countries, because (1) public health care is limited and (2) climate conditions are favourable for mosquito development. However, climate conditions are changing worldwide due to global warming. Extreme weather scenarios, like heavy rainfalls on the one hand and drought periods on the other hand, will increase (Dubrovsky et al., 2009). Mosquito development is forwarded by those climate conditions (Chase and Knight, 2003) and consequently the risk for vector-born diseases is rising (Blashki et al., 2007). The result of such development was reported in North America 2002, where an epidemic of the West-Nile-Virus, transmitted by the mosquito species *Culex pipiens*, was associated with an increase in annual rainfall (Wang et al., 2010).

The spread of vector-born diseases certainly is not only favoured by climate change, but also by global transport networks. The tiger mosquito, *Aedes albopictus*, (vector for West-Nile-Virus, Yellow fever, Dengue and Chikungunya fever) was originally located in tropical regions of Southeast Asia. However, larvae of *Aedes albopictus* were transported in used tires and therefore spread into Africa, Europe and North America (Mitchell, 1995; Juliano and Philip Lounibos, 2005). The invasive mosquito species was able to develop rapidly at the new areas, causing strong plagues and even outbreaks of Chikungunya fever (Rezza et al., 2007).

Both climate change and global transport hence increase the risk of vector born diseases. To limit the impact of mosquitoes on human health, various control strategies have been developed.

1.2 Mosquito control: from chemical insecticides towards integrated management

Mosquito control is a very old issue in human societies, dating back to the Greeks about 480 B.C. (Patterson, 2004). However, mosquito control gained a new dimension at the turn of the 20th century, when mosquitoes were detected as vectors for diseases (Mattingly, 1969). Since that time mosquito control programs were included into human health programs of many states and countries (Floore, 2006). Commonly used agents against mosquito larvae were synthetic organic insecticides with a broad spectrum of activity (e.g. DDT, Malathion, Pyrethrins). However those insecticides often had strong side effects on non- target organisms, heavily affecting human health and ecological balance of aquatic biotopes (Mestres and Mestres, 1992; Mian and Mulla, 1992). In addition, many mosquito species developed resistances against those insecticides (Raymond et al., 1991; Hemingway and Ranson, 2000; Weill et al., 2003; Suman et al., 2010). Consequently new strategies for mosquito control were required, which act environmental friendly and avoid resistance development.

1.2.1 Biological insecticides: development of *Bacillus thuringiensis israelensis* (Bti)

An important step in this direction was done, when Prof. Margalit isolated the Bacterium *Bacillus thuringiensis israelensis* (Bti) from dead mosquito larvae in Israel (Margalit and Dean, 1985). During the following years some proteins of Bti were detected being heavily toxic against mosquito larvae (Diptera: Culicidae) and blackfly larvae (Diptera: Simuliidae), when used as stomach insecticide. The mode of action of Bti was described by (Gill et al., 1992) as follows: “B.thuringiensis, a gram-positive bacteria, produces a proteinaceous parasporal crystalline inclusion during sporulation. Upon ingestion by insects, this crystalline inclusion is solubilized in the midgut, releasing proteins called δ-endotoxins. These proteins (protoxins) are activated by midgut proteases, and the activated toxins interact with the larval midgut epithelium causing a disruption in membrane integrity and ultimately leading to insect death.” An important fact of the mode of action of Bti is the interaction of four toxic components (CrylVA, CrylVB, CrylVD and CytA), causing the lethal effect of target organism. This is in contrast to common synthetic insecticides, which usually act with only one toxic component. The complex mode of action of Bti has two advantages: 1) Development of resistance against Bti is suppressed, because resistance evolves much slower, when several toxic components have to be knocked out (Georghiou and Wirth, 1997) 2) Bti acts very specific on mosquito larvae. The midgut ephitel of other species vary from those of mosquitoes and therefore toxic components of Bti do not act in other species (Ali, 1981).

Although Bti seems to be the perfect solution for mosquito control, there are also some disadvantages. Bti act as stomach insecticides and therefore mosquito larvae have to ingest it. However, filtering behaviour of mosquito larvae varies between species and depends on age, density of larvae and habitat factors (temperature, depth of water, turbidity, presence of vegetation, ect.) (Becker et al. 1992; Lacey 2007). In addition sedimentation as well as natural degradation limits the persistence and efficacy of Bti (Sheeran and Fisher, 1992). Indeed, Bti is active only for several days or few weeks (Aldemir, 2009). In consequence Bti application has to be repeated several times during one season. This practice is not only expensive, but can promote resistance. Indeed, intensive applications of an insecticide causes strong selective pressure on the target populations and accelerates resistance evolvement (Melo-Santos et al., 2010). Changes in mosquito gene expression have already been reported for populations in areas with regular Bti applications (Goldman et al., 1986; Akiner et al., 2009). Therefore development of Bti resistance could only be a matter of time.

Another aspect regards the environmental sustainability of Bti. Although Bti is most affective against Culicidae and Simuliidae, there was also mortality reported for non-target species such as Chironomidae (Kondo et al., 1995; Boisvert and Boisvert, 2000), Muscidae (Entwistle, 1993) or Tephritidae (Robacker et al., 1996). However, most of these studies were conducted under lab conditions and negative side-effects of Bti were mainly reported for Bti overdosages (Boisvert and Boisvert, 2000). Studies on side effects of Bti on non-target species under field conditions are rare and produced contradictory results. For example Bti application in the field was found to decrease chironomid larval densities and negatively affected larval emergence (Pont et al., 1999), while chironomid populations remained unaffected by Bti at other studies (Molloy, 1992; Lundström et al., 2010). However, Bti could cause not only direct effects on non-targte species (i.e. mortality), but also influence populations structures via indirect effects. Reduction of mosquitoes, which serve as prey for other aquatic and terrestrial predators, could disturb food web structures. Indeed, reduced abundances of mosquito predators, such as spiders and dragonflies, were found to reduce bird breeding success at areas treated with Bti (Poulin et al., 2010; Poulin, 2012). However, such indirect effects of Bti were not found for other higher-tier predators, such as anurans and bats (Blum et al., 1997; Arnold, 1999). More investigations are necessary to clarify the importance of mosquitoes within food web structures.

In conclusion Bti is in fact an effective insecticide with low risk of resistance development. In addition Bti acts more specific compared with other insecticides commonly used for mosquito control. However environmental factors influence Bti effectiveness and intensive and exclusive application could cause resistance development, even for this complex insecticide. Potential side-effects of Bti on non-target species are not yet sufficiently investigated.

The negative effects of one- sided Bti application could be buffered, when Bti is used in an integrated control management. Therefore a complementary control agent is necessary, which compensates the short period of Bti effectiveness and lowers the risk of resistance development. In addition this control agent should act environmentally friendly and therefore a natural antagonist of mosquito larvae seems to be most suitable.

1.1.1 Biological control using natural antagonists

In 1960 a symposium in Washington, D.C. emphasized the importance of biological mosquito control, to face the problem of insecticide resistance (Legner, 1995). Substantial increase in research on natural enemies followed. About 1500 natural antagonists of mosquitoes (parasites, pathogens and predators) were known at this point of time (Jenkins, 1964). However, many of these antagonists (i.e., pathogenic fungi, parasitic nematodes and protozoa) are of lower interest for commercial mosquito control, because of intolerance to environmental factors (Petersen, 1973; Jaronski and Axtell, 1982), inappreciable persistence at the sites (Lacey and Undeen, 1986) or difficulties during mass production (Legner, 1995).

Predators

The main interest of research was focused on natural predators of mosquito larvae. The mosquitofish, *Gambusia affinis*, has been used for mosquito since the early decades of the 1900s (Legner and Sjogren, 1984). However, mosquitofish was found to affect not only mosquito larval populations, but also reduce or even displace other native species (Miura et al., 1984; Goodsell and Kats, 1999; Pyke, 2008). In addition, mosquitofish sometimes failed to control mosquito larval populations, most likely due to reduction of other natural antagonists (Blaustein, 1992). Predatory insects and their larvae (e.g. Dystiscidae, Notonectidae, Odonata) do not only prey on mosquito larvae, but also prevent adult mosquitoes from oviposition (Fincke et al., 1997; Stav et al., 2000; Lundkvist et al., 2003; Fischer et al., 2012). However, difficulties in colonization and management of insect predators, as well as a lack of synchrony between predator and prey life cycle, impeded their deployment (Bay, 1974; Sebastian et al., 1980). Both mosquitofish and insect predators have in common, that they occur mainly in large, permanent ponds, while most mosquito species prefer temporary ponds as breeding sites (Batzer and Wissinger, 1996). Therefore their impact on natural mosquito larval populations could be overestimated, anyway. Predaceous crustaceans (i.e., Copepoda) show more suitable biotope preferences, occurring in high numbers at several types of temporary ponds (Frisch et al., 2006; Williams, 2006; Frisch and Green, 2007). Some species of Copepoda are known to be very efficient against mosquito larvae (Lardeux et al., 1992; Russell et al., 1996; Murugan et al., 2011). However, the benthic feeding behaviour of the many copepod species limit their impact on benthos foraging mosquito species like *Aedes* sp., excluding surface foraging mosquitoes like Culex (Lardeux et al., 1992). The effectiveness of Copepoda is thereby not only a result of predation but includes also competition for food (Rivière et al., 1987). Many species of Copepoda are not exclusively predators, but also filter feeders. Therefore they use also partly the same food resources compared to mosquito larvae. Indeed, food competition is a very important mechanisms affecting mosquito larval development.

Competitors

Mosquito larvae occur preferably in small, temporary ponds and those biotopes are often limited in number of trophic levels due to lack of primary productivity (Washburn, 1995). Therefore not predation but competition could be the major factor driving population dynamics in those biotopes (Schneider and Frost, 1996).

Competition is known to play an important role in mosquito larval development. The problem of invasive mosquito species (e.g. *Aedes albopictus*) caused a series of studies, investigating the competitive effect of invasive mosquito species on native mosquito species. Many mosquito species were found to react very sensitive to interspecific competition with other mosquito species, often resulting in competitive exclusion of one species (Juliano, 1998; Braks et al., 2004; Costanzo et al., 2005). However, in terms of mosquito control, it makes limited sense to replace one mosquito species by another, particularly when both species carry vector abilities. Mosquito larvae are also sensitive towards intraspecific competition (Spencer et al., 2002; Kiflawi et al., 2003). Again, this is not useful for mosquito control. Other competitor species, like crustaceans (e.g. Cladocera, Ostracoda), could be more useful for that purpose. Crustaceans are very common in nearly all kind of ponds (Williams, 2006); they occur in high numbers and can colonize new biotopes within short time periods. Many crustacean species are filter feeders, using similar food resources as mosquito larvae. Previous studies demonstrated a negative correlation between crustaceans and mosquito larvae in the field (Chase and Knight, 2003; Meyabeme Elono et al., 2010). Populations of *Daphnia magna* reduced mosquito oviposition and larval abundances at two-species test systems (Duquesne et al., 2011). In addition *Daphnia* sp. increased time to mosquito larval metamorphosis and larval size at metamorphosis (Stav et al., 2005). Hence *Daphnia* sp. has a strong negative effect on mosquito larval populations, but is this effect also relevant under field conditions? Do mosquito larvae and Daphnia (or other Cladoceran species) develop synchronously in terms of temporal and spatial occurrence? Does the presence of other crustacean species increase or decrease the impact of a certain crustacean species on mosquito larval populations? In addition we need to know, if the presence of crustaceans influence the effect of insecticide application in order to assess their relevance within integrated mosquito control.

1.1.2 Integrated pest management

The two different control strategies, using insecticides or natural antagonists respectively, were used exclusively in most cases. The development of chemical insecticides in fact lowered the interest in biological control strategies, because chemical insecticides were much easier to produce and to handle compared to natural antagonists (Legner, 1995). However, when the problem of resistance evolvement came up, biological control gained importance again. Nevertheless, chemical insecticides and natural antagonists were incompatible for a long time, because insecticides with a broad spectrum of activity did not only affect the target organism, but also the natural enemies (Gentz et al., 2010). Newer insecticides, like Bti, act with higher selectivity and are therefore more suitable for integrated pest management. The advantages of tandem application of insecticides and natural enemies were already demonstrated in agriculture and urban settings (Batra, 1982; SU and Scheffrahn, 1998). However, integrated pest management in mosquito control is poorly investigated. Few studies investigated the effect of “biorational” compounds (e.g., Bti or methoprene) in combination with predatory copepods against container breeding mosquitoes, clearly demonstrating an increase in effectiveness of the joint application compared to single application (Rivière et al., 1987; Tietze et al., 1994). However it remains unclear, if competing crustaceans, such as Cladocerans, or mixed communities consisting of different crustacean taxa are also useful for integrated mosquito management. The following study focused on this question, investigating the effect of different crustacean taxa in combination with the biological insecticide Bti.

1.2 Concept and aim of the study

The aim of the dissertation was to evaluate the role of antagonistic crustaceans for mosquito larval control, either alone or in combination with the biological insecticide Bti. Changing impact of crustacean communities on mosquito larval populations according to variations in crustacean taxa composition were addressed.

We first investigated the biological niche overlap between different crustacean taxa and different mosquito species under field conditions in order to assess the relevance of crustaceans for mosquito control.

Second we investigated the effect of altering crustacean community structures (i.e. changes in crustacean abundances and crustacean taxa diversity) on mosquito larval populations during the process of pond colonization. Bti was applied in order to assess, if different types of crustacean communities alter sensitivity of mosquito larvae towards Bti or change mosquito recolonization behaviour after Bti treatment.

In a final step we evaluated under field conditions, if the combined application of crustaceans and Bti was more effective and sustainable against mosquito larval populations compared with single applications.

Results of the three studies are reported within three publications. Concepts and aims of those publications are presented below, while the results are summarized in chapter 5.

Publication 1:

The first study was designed to assess temporal and spatial niche overlap between larval populations of different mosquito species and antagonistic crustaceans under field conditions. Therefore a field monitoring was conducted to answer the following questions:

- Which crustacean taxa co-occur synchronously with larvae of most abundant mosquito species in terms of spatial and temporal appearance?
- Do environmental conditions, like biotope structure or duration of hydroperiod, influence community structure by giving developmental advantage to certain species?
- Is the negative relationship between crustaceans and mosquito larvae visible under field conditions?

Publication 2:

An outdoor microcosm experiment was designed to investigate the impact of crustacean communities, consisting of multiple species, on colonization behaviour of the mosquito species *Culex pipiens* (i.e. oviposition, larval abundances and larval development). We thereby compared set-ups including crustacean communities at different levels of colonization, i.e. set-ups recently inocculated with crustaceans and set-ups including crustaceans after 2 weeks of propagation. The following questions should be answered:

- Does the adverse effect of crustacean communities on mosquito larval populations change with alteration in crustacean species composition and crustacean abundances during the process of pond colonization?
- Which characteristics of crustacean communities are more important for mosquito larval control, crustacean abundances or crustacean taxa diversity? How do these two factors change during the process of pond colonization?

In addition we treated the set-ups with different concentrations of the biological insecticide Bti (*Bacillus thuringiensis israelensis*) to answer the following questions:

- How does the sensitivity of mosquito larvae towards Bti-treatment change in presence of crustacean communities at different levels of colonization?
- Do crustacean communities prolong the effect of Bti-treatment on mosquito larvae and inhibit recolonization after Bti-treatment?

Publication 3:

The first two studies of this dissertation showed, that crustaceans have a strong negative effect on mosquito larval populations, additionally increasing the effect of Bti application. In a third step we evaluated a combined approach, using Bti and crustaceans for mosquito control in the field.

The following questions should be answered:

- Is it possible to establish crustacean communities via active application in the field?
- Is joint treatment, using Bti and crustaceans, more effective compared to single treatment in terms of actute mortality and time of recolonization?

1.3. Study design

The present investigations were performed using different study designs according to the research questions addressed. The first study was performed to show, if certain crustacean taxa cooccur with mosquito larval populations under field conditions. Field conditions, however, are highly variable, changing with biotopes, geographical regions, seasonal change etc. In order to generate a representative dataset, a large-scaled field monitoring was performed, investigating various types of ponds at different locations (Fig. 1.1). Data of different ponds were hardly comparable and hence statistical power was sometimes low.

Figure 1.1. Examples of ponds investigated during large-scales field monitoing (i.e. first study).

The second study was conducted to investigate in detail, how crustacean communities affect mosquito oviposition and larval development after treatment with Bti. The study required more controlled conditions in order to generate replicates characterised by similar crustacean communities. However, undisturbed oviposition behaviour of mosquitoes was required. Therefore an outdoor microcosm study was designed, which allowed for regulation of crustacean communities via artificial introduction, equal environmental conditions supporting equal development of crustacean communities, and natural oviposition behaviour of mosquitoes (Fig. 1.2.). Results of different set-ups were well comparable and some important mechanism driving mosquito-crustacean interactions became visible. However the artificial design as well as the occurrence of only one mosquito species limited extrapolation of results.

Figure 1.2. Outdoor pond microcosm at the UFZ area, designed for second study.

The third study was conducted to investigate the effect of Bti-treatment and introduction of crustaceans on mosquito larval populations, either alone or within a combined approach. The study required a set of ponds characterised by similar starting conditions (i.e. prior colonization by the same mosquito species but no colonization of crustaceans). However, the study was performed in the field in order to prove, that applied mosquito control strategies work under natural conditions. Hence a small scaled field study was conducted, investigating small ponds (< $8m^2$) at one biotope and one geographical location (Fig. 1.3). Results of different set-ups were well comparable and representative for this certain environment. However, only one mosquito species and only one crustacean taxa became established at those ponds. Hence applicablility of investigated mosquito control strategies on lage-scaled field conditions (including several mosquito species and different environmental conditons) still needs to be proven.

Figure 1.3. Examples of ponds investigated at small scaled field study (i.e. third study).

The small scaled analyses of mechanisms and effects driving the crustacean impact on mosquito larval populations was complemented by large-scaled field investigation, demonstrating the relevance of findings for mosquito control. The combination of different stuy designs was most useful to compensate disadvantages of single designs; e.g. trends observed at large scaled field investigation became significant under more controlled conditions.

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2 Temporal and spatial habitat preferences and biotic interactions between mosquito larvae and antagonistic crustaceans in the field

Kroeger, I.[1,2], Liess, M.[1] & Duquesne, S.[1,3]

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[1]Department of System Ecotoxicology, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany
[2]Quantitative Landscape Ecology, Institute for Environmental Science, University of Koblenz-Landau, Fortstraße 7, D-76829 Landau, Germany
[3]Department of Biology Conservation, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany

2.1 Abstract

Investigations on natural antagonists of mosquito larvae found that micro-crustaceans (e.g., Cladocera) control mosquito populations under experimental conditions. However, their relevance for mosquito control under field situations remains widely unclear because important information about habitat preferences and time of occurrence of crustaceans and mosquito larvae are still missing. In order to fill this knowledge gap, a field study was undertaken in different wetland areas of Saxony, Germany, in different habitats (i.e., grassland, forest, and reed-covered wetlands). We found negative interactions between larvae of *Ae. vexans* and predatory Cyclopoida (Crustacean: Copepoda), which both were dominant during the first two weeks of hydroperiod, at ponds located at grassland habitats. Larvae of *Cx. pipiens* were spatially associated with competing Cladocera, but they colonized ponds more rapidly. Populations of Cladocera established from the third week of hydroperiod and prevented *Cx. pipiens* colonization thereafter. Ostracoda were highly abundant during the whole hydroperiod, but their presence was restricted to habitats of reed-covered wetland at one geographical area. Mosquito larvae hardly occurred at those ponds. In general, we found that ponds at the reed-covered wetlands provided better conditions for the initial development of crustaceans and hence, mosquito larval colonization was strongly inhibited. Grassland habitat, in contrast, favored early development of mosquito larvae. This study showed that micro-crustaceans are relevant for mosquito management but their impact on mosquito larvae varies between species and depends on environmental conditions.

KEY WORDS antagonists, competition, predation, Culicidae, hydroperiod,

2.2 Introduction

Given their roles as vectors for diseases such as dengue and West Nile virus, mosquitoes continue to have a considerable impact on human societies. Most programs to control mosquitoes involve the application of insecticides. However, biological control strategies have become increasingly important, given the unwanted effects of intensive use of insecticides on non-target species; these effects include disturbance of the structures of communities and the functioning of ecosystems (Mian and Mulla 1992, Suma et al. 2009, Mommaerts et al. 2010), as well as the risk that mosquitoes develop insecticide resistance (Akiner et al. 2009, Melo-Santos et al. 2010).

The use of natural antagonists for mosquito larval control, however, requires synchronous development and similar habitat preferences for the target species (i.e. mosquito larvae) and the antagonist species. Many predators, such as fish, Odonata, or Coleoptera, are highly efficient against mosquito larvae under artificial conditions (Shaalan and Canyon 2009, Saha et al. 2010). However, investigations on species occurrence along a freshwater habitat gradient from small ephemeral ponds to large permanent lakes concluded that large predators are mostly common in more permanent ponds (Wellborn et al. 1996), while mosquito larvae breeds preferably in temporary ponds (Chase and Knight 2003, Becker et al. 2010). Life traits (e.g., long generation time and low reproduction rates) make large predators susceptible to drying conditions; thus their impact on community structures in such habitats is restricted.

The simplified community (reduced numbers of trophic levels) at temporary ponds suggests that competition is an important factor influencing community structures (Schneider and Frost 1996). Several controphic competitors of mosquito larvae, such as snails, tadpoles and cladocerans, were identified (Blaustein and Chase 2007) and their negative impact on mosquito larval populations was demonstrated under artificial conditions (Blaustein and Margalit 1996, Knight et al. 2004, Duquesne et al. 2011). However, in the field the impact of competition is expected to be limited at the beginning of hydroperiod because resources are likely to be high while abundances of competitiors are low (Wellborn et al. 1996, Wilbur 1997). Hence, mosquito larvae could complete their metamorphosis and leave the water body before populations of competitors had established. Therefore, small predators, rather than competitors, are likely to play an important role at the early phase of pond colonization. Predatory copepods, for example, were already successfully applied against mosquito larval populations in laboratory settings (Tietze et al. 1994, Russell et al. 1996, Rey et al. 2004) and field microhabitats like tree-holes and land crab burrows (Rivière et al. 1987, Lardeux et al. 1992). Copepods were reported to colonize newly established habitats rapidly (Frisch and Green 2007), and could affect mosquito larval populations from the beginning on. However, the impact of both predation and competition varies according to environmental conditions and presence of other species. For example, the presence of alternative prey reduces predation pressure on one species (Blaustein and Karban 1990), while high food resources alter competition impact (Bevins 2007). More information is necessary to characterize both the habitats and the temporal windows, which allow for natural mosquito control via predators or competitors.

In the present field investigation, we monitored mosquito larvae of different species and controphic micro-crustaceans at different types of habitats during the colonization process of ephemeral ponds. The study aimed to characterize habitat preferences of the different species and the time window of predatory or competitive interactions.

2.3 Materials and Methods

2.3.1 Characterization of ponds

Field investigations were performed in three different federal states of Germany. The sites in Rosslau (Saxony-Anhalt) were located on the floodplain of the River Elbe. The sites in Spreewald (Brandenburg) were located in a region of meadowy countryside and moorlands characterized by channels of the River Spree. The sites in Leipzig (Saxony) were located on the floodplain of the River Parthe.

Investigations were performed in three different types of habitat: grassland (n = 16), reed-covered wetland (n = 7), and forest (n = 3). The grassland areas were characterized by vegetation less than 50 cm in height (i.e., without trees and bushes). In contrast to the other habitats, all grassland areas were cropped by farmers twice a year. The cut grass was removed, which resulted in only a limited amount of degraded plant material on the ground. The reed-covered wetland areas were characterized by the presence of reeds of approximately 1 to 3 m in height. In these areas, degraded plant material covered the ground. Finally, the forested areas were characterized by trees of approximately 6 m in height. In these areas, shrubs were scarce and dead leaves covered the ground.

Twenty-six ponds in total were investigated, which included 11 ponds at Rosslau (seven in grassland, two in reed-covered wetland, two in forest), nine ponds at Spreewald (seven in grassland, one in reed-covered wetland, one in forest), and six ponds at Leipzig (two in grassland, four in reed-covered wetland).

The monitored ponds were classified into two categories according to the duration of their hydoperiod. Ponds that dried out latest after two weeks and were naturally refilled by rainwater several times during the observation period were classified as “short-term flooded ponds” (N=16). Ponds, with a hydroperiod >two weeks were classified as “medium-term flooded ponds”; those ponds were not refilled after drying. Most of those ponds dried out after seven weeks and therefore only data from a hydroperiod ≤ seven weeks are reported in the following.

2.3.2 Sampling and determination methods

A water sample with a total volume of one to three liters (depending on the pond size) was collected once a week from each pond from June 11 until September 7 of 2007. This water sample consisted of several 300-ml subsamples that were collected randomly with a scoop from both bank and mid-water parts of the pond. The samples were filtered through a plankton net (55-µm mesh size) and preserved in 70% ethanol (a volume of approximately 30 ml). The composition of the zooplankton was then analyzed using a binocular Leica S6D microscope (Leica, Wetzlar, Germany). Mosquito larvae were characterized to the species level using the key of Becker et al. (2010). All other invertebrates were characterized to the suborder or family level using the key of (Stresemann 1957).

Water parameters were measured once a week between 09:00 and 14:00, including dissolved oxygen (Oxi340 oxygen meter, WTW, Weilheim, Germany), as well as pH, conductivity, and temperature (pH/EC/TDS Combo testing meter, Hanna Instruments, Germany). The percentage of emergent vegetation cover (water surface covered by vegetation in relation with total water surface) and the surface area of the water were estimated visually. The depth of the water was assessed with a ruler as the mean value of three random measurements at different points in each pond. The surface areas of the ponds varied from 5 to more than 1,000 m2 and water depth varied from 3 to 70 cm.

2.3.3 Data analysis

In a first step, the relevant environmental parameters driving distribution of mosquito species and micro-crustacean taxa were identified. Therefore, the impact of physiochemical parameters (water temperature, pH), spatial parameters (geographical location and habitat structure), and temporal parameters (duration of hydroperiod and date) on species abundances were analyzed using redundancy analysis (RDA) in accordance with a previous report (Legendre and Legendre 1998). Only species encountered frequently (species present in >20% of ponds) were included, and non-significant parameters were excluded from the model during backwards selection. As a result, species sharing similar habitat preferences were detected. All count data were log(x+1)- transformed prior to analysis.

In a second step, the interaction between mosquito larvae and associated crustacean taxa (identified above) at different habitats was analyzed. Habitats characterized by highly ephemeral ponds (hydroperiod <two weeks) were represented by a maximum of two data sets per pond, but ponds were refilled several times during the observation period. Multiple measurements did not represent a consecutive time line but rather sporadic events during the course of observation. Physiochemical parameters changed markedly within data sets of ponds over time, but community structure, such as the occurrence of certain species and magnitude of abundances (rather low or elevated abundances) was similar. Therefore, we used mean values of each pond to produce a more homogenous data set and strengthen analysis on species interactions. We are aware that we thereby lost information about the impact of physiochemical parameters on species occurrence, but the sporadic measurements of those parameters did not allow for statistical analysis.

More temporary ponds were defined as scenarios corresponding to hydroperiod >five weeks. In such scenarios, multiple measurements represented changes in physiochemical parameters and community structures along a consecutive time gradient; therefore, all data points were included in the analysis. The use of multiple measurements of ponds during hydroperiod violated the statistical assumption of independence of observations for standard testing (West et al. 2006). Therefore, a generalized least squares (GLS) mixed model approach was performed. Whereas our response variable was “mosquito larval abundances,” the predictor variable was “crustacean abundances.” The analysis used multiple measures over time in multiple ponds. We thus used the factorial variable “pond” as a random effect. Given that temporal autocorrelation of subsequent measures in the same pond had to be expected, we additionally inserted an autocorrelation structure (AR1-autoregressive model of order 1; Zuur et al. 2009). The model was generated using the restricted maximum likelihood (REML) calculation. Analyses were performed using the ‘R’ statistical and programming environment and the ‘nlme’ and ‘lattice’ packages.

2.4 Results

2.4.1 Mosquito species and their associated communities

In total, 13 species of mosquito larvae were observed (*Ochlerotatus mercurator, Ochlerotatus euedes, Aedes vexans, Aedes cinereus, Culex pipiens, Culex territans, Culex martinii*, *Culex hortensis*, *Anopheles claviger*, *Anopheles hycranus*, *Anopheles maculipennis, Culisetta annulata*, and *Culisetta longiareolata*). However, given that only two species (*Ae. vexans, Cx. pipiens*) occurred frequently (presence at more than 30% of ponds), these were the only species included in the statistical analyses.

The associated crustacean community consisted of Ostracoda, Copepoda (Cyclopoida and Harpacticoida), and Cladocera (Chydoriidae and the Daphniidae genera *Daphnia*, *Ceriodaphnia*, *Simocephalus*, S*capholeberis*). In addition, Asselidae, Gastropoda, as well as larvae of Ephemeroptera and Chironomidae were frequently observed. However, we did not find any negative correlation between non-crustacean taxa and mosquito species (data not shown); hence, those non-antagonistic species were not further reported. Different species of predatory insects (i.e., Odonata, Dytiscidae, Chaoboridae, Notonectidae, and Corixidae) were sporadically found in the samples (presence at less than 30% of samples). Our question was focused on crustaceans and so was the sampling method adapted; hence, only crustaceans were included in the statistical analysis.

2.4.2 Habitat preferences of mosquito larvae and antagonistic crustaceans

In a first step, we analyzed which crustacean taxa preferred the same habitat compared with mosquito larvae in terms of physiochemical and temporal parameters. Results of multivariate analysis (RDA) identified the following parameters relevant for species distribution: water temperature (variance 0.350, p = 0.005), water level (variance = 0.173, p = 0.005), pH (variance = 0.538, p = 0.005), hydroperiod (variance = 0.460, p=0.005), season (variance = 0.287, P=0.005), habitat (variance = 0.138, p=0.020), and location (variance = 0.470, p=0.005). The model explained 50% of the total variance (Figure 2.1). Larvae of *Aedes vexans* were most strongly and negatively correlated with duration of hydroperiod and preferred forest and grassland habitats and Rosslau locations (Figure 2.1). They tended to be associated with the crustacean taxa of Cyclopoida. Larvae of *Culex pipiens* were positively correlated with water temperature, season, and duration of hydroperiod (Figure 2.1). They tended to be associated with Daphniidae and occurred at Rosslau and Spreewald locations. Ostracoda and Harpacticoida were positively correlated with pH and occurred preferably in reed habitats of the Leipzig area (Figure 2.1). They were unaffected by duration of hydroperiod and developed apart from mosquito larvae.

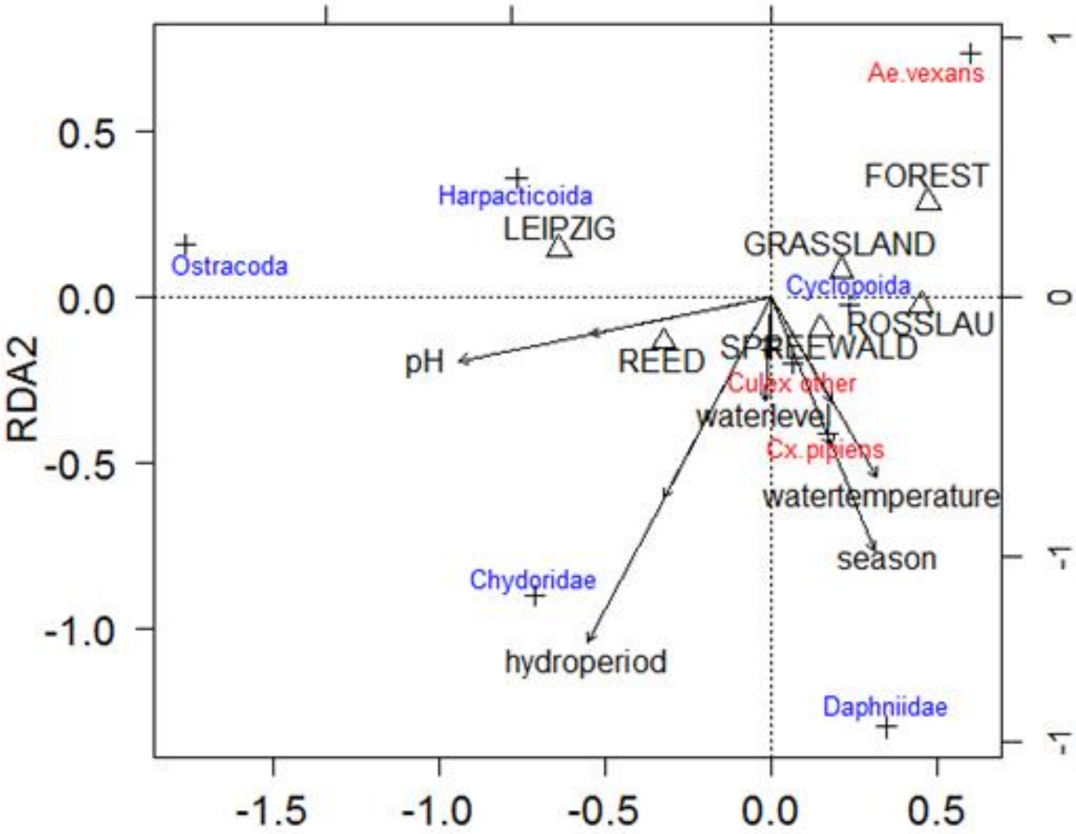

Figure 2.1: Ordination plot for redundancy analysis (RDA). Rare taxa (present in <20% of ponds) were excluded. Arrows represent independent variables (water parameters), whereas crosses represent dependent variables (mosquito species and crustacean taxa). For clarity, only taxa that were well explained by the model (species score >0.3 for one of the first two axes) are labeled (red: mosquito species, blue: crustacean taxa).

2.4.3 Biological interactions between mosquito larvae and associated species

In a second step, we analyzed if the species sharing a same habitat are interacting. Species were unequally distributed between different habitats and along the temporal gradient (Figure 2.1). In order to gain more homogenous data sets suitable for statistical analysis, we split our data set into three parts according to the information about habitat preferences identified above. Three main habitats were identified. We classified all short-term flooded ponds as “habitat 1” (N=16), all medium-term with Ostracoda being subdominant as “habitat 2” (N= 5), and all medium-term flooded ponds dominated by Ostracoda as “habitat 3” (N= 4). Abundances of crustaceans and mosquito larvae as well as habitat characteristics of the different habitats are listed in Table 2.1.

Ponds at habitat 1 were mainly located in grassland habitats (n = 11), with only a few ponds located in either forest (n = 3) or reed-covered wetland habitats (n = 2). *Aedes vexans* was the dominant mosquito species (Table 2.1), which was negatively correlated with Cyclopoida (Figure 2.2).

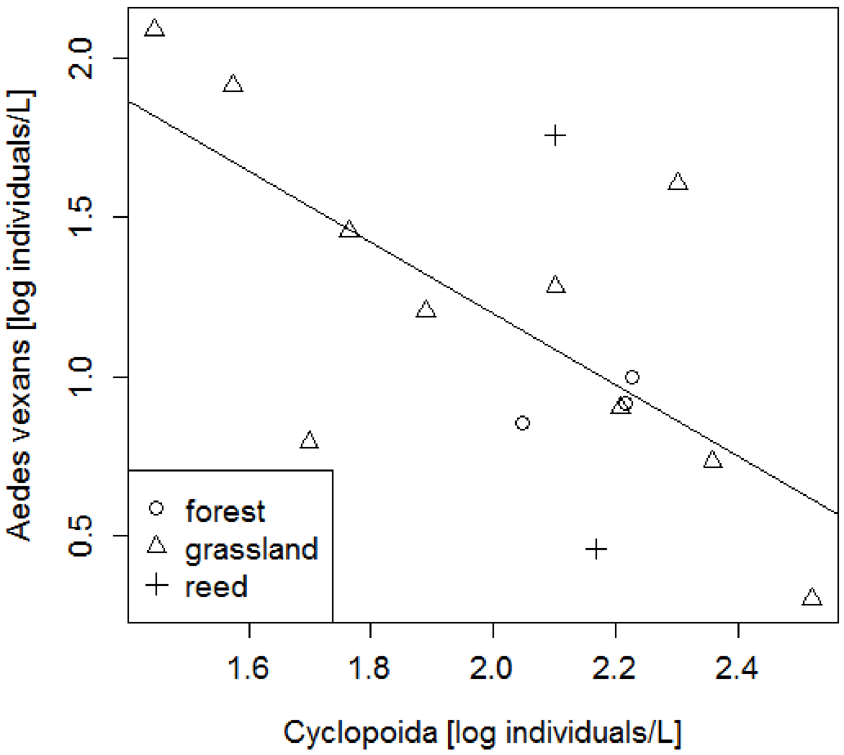

Figure 2.2. Correlation between larvae of *Aedes vexans* and Cyclopoida at short-term flooded ponds (hydroperiod ≤ 2 weeks). Regression line added (y=3.44-1.12x, P=0.009, R2=0.422).

Ponds at habitat 2 were mainly found in grassland habitats (grassland n=3, reed n=1), located at Rosslau (n=1) and Spreewland (n=3). Ponds at habitat 2 were initially colonized by low abundances of crustaceans and high numbers of mosquito larvae, with the abundances of crustaceans (Cyclopoida, Daphniidae, and Chydoriidae) increasing (Figure 3B) and those of mosquito larvae decreasing over time (Figure 2.3A). *Cx.pipiens* was the dominant mosquito species (Table 2.1). The abundances of larvae of *Cx. pipiens* were negatively correlated with those of larvae of *Ae. vexans*, and larval abundances of both mosquito species were negatively correlated with abundances of Cladocera (Table 2.2).

Table 2.1: Differences in crustacean and mosquito larval abundances (Ind./L) and physiochemical parameters of water among ponds at different habitats (habitat 1 = short term flooding ponds at all locations, habitat 2 = medium-term flooded ponds not dominated by Ostracoda, habitat 3 = medium-term flooded ponds dominated by Ostracoda) using mean value (mean ± SD) over flooding period. ANOVA was performed using log-transformed (log x+1) data, and pairwise t-test was used for post-hoc analysis.

Parameter	Niche 1	Niche 2	Niche 3	Pairwise t-test
Hydroperiod [week]	1.34 ± 0.16	3.57 ± 0.80	5.47 ± 1.79	$P_{1:2}<0.001$ $P_{1:3}<0.001$ $P_{2:3}<0.001$
season	22.1 ± 0.83	22.3 ± 1.24	21.7 ± 0.40	$P_{1:2}=0.722$ $P_{1:3} = 0.428$ $P_{2:3} = 0.694$
pH	6.31 ± 0.78	6.56 ± 0.53	7.62 ± 0.11	$P_{1:2}=0.914$ $P_{1:3} = 0.002$ $P_{2:3} = 0.010$
Water temperature	18.1 ± 1.44	19.9 ± 2.58	17.4 ± 0.41	$P_{1:2}= 0.005$ $P_{1:3} = 0.404$ $P_{2:3} = 0.005$
Water level	8.72 ± 3.56	11.7 ± 2.39	9.40 ± 3.23	$P_{1:2}= 0.031$ $P_{1:3} = 0.667$ $P_{2:3} = 0.200$
Cladocera [% of total crustacean]	4.16 ± 6.28	46.7 ± 27.8	10.1 ± 7.69	$P_{1:2}< 0.001$ $P_{1:3} = 0.027$ $P_{2:3} < 0.001$
Copepoda [% of total crustacean]	84.1 ± 22.8	47.1 ± 26.6	16.1 ± 5.20	$P_{1:2} < 0.001$ $P_{1:3} < 0.001$ $P_{2:3} = 0.074$
Ostracoda [% of total crustacean]	11.7 ± 19.1	6.19 ± 6.22	75.1 ± 14.7	$P_{1:2} = 0.435$ $P_{1:3} < 0.001$ $P_{2:3} < 0.001$
Crustacean [Ind./L]	193 ± 236	251 ± 142	477 ± 75.2	$P_{1:2} = 0.667$ $P_{1:3} = 0.003$ $P_{2:3} = 0.025$
Aedes [Ind./L]	25.1 ± 34.1	8.60 ± 10.6	1.89 ± 1.99	$P_{1:2} = 0.501$ $P_{1:3} = 0.011$ $P_{2:3} = 0.084$
Culex [Ind./L]	7.84 ± 14.9	44.8 ± 57.4	4.45 ± 1.57	$P_{1:2} = 0.009$ $P_{1:3} = 0.213$ $P_{2:3} = 0.272$

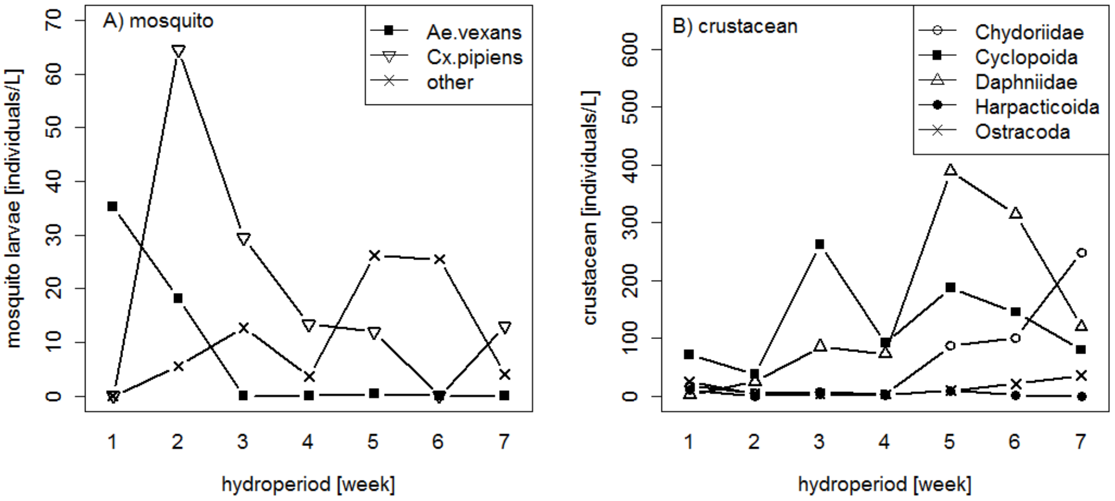

Figure 2.3: development of mosquito larvae (A) and crustaceans (B) at ponds of habitat 2 (medium-term flooded ponds not dominated by Ostracoda) over duration of hydroperiod. Error bars were omitted for clarity.

Ponds at habitat 3 (dominated by Ostracoda) were only found at reed habitats located at Leipzig (n=4). Abundances of Ostracoda were consistently high during the whole duration of hydroperiod (Figure 2.4B). Other species, either mosquito larvae or other crustacean taxa, did not occur at considerable numbers (Figures 2.4A and 2.4B). No correlation between mosquito larvae and any crustacean taxa was observed (multiple linear regression,$p>0.05$)

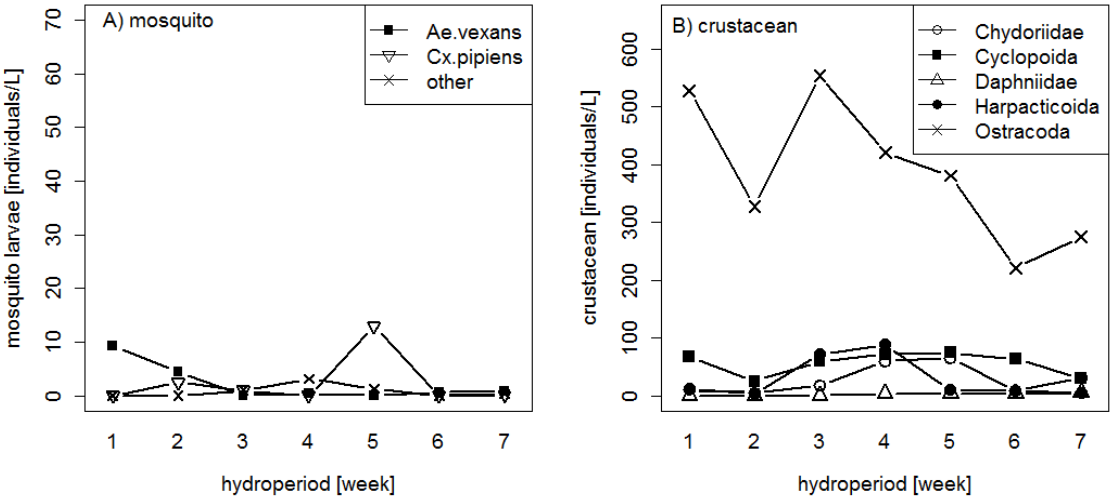

Figure 2.4: Development of mosquito larvae (A) and crustaceans (B) at ponds of habitat 3 (medium-term flooded pond dominated by Ostracoda) over duration of hydroperiod. Error bars were omitted for clarity.

2.4.4 Impact of biotope structure on species development

Results indicated that early establishment of micro-crustaceans prevented mosquito larval development (see above). Indeed, total abundances of mosquito larvae (mean abundances during the whole duration of hydroperiod) were significantly and negatively correlated with early abundances of crustaceans (mean abundances of the first week of hydroperiod) (Figure 2.5). Habitats at reed-covered wetlands contained initially higher abundances of crustaceans and consequently lower abundances of mosquito larvae compared to grassland habitats (Figure 2.5).

Table 2.2: Impact of antagonist crustaceans on mosquito larval abundances in ponds of habitat 2 (medium-term flooded ponds not dominated by Ostracoda) using generalized least squares model (see methods)

Response variable	Predictor variable	Regression Coef.	SE	t-value	p-value
Cx. pipiens	Ae. Vexans	-0.928	0.217	-4.276	<0.001
	Cladocera	-0.442	0.191	-2.311	0.031
Ae. vexans	Cx. pipiens	-0.508	0.109	-4.652	<0.001
	Cladocera	-0.389	0.131	-2.960	0.008

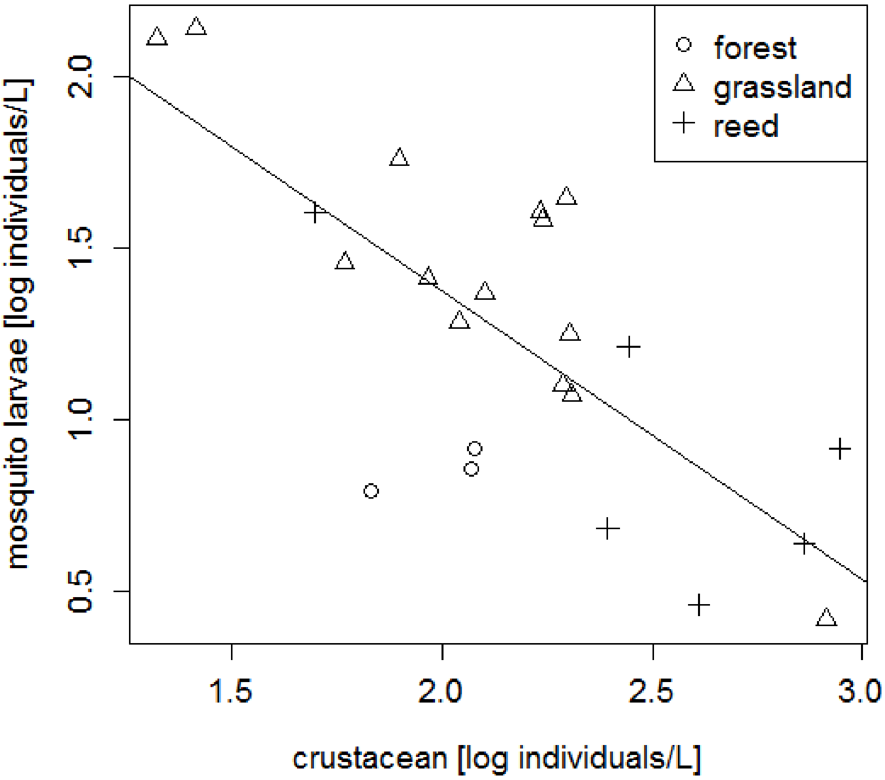

Figure 2.5. Correlation between total abundances of mosquito larvae (i.e. mean abundances during the whole duration of hydroperiod) and early abundances of crustacean (i.e. abundances during the first week of hydroperiod). Regression line added (y= 3.06-0.84x, P<0.001, R2=0.559).

2.5 Discussion

Our results showed that during the first two weeks of hydroperiod, pond communities were mainly dominated by larvae of the mosquito *Ae. vexans* and Cyclopoida (Crustacean: Copepoda). Indeed, both types of organisms showed reproductive traits that are adapted to highly ephemeral ponds. Many species of Cyclopoida can survive desiccation in a dormant condition and later propagate rapidly due to their short generation time and the ability to store sperm (Frisch and Green 2007). Adult females of *Ae. vexans* lay their eggs in the soil, so the larvae are able to hatch immediately after ponds are refilled (Becker et al. 2010). In our study, we observed a negative relationship between Cyclopoida and larvae of *Ae. vexans* but not between Cyclopoida and larvae of *Cx. pipiens*. This is in line with previous studies, demonstrating the negative effect of Cyclopoida on population abundances of mosquito larvae of the genus *Aedes*, particularly *Ae. aegypti* (Rivière et al. 1987, Chansang et al. 2004, Rey et al. 2004), *Ae. albopictus* (Rey et al. 2004), and *Ae. polynesiensis* (Rivière et al. 1987, Lardeux et al. 1992) but not on mosquito larvae of the genus *Culex*, particularly *Cx. roseni* and *Cx. quinquefasciatus* (Rivière et al. 1987). Indeed, the bottom-feeding Cyclopoida does not affect the surface and column-feeding *Culex* larvae but does affect the bottom-feeding *Aedes* larvae (Rivière et al. 1987). Hence, micro-crustaceans other than Cyclopoida are needed to interact with *Culex* larvae to potentially be useful as mosquito control agents.

In contrast to *Ae. vexans*, *Cx. pipiens* oviposits directly on the water surface. Hence, it oviposits at a time when the larvae of *Ae. vexans* have already hatched. Indeed, we observed larvae of *Cx. pipiens* in the second week of the hydroperiod at the earliest, present thereafter together with larvae of *Ae. vexans* and with Cladocera. Cladoceran populations increased continuously over time, reaching their peaks of abundance after five weeks of hydroperiod. This is consistent with the outcomes of laboratory and outdoor studies that reported a period of a few weeks for the establishment of Cladoceran populations (Kroeger et al. in press, Duquesne et al. 2011). In the present study, *Cx. pipiens* and Cladocera co-occurred only at an advanced stage of hydroperiod after more than three weeks. This is in line with the field study of Kroeger et al. (2013), showing that established populations of micro-crustaceans prevent the colonization of ponds by mosquito larvae at a late stage of hydroperiod but are insufficient for mosquito control during an earlier stage. In general, our study supports the hypothesis that predation drives community structures during the early stage of hydroperiod, while competition occurred later in time (Wellborn et al. 1996, Blaustein and Chase 2007).

In addition to ponds colonized at a late stage by Cladocerans, we observed at one of the three studied locations (Leipzig area), medium-term flooded ponds (hydroperiod = 7 weeks) colonized mainly by Ostracoda. At those ponds, no populations of mosquito larvae, neither *Ae. vexans* nor *Cx. pipiens*, were established. One reason may be that at the time of our study, overall mosquito abundances were low at this location, although high abundances of *Ae. vexans* were found at short-term flooded ponds of this location and high abundances of *Cx. pipiens* populations had been previously observed (Duquesne et al. 2011, Kroeger et al. 2013)and should be less efficient competitors. To test this hypothesis, the establishment and development of larval populations of wild Culex pipiens were investigated in outdoor microcosms varying in terms of Daphnia magna populations. When the population was well established (i.e., high densities of D. magna. Physiochemical water parameters characteristic for that habitat (e.g., elevated pH) could have prevented mosquito larval colonization. However, larvae of at least *Cx. pipiens* tolerate a wide spectrum of physiochemical parameters (Vinogradova 2000), hence water chemistry may play a minor role in species distribution. Another reason may be that

Ostracoda have a strong negative impact on the development of mosquito larval populations compared to Cyclopoida or Cladocera. Indeed, previous studies have indicated that Ostracoda are both predators and food competitors for mosquito larvae (Brauer 1909, Rossi et al. 2011); hence, they can affect mosquito larval populations due to these two strategies. However, Ostracoda feed preferably at the bottom of ponds, while, as reported above, larvae of *Cx. pipiens* feed preferably in the water column (Rivière et al. 1987). Hence, the impact of Ostracoda on at least *Cx. pipiens* larvae is spatially limited. An alternative explanation is that in our study, populations of Ostracoda were abundant in ponds from the beginning of hydroperiod. All ponds were in reed habitats and layers of detritus probably prevented full drying of the ground and it was shown that Ostracoda are adapted to survive relatively dry conditions by closing their shells (Brauer 1909). In addition, decomposition processes could have influenced water chemnistry, providing suitable conditions for Ostracod development. Due to favorable environmental conditions as well as species adaptation, Ostracoda may be able to colonize refilled ponds before development of mosquito larvae takes place. Time of colonization is highly important for species distribution, with the species occurring first in the ponds gaining advantage on the species colonizing later (Sredl and Collins 1991, Lawler and Morin 1993, Blaustein and Margalit 1996). In ponds located in grassland habitats and thus exposed to sunlight, the risk of drying out increases. Hence, the survival of certain crustacean taxa is impeded, thus giving advantage to the early development of mosquito larvae.

2.6 Conclusion

Our study demonstrated that, in general, micro-crustaceans are relevant for mosquito larval control under natural conditions. Predatory interactions were thereby most important during the first two weeks of hydroperiod, while competitive interactions took place later in time. Various types of habitats provide different conditions for colonizers, which gives some species a certain advantage on others. Synchronized development between target mosquito species and their antagonists as well as favorable conditions for crustacean development are necessary for natural mosquito management.

Acknowledgments

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3 Crustacean biodiversity as an important factor for mosquito larval control

Kroeger, I.^{1,2}*, Duquesne, S.*^{1,3}*, & Liess, M.*^{1}

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[1]Department of System Ecotoxicology, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany
[2]Quantitative Landscape Ecology, Institute for Environmental Science, University of Koblenz-Landau, Fortstraße 7, D-76829 Landau, Germany
[3]Department of Biology Conservation, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany

3.1 Abstract

Mosquitoes prefer to breed in newly established ponds, which are highly dynamic systems with changing levels of biological interactions between species. This study investigated the impact of crustacean abundances and crustacean taxa diversity on mosquito oviposition and larval development. The effects of the biological larvicide *Bacillus thuringiensis israelensis* (Bti) on mosquito larvae were monitored according to fluctuations in crustacean communities.

Populations of the mosquito *Cx. pipiens* colonised artificial ponds that contained crustacean communities at different time points of colonization by crustaceans: 1) "no colonization" (no crustaceans), 2) "simultaneous colonization" by crustaceans and mosquitoes, and 3) "head-start colonization" by crustaceans (preceding colonisation by mosquitoes). All types of pond were treated with three concentrations of Bti (10, 100, or 1,000 μg L^{-1}).

Colonization of all ponds by *Cx. pipiens* (in terms of oviposition, larval abundance and larval development) decreased significantly with increasing diversity of crustacean taxa. The total abundance of crustaceans had a minor effect on colonization by *Cx. pipiens*.

The presence of crustaceans increased the sensitivity of *Cx. pipiens* larvae to Bti treatment by a factor of 10 and delayed the time of recolonization. This effect of Bti was relevant in the short term. In the long term, the presence of *Cx. pipiens* was determined by crustacean biodiversity.

Keywords: Biological control, Cladocera, Combined effect, Competition, Culicidae, Pond colonization

3.2 Introduction

The control of mosquitoes is important for human societies in order to combat vector-borne diseases and to control the spread of invasive mosquito species. Although extensive resources are already employed for mosquito control, adverse effects associated with mosquitoes are becoming increasingly severe. Factors such as climate change and global trade promote the dispersion of mosquitoes (Schäfer and Lundström, 2009; Weaver and Reisen, 2010); in addition, mosquito control strategies have been thwarted by the development of insecticide resistance (Akiner et al., 2009; Melo-Santos et al., 2010).

Consequently, there is a need for more efficient strategies to reduce adverse effects associated with mosquitoes in a changing environment.

A common approach for mosquito control involves the use of the biological insecticide *Bacillus thuringiensis israelensis* (Bti). This insecticide is considered to target larvae of mosquitoes (Culicidae) and non-biting midges (Chironomidae) specifically, and thus to avoid adverse effects on other species (Boisvert and Boisvert, 2000; Becker, 2003; Russell et al., 2009). However, a disadvantage of the use of Bti is that it remains effective for only a few days owing to sedimentation and natural degradation (Becker et al., 1992; Aldemir, 2009). This necessitates the repeated application of Bti during each mosquito breeding season, which is expensive and promotes the emergence of resistance (Boyer et al., 2007).

The effect of Bti can be improved by combining the insecticide with natural antagonists. Indeed, two studies demonstrated that combining Bti use with the introduction of predators was more effective for the long-term elimination of mosquito larvae than Bti treatment alone (Neri-Barbosa et al., 1997; Chansang et al., 2004). Recent studies demonstrated that not only predators, but also competitors, are highly effective in controlling the growth of populations of mosquito larvae (Blaustein and Chase, 2007; Meyabeme Elono et al., 2010; Duquesne et al., 2011). In particular, crustaceans are promising candidates for mosquito control because they reduce the size of populations of larval mosquito populations (Chase and Knight, 2003; Meyabeme Elono et al., 2010) through supressing both mosquito oviposition and larval development (Duquesne et al., 2011). In contrast to many predators, crustaceans are able to colonise not only permanent but also temporary ponds (Williams, 2006). As a consequence, crustaceans are found at the breeding sites that mosquitoes prefer to colonise. However, given that mosquitoes colonise newly established ponds rapidly, the question arises whether crustacean communities can grow rapidly enough to limit populations of mosquito larvae. In addition, crustacean populations can change markedly during the process of pond colonization in terms of both species abundances and community composition (Williams, 2006). Little is known about how these changing characteristics of crustacean communities influence their adverse effects on mosquito larval populations. (Duquesne et al., 2011) showed that populations of Daphnia magna adversely impacted on oviposition and larval development of Cx.pipiens, and that this relationship was density dependent, with higher D.magna densities increasingly inhibiting mosquito population development. However, this two-species study did not investigate how the dynamics of multiple crustacean species influences populations of *Cx. pipiens*. In addition, there is a lack of knowledge on the extent to which changes in crustacean community structure alter the sensitivity of mosquito larvae to insecticides. (Kroeger et al., 2013) demonstrated a reduction in the recovery of mosquito larval populations after treatment with Bti in the presence of *Daphnia* sp.; however, this investigation did not include crustacean communities that consisted of multiple crustacean taxa.

The current study used outdoor pond microcosms to investigate the impacts of crustacean communities on populations of mosquito larvae. The effects of several taxa of crustaceans at different degrees of colonization were studied in combination with the effects of different levels of Bti treatment. Specifically, we wanted to answer the several questions. First, does the impact of crustacean communities on mosquito larval populations change during the process of pond colonization, and do changes in crustacean species diversity contribute to this? Second, does Bti treatment reduce mosquito larval populations at lower concentrations when crustaceans are present? Finally, which control mechanism is the most effective and sustainable: crustaceans alone, insecticide treatment alone, or a combination of both?

3.3 Material and Methods

3.3.1 Outdoor microcosms

This study was carried out in the outdoor area of the Helmholtz Centre for Environmental Research (UFZ), Leipzig, Germany. Forty-five round, black plastic containers (hight 36cm, diameter 70cm, each with a volume of 90 L) were placed into the ground to provide a temperature regime close to that observed under natural conditions in this area. The distance between each container was approximately 0.5 m. The containers were filled with a layer of natural sediment (1-cm thick), which was collected from a pond in a forested area (Abtnaundorfer Lake; 51°22’29.66”N, 12°25’40.39”E). The sediment was a mixture of 70% sandy sediment and 30% organic sediment (containing leaves and degrading particles). A culture of the alga *Desmodesmus subspicatus* (3×10^9 cells mL^1) was added as an initial food resource (200 mL $container^{-1}$). Containers were topped up with tap water to a final volume of 60 L.

Three different conditions were randomly established:

(1) “Head-start colonization” – On July 13, 2006, 15 containers were filled with 19 L of water from Abtnaundorfer Lake, which contained zooplankton and phytoplankton. In addition, 1 L of lake water enriched with plankton (obtained using a plankton net, mesh size 500 µm, from Abtnaundorfer Pond) was added to the containers. The containers were then covered with gauze for two weeks to avoid oviposition by mosquitoes and other insects. Hence, the crustacean community was allowed to become established for two weeks in the absence of the influence of insect larvae. On July 27, 2006, the gauze was removed from the containers, so that mosquitoes and other insects were able to colonise the biotopes. This was considered as the start of the experiment.

(2) “Simultaneous colonization” – The set-up for this group was comparable to that for “head-start colonization”, but ponds were established on July 27, 2006 (considered as the start of the experiment), and the containers were not covered with gauze after the initial colonization with plankton. 'This enabled simultaneous colonization by crustaceans, mosquitoes, and other insects..

(3) “No colonization” – On July 27, 2006, 15 containers were filled only with tap water and no crustaceans were added; the containers were not covered, so mosquitoes and other insects were thus able to oviposit from the first day. The lake sediment used in these containers was sterilised (at 100°C for 10 h) to eliminate any autochthonous organisms. The alga *D. subspicatus* was introduced as described above.

All three experimental set-ups were monitored until September 3, 2006.

3.3.2 Treatment with Bti

Twenty-one days after the start of the experiments (August 16, 2006), the microcosms were treated with Bti. A liquid stock solution (Vectobac 12 AS) at a concentration of 1,200 International Toxin Units mg^{-1} was used. Three concentrations were established (10, 100 and 1,000 µg L^{-1}), with four replicates per concentration and for each condition. Controls (no inoculation with Bti) had only three replicates.

3.3.3 Sampling method

Monitoring of the microcosms started on Day 0 of the experiment, that is, July 27, 2006. The numbers of mosquito eggs and larvae, as well as the species compositions and densities of the associated communities of crustaceans and other insects and insect larvae, were monitored. Given that species of the insect families Gerridae and Notonectidae, as well as larvae of the order Odonata, were observed only sporadically (colonising <11% of microcosms), their numbers were excluded from statistical analyses. Larvae of the order Ephemeroptera were observed more frequently (colonising 65% of ponds), but their abundances were low (0.95±1.06 individuals L^{-1}), and they were not affected significantly by any of the conditions or Bti treatment (two-way ANOVA, $P_{\text{condition}} = 0.125$, $P_{\text{Bti-treatment}} = 0.235$). Hence, only the frequencies of mosquito oviposition and the numbers of mosquito larvae and crustaceans are outlined here.

Clutches of mosquito eggs on the water surface were counted every second day. Mosquito oviposition decreased over time in all microcosms due to seasonal decline. Mosquito larvae were sampled using a white plastic bowl (length, 37 cm; width, 31 cm; height, 7 cm; volume, 2.5 L). The bowl was dipped into the water to catch the larvae, which were floating below the water surface. The number of mosquito larvae in the bowl was counted and their instar was estimated by classifying them as belonging to one of two different size classes (small, larvae of first or second instars; large, larvae of third or fourth instars). The pupae of mosquitoes were not included in the analysis, because their rapid movement would have under-estimated their abundances in the samples. Samples of mosquito larvae were collected every two days.

Samples of other taxa of associated communities within the microcosms were collected twice a week using a plastic tube (diameter, 7 cm; length, 31 cm), which was placed into the water and then closed using a cap, so that a water column of 1 L remained in the tube. The water in the containers was stirred with a wooden stick before sampling to ensure that the associated community was distributed evenly throughout the volume of water. The water, together with the associated community, was filtered through a plankton net (180 μm mesh size) and the filtered organisms were preserved in 70% ethanol. The abundance and composition of associated communities were determined in the laboratory using a binocular Leica S6D microscope (Wetzlar, Germany). In addition, mosquito larvae were characterised to the species level using the determination key of (Becker et al., 2010). All other invertebrates were characterised to the suborder or family level using various determination keys (Brauer, 1909; Lieder, 1999; Meisch, 2000; Klausnitzer, 2009).

3.3.4 Physiochemical parameters of water

The pH and concentrations of ammonium, phosphate, nitrite, and nitrate were monitored in the microcosms every two weeks using a pH/EC/TDS Combo testing meter (Hanna Instruments, Woonsocket, Rhode Island, USA) and Aquamerck colorimetric tests (Merck, Darmstadt, Germany). Ammonium and nitrite were detected only sporadically, and consequently were excluded from the analyses. Turbidity and chlorophyll were measured once a week. Turbidity was measured using a turbidimeter (Turbiquant 1100IR, Merck, Darmstadt, Germany), and chlorophyll was measured using a spectrofluorometer (Gemini EM Spectramax, USA; wavelengths of 400 nm for excitation, 700 nm for emission and 690 nm as a cut-off), in relative fluorescence units. Water temperature was measured every hour (DK 501-PL, Driesen & Kern GmbH, Bad Bramstedt, Germany), as were air temperature and humidity (data obtained from the Department of Bioremediation, UFZ Leipzig).

The initial water level was 25 cm (equivalent to a volume of 60 L) in all microcosms. Owing to evaporation and rain, the water level varied between 22 and 28 cm throughout the course of the experiment. Before Bti treatment, the water level was returned to 25 cm (by the addition of tap water or the removal of water).

3.3.5 Statistical analyses

Data were divided into the time period before treatment with Bti (early period, days 1–19) and the time period after this treatment (late period, days 20–38). The separate datasets were analysed using a generalized least squares (GLS) mixed model approach. Analysis used multiple measures over time in multiple microcosms and temporal autocorrelation of subsequent measures in the same microcosm was to be expected. Therefore an autocorrelation structure (autoregressive model of order 1 according to (Zuur et al., 2009)) was implemented within the model. The final model was presented using the restricted maximum likelihood (REML) calculation. Homogeneity of variance was evaluated by plotting residuals versus fitted values.

First we investigated the impact of conditions (“no colonoization”, “simultaneous colonization” and “head-start colonization”) on zooplankton communities. Analysis of the early time period included data from all microcosms, while analysis of the late time period included only data of those microcosms not treated with Bti. Second we analyse two sets of impacts on *Cx. pipiens* colonization. These were a) the impact of crustacean abundance and the diversity of taxa in the early time period (set-ups with no colonization were excluded because all explanatory variables were zero) and b) the impacts of crustacean abundance, the diversity of taxa, and Bti treatment in the late time period (all set-ups included). Both dependent and explanatory variables were Z-transformed. The Simpson index was used as an indicator of the diversity of crustacean taxa according to Simpson (1949). Development of *Cx. pipiens* larvae was analysed by determining the ratio of large larvae (third and fourth instars) to small larvae (first and second instars). If the abundances of both small and large larvae were zero, the ratio was also set to zero.

The impact of Bti treatment on zooplankton in the different conditions was analysed in terms of mortality (one day after treatment) and recovery (in the long term, using the mean value of the last two sampling points: on days 34 and 38) separately using two-way ANOVA. In addition, a dose-response analysis was performed for the abundances of mosquito larvae, using log(x+1)-transformed data that had been normalised using the control.

Physiochemical water parameters were less often monitored compared with zooplankton abundances (see above) and hence mean values of a certain time period (i.e. before and after Bti treatment) were used for analysis. Differences in water parameters between conditions were analysed using ANOVA followed by pairwise t-test. Correlations between water parameters and zooplankton abundances were analysed using linear regression. Data were tested for a normal distribution (using the Shapiro–Wilk Normality Test) and homogeneity (F-test) to verify that underlying statistical assumptions were not violated.

Analyses were performed using the ‘R’ statistical and programming environment (R Development Core Team 2010) and the ‘nlme’ (Pinheiro et al., 2009), ‘lattice’(Sarkar, 2009) packages.

3.4 Results

3.4.1 Development of mosquito larval populations according to colonization level of crustacean communities

Mosquito oviposition was largely dominated by *Culex* spp. (>99%), and decreased with time in all microcosms (Fig. 3.1); a few *Anopheles* spp. also oviposit in the microcosms. Larval populations were dominated by *Cx. pipiens* (>99%), whereas the level of *Anopheles* spp. colonization was so low that it was not analysed.

During the early time period (i.e. before treatment with Bti) *Cx. pipiens* oviposition, larval abundance, and the larval size ratio (the ratio of large larvae to small larvae) differed significantly between conditions (Table 3.1). In microcosms with no colonization, *Cx. pipiens* oviposition and larval abundance were the highest (Fig. 3.1 and 3.2a) and a larval size ratio of around 1 (Fig. 3.3) indicated that all small larvae were able to develop into large larvae. Upon simultaneous colonization, oviposition (Fig. 3.1) and larval abundance (Fig. 3.2c) were found to decrease, and a larval size ratio of about 0.5 (Fig. 3.3) indicated that only half of the small larvae had developed. Upon head-start colonization, oviposition and larval abundance were the lowest (Fig. 3.1 and 3.2e), and the small larvae present in the microcosms were not able to develop into late instars (Fig. 3.3).

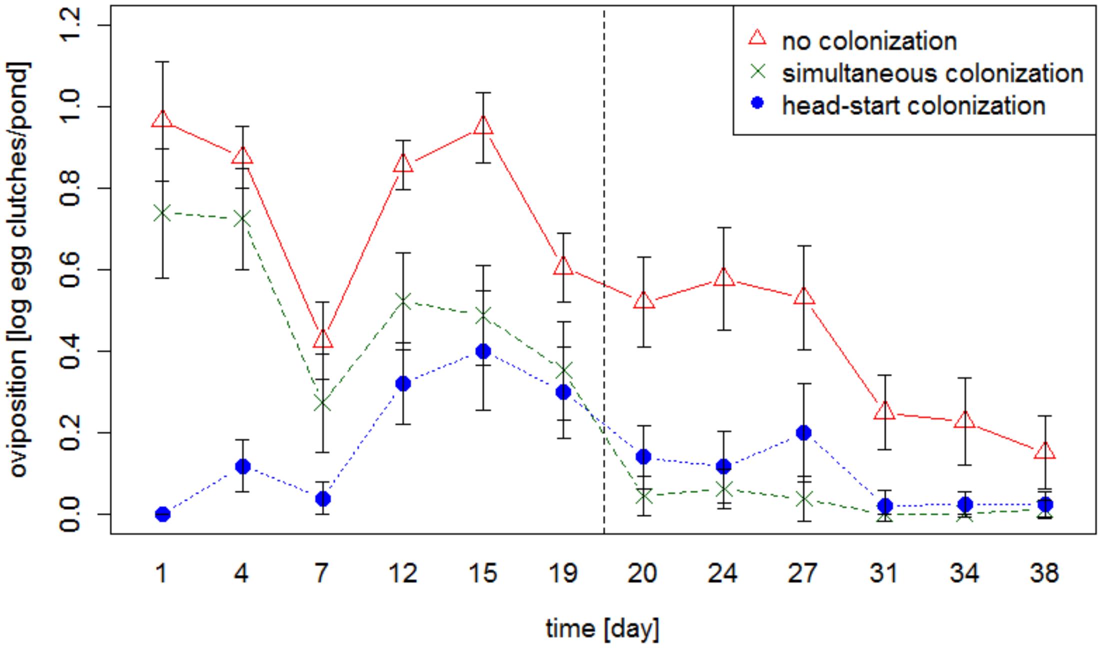

Figure 3.1: Oviposition in set-ups at different time points of crustacean colonization (no colonization = crustacean absent; simultaneous colonization = crustacean development started at the same time as insect colonization; headstart colonization = crustacean development started two weeks prior to insect colonization) over time. The vertical dahed line marks treatment with Bti. Error bars represent ± SE.

Table 3.1: Differences in zooplankton communities between different conditions of crustacean colonization (no colonization = no crustaceans were introduced; simultaneous crolonization = crustaceans were introduces at the same time as when insect colonization started; head-start colonization = crustaceans were introduced twoweeks prior to insect colonization) during different time periods (eraly = before treatment with Bti, late = after treatment with Bti) using gls model (see methods)

Object	Time period	N	ANOVA	Summary of gls-model			
			P	Condition	Regression Coef.	SE	P
Cx.pipiens oviposition	early	45	<0.001	no - simul.	-3.125	0.486	<0.001
				no - head	-5.412	0.486	<0.001
				simul. - head	-2.287	0.486	<0.001
	late	9	<0.001	no - simul.	-1.935	0.321	<0.001
				no - head	-1.583	0.321	<0.001
				simul. - head	0.351	0.321	0.278
Cx.pipiens larval abundances	early	45	<0.001	no - simul.	-132.8	15.71	<0.001
				no - head	-188.7	15.71	<0.001
				simul. - head	-55.85	15.71	<0.001
	late	9	<0.001	no - simul.	-247.9	28.12	<0.001
				no - head	-252.3	28.12	<0.001
				simul. - head	-4.370	28.12	0.877
Cx.pipiens larval size ratio	early	45	<0.001	no - simul.	-0.549	0.153	<0.001
				no - head	-1.082	0.153	<0.001
				simul. - head	-0.533	0.153	<0.001
	late	9	0.012	no - simul.	0.715	0.543	0.194
				no - head	-0.966	0.543	0.081
				simul. - head	-1.680	0.543	0.003
Crustacean abundances	early	45	<0.001	no - simul.	194.4	17.37	<0.001
				no - head	304.4	17.37	<0.001
				simul. - head	-8.104	17.37	0.649
	late	9	<0.001	no - simul.	300.6	41.18	<0.001
				no - head	495.4	41.18	<0.001
				simul. - head	194.8	41.18	<0.001
Crustacean taxa diversity [simpson index]	early	45	<0.001	no - simul.	0.129	0.018	<0.001
				no - head	0.513	0.018	<0.001
				simul. - head	0.384	0.018	<0.001
	late	9	<0.001	no - simul.	0.505	0.032	<0.001
				no - head	0.662	0.032	<0.001
				simul. - head	0.157	0.032	<0.001

During the late time period (i.e. after treatment with Bti) both *Cx. pipiens* oviposition (Fig. 3.1) and larval abundance (Fig. 3.2c, 3.2e) became similar between the simultaneous and head-start colonization in microcosms not treated with Bti (Table 3.1). In microcosms with no colonization, mosquito colonisation was still significantly higher than in microcosms with simultaneous and head-start colonization (Table 3.1). The larval size ratios of *Cx. pipiens* larvae were around 1 both at no colonization (mean size ratio over time = 1.53 ± 1.12) or simultaneous colonization (mean size ration over time = 2.24 ± 0.89), but significantly reduced at head-start colonization (mean size ration over time = 0.11 ± 0.19, Table 3.1).

3.4.2 Development of crustaceans in different set-ups

In microcosms with no colonization, the abundance of crustaceans was close to zero throughout the observation period (Fig. 3.2b). During the early time period (i.e., before treatment with Bti), crustacean abundances were similar between simultaneous colonization and head-start colonization (Table 3.1). However, the composition of the taxa (i.e. simpson index) differed significantly between these two conditions (Table 3.1), with simultaneous colonization being largely dominated by *Scapholeberis* (>75%, Fig. 3.2d), while crustacean communities with head-start colonization were more diverse (Fig. 3.2f). Indeed, throughout the entire observation period, the abundances of all crustacean taxa except *Scapholeberis* were significantly lower following simultaneous colonization compared with the levels observed for head-start colonization (Fig. 3.2d and 3.2f).

During the late time period (i.e., after Bti treatment) microcosms of the two conditions differed significantly in terms of crustacean abundances and taxa diversity (Table 3.1), with the highest abundances and highest diversity of taxa for head-start colonization (Fig. 3.2f). However, the diversity of crustacean taxa for simultaneous colonization reached a level similar to that for head-start colonization during the early time period ($\text{simpson}_{\text{head-start/early}}=0.53 \pm 0.10$, $\text{simpson}_{\text{simultaneous/late}}=0.53 \pm 0.06$); hence, microcosms of both conditions showed an elevated diversity index at the late time period.

In a second step we analysed the impact of crustacean abundances and crustacean taxa diversity on mosquito colonization. The diversity of crustacean taxa had a strong and significant negative impact on *Cx. pipiens* oviposition, both before (gls model, Beta = -0.276, SE = 0.065, P<0.001) and after Bti treatment (gls model, Beta = -0.690, SE = 0.125, P<0.001), as well as on *Cx.pipiens* larval abundances both before (gls model, Beta = -0.179, SE = 0.045, P<0.001) and after treatment with Bti (gls model, Beta = -0.822, SE = 0.102, P<0.001). In contrast, crustacean abundances did not affect neither *Cx.pipiens* oviposition or larval abundances (gls-model, p>0.05). There was no interaction between crustacean abundances and crustacean taxa diversity observed (gls model, p>0.05).

A low level of diversity of taxa in simultaneous colonization microcosms was always associated with a low abundance of crustacean taxa, except for *Scapholeberis* (see above). It is likely that *Scapholeberis* has only a low impact on mosquito larval populations (see discussion). Hence, the strong impact of the presence of diverse crustacean taxa on mosquito larval populations could be assumed to be a masked density-dependent effect of a certain genus, for example, *Daphnia*. However, if this was the case, we would expect higher levels of *Cx. pipiens* oviposition and larval development upon simultaneous colonization than in head-start colonization throughout the whole observation period. This was not observed; instead, equivalence in terms of *Cx. pipiens* oviposition and larval abundance developed between these two conditions as soon as the diversity of taxa increased for simultaneous colonization.

The size ratio of *Cx. pipiens* larvae was negatively affected by the diversity of crustacean species before treatment with Bti (gls model, Beta = -0.280, SE = 0.054, p<0.001), and negatively affected by crustacean abundance (gls model, Beta = -0.195, SE = 0.091, P=0.033) and Bti treatment (gls model, $\text{Beta}_{10\mu g/LBti}=-0.391$, $P_{10\mu g/LBti}=0.024$, $\text{Beta}_{100\mu g/LBti}=-0.563$, $P_{100\mu g/LBti}=0.001$, $\text{Beta}_{1000\mu g/LBti}=0.702$, $P_{100\mu g/LBti}<0.001$) after such treatment.

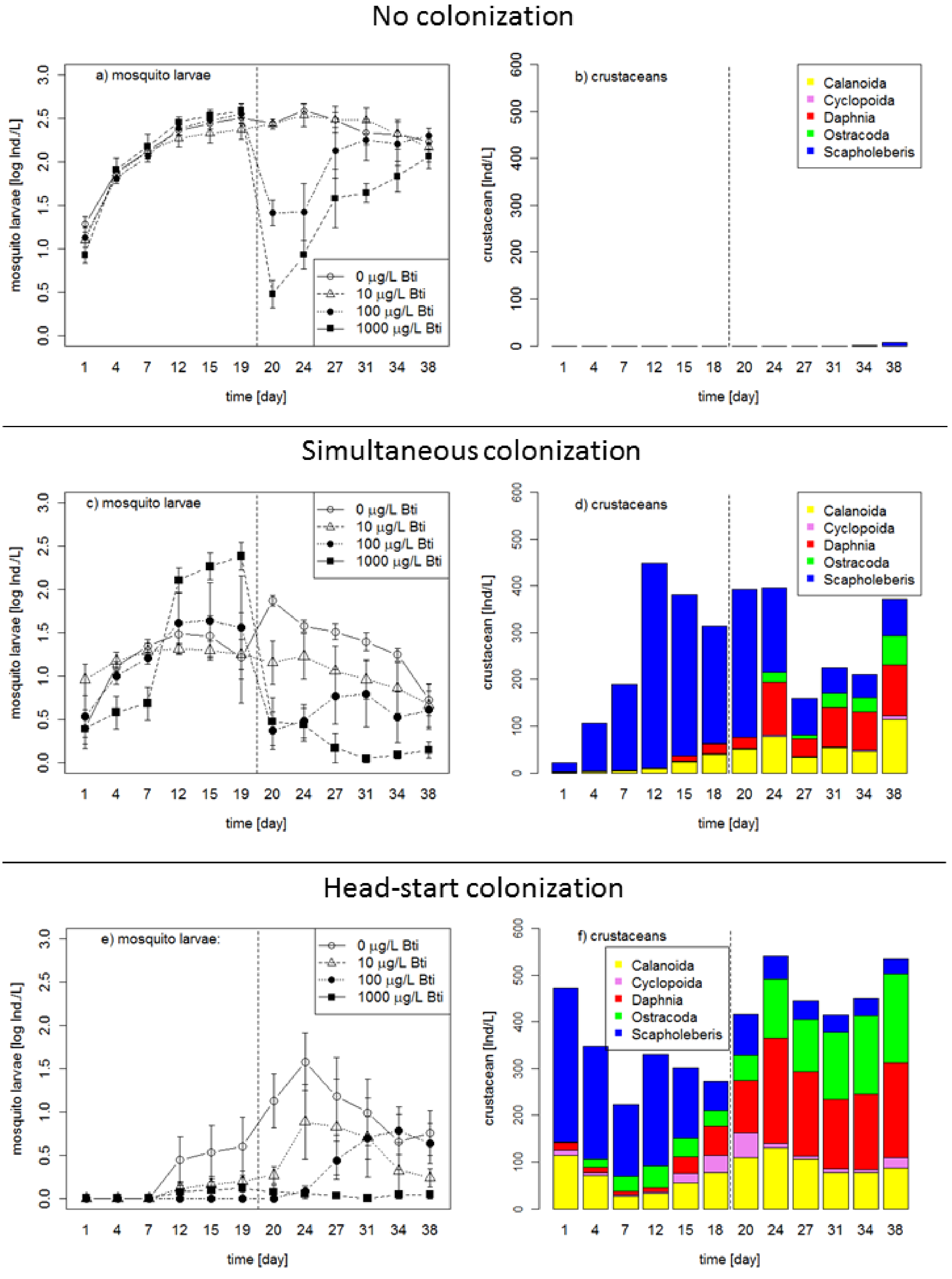

Figure 3.2: Differences in mosquito larval abundances as well as crustacean abundances and composition in set-ups with different time points of crustacean colonization (no colonization, crustaceans absent; simultaneous colonization, crustacean development started at the same time as insect colonization; head-start colonization, crustacean development started two weeks prior to insect colonization).

The vertical dashed line marks the treatment with Bti. Error bars represent ± SE.

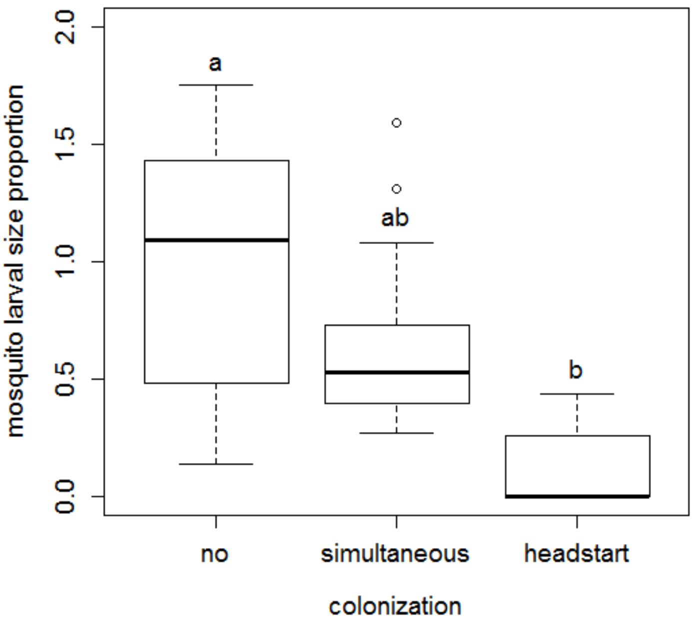

Figure 3.3: Mosquito larval size distribution (ratio of large to small larvae) in set-ups at different time points of crustacean colonization (no colonization, crustaceans absent; simultaneous colonization, crustacean development started at the same time as insect colonisation; head-start colonization, crustacean development started two weeks prior to insect colonization) using mean values of the time period before treatment with Bti (days 1–19). Different letters above bars signify statistically significant differences by pairwise t-test ($p<0.05$). Error bars represent ± SE.

3.4.3 Impact of Bti treatment

The effect of Bti on the development of *Cx. pipiens* larvae increased significantly in the presence of crustaceans. Indeed, in microcosms with no colonization, the abundance of mosquito larvae was significantly reduced in the short term at both medium (100 µg/L) and high (1,000 µg/L) concentrations of Bti, whereas the lowest concentration of Bti tested (10 µg/L) also had an effect following both simultaneous and head-start colonization (Fig. 3.4a). A long-term effect of Bti was only observed for populations of *Cx. pipiens* larvae following either simultaneous or head-start colonization for the highest (1,000 µg/L) concentration of Bti tested (Fig. 3.4b).

However, the impact of Bti on the abundance of *Cx. pipiens* larvae (gls model, $\text{Beta}_{10\mu g/L Bti}=0.069$, $P_{10\mu g/L Bti}=0.545$, $\text{Beta}_{100\mu g/L Bti}=-0.414$, $P_{100\mu g/L Bti}<0.001$, $\text{Beta}_{1000\mu g/L Bti}=-0.688$, $P_{100\mu g/L Bti}<0.001$) was outweighed by the effect of crustacean species diversity, as indicated by the significant interaction between Bti and diversity of crustacean taxa (gls model, $\text{Beta}_{10\mu g/L Bti*simpson}=-0.112$, $P_{10\mu g/L Bti*simpson}=0.326$, $\text{Beta}_{100\mu g/L Bti*simpson}=0.268$, $P_{100\mu g/L Bti*simpson}=0.022$, $\text{Beta}_{1000\mu g/L Bti*simpson}=0.635$, $P_{100\mu g/L Bti*simpson}<0.001$). Indeed, the abundance of mosquito larvae was extremely low for head-start colonization (Fig. 3.2e); hence, the long-term effect of Bti was hardly detectable.

Administration of Bti had no significant short- or long-term effect on *Cx. pipiens* oviposition or the abundance of crustacean taxa (gls model, $P > 0.05$, data not shown).

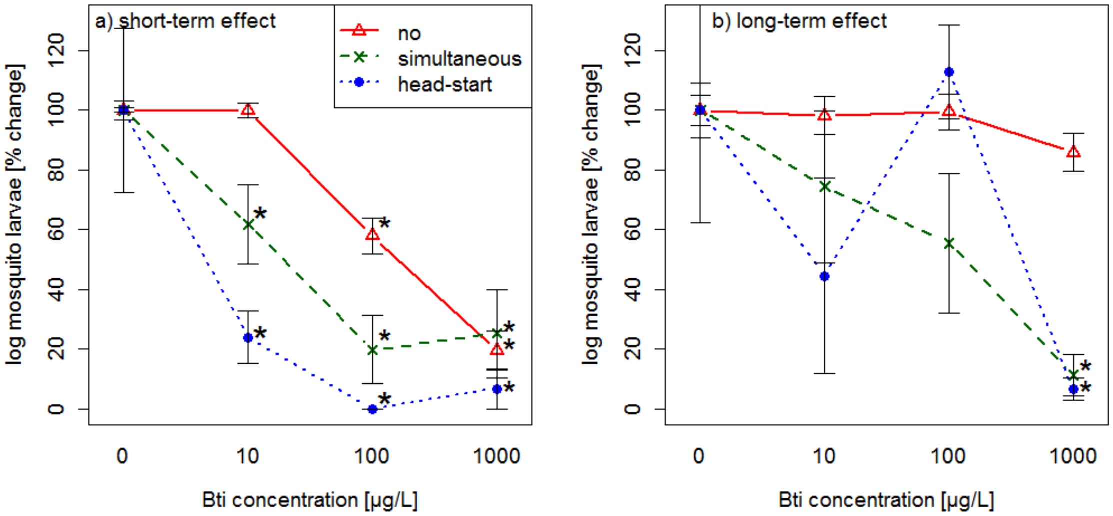

Figure 3.4. Dose-response relationship between the abundance of mosquito larvae and Bti treatment at different concentrations in the a) short term (i.e., one day after treatment) and b) long term (i.e., mean effect on the last two days of sampling). Asterisks indicate significant differences compared with the control, that is, 0 µg/L Bti (t-test, P < 0.05). Error bars indicate ± SE.

3.4.4 Water parameters related to colonization set-ups

Phosphate, chlorophyll, turbidity and dissolved oxygen were significantly higher in microcosms with no colonization than in those with simultaneous and head-start colonization both before and after Bti treatment (Table 3.2). The pH was highest in head-start colonization, with significant differences compared with the other two conditions (Table 3.2).

Treatment with Bti was followed by a significant reduction of chlorophyll (two-way ANOVA, $P_{\text{condition}} < 0.001$, $P_{\text{Bti}} = 0.001$), but the other water parameters tested were unaffected. However, this impact of Bti was only detectable for microcosms with no colonization, with significant differences between control and intermediate-Bti treatments (pairwise t-test, $P < 0.001$) and between control and high-Bti treatments (pairwise t-test, $P < 0.001$). Chlorophyll was significantly positively correlated with the abundance of *Cx. pipiens* larvae before Bti treatment (linear regression, t-value = 7.729, $P < 0.001$, $R^2 = 0.410$). After treatment with Bti, chlorophyll was still significantly positively correlated with the abundance of *Cx. pipiens* larvae in microcosms not treated with Bti (linear regression, t-value = 6.253, $P < 0.001$, $R^2 = 0.848$).

Analysis of only microcosms that included crustaceans (i.e., simultaneous and head-start colonization) before treatment with Bti revealed that chlorophyll was significantly and negatively correlated with the diversity of crustacean taxa, but not with crustacean abundance (multiple linear regression, $P_{\text{diversity}} < 0.001$, $P_{\text{abundances}} = 0.682$, $R^2 = 0.239$).

Table 3.2: Differences in water parameters between set-ups using mean values of different time points (early = days 1-19, late = days 20-38) of crustacean colonization (no colonization, crustaceans absent; simultaneous colonization, crustaceans introduced at the same time as when insect colonization started; head-start colonization, crustaceans introduced two weeks prior to insect colonization) using ANOVA.

Parameter	Time period	Condition	Mean ± SD	ANOVA P	Pairwise t-test	
					condition	P
Chlorophyll [µg L^{-1}]	early	no	0.92 ± 0.18	<0.001	no - simul.	<0.001
		simul.	0.72± 0.16		no - head	<0.001
		head	0.64 ± 0.39		simul. - head	0.264
	late	no	3.41 ± 1.16	<0.001	no - simul.	0.002
		simul.	0.95 ± 0.77		no - head	<0.001
		head	0.64 ± 0.06		simul. - head	0.610
Oxygen [%]	early	no	19.79 ± 1.43	<0.001	no - simul.	<0.001
		simul.	15.82 ± 0.81		no - head	0.025
		head	17.55 ± 4.28		simul. - head	0.082
	late	no	18.66 ± 0.59	<0.001	no - simul.	0.003
		simul.	14.93 ± 0.50		no - head	0.066
		head	16.70 ± 2.16		simul. - head	0.090
pH	early	no	8.10 ± 0.12	<0.001	no - simul.	0.350
		simul.	8.03 ± 0.09		no - head	<0.001
		head	8.83 ± 0.35		simul. - head	<0.001
	late	no	8.15 ± 0.06	<0.001	no - simul.	0.115
		simul.	7.98 ± 0.04		no - head	<0.001
		head	8.65 ± 0.21		simul. - head	<0.001
Phosphate [mmol L^{-1}]	early	no	2.18 ± 0.50	<0.001	no - simul.	<0.001
		simul.	0.21 ± 0.15		no - head	<0.001
		head	0.12 ± 0.14		simul. - head	0.429
	late	no	1.90 ± 0.25	<0.001	no - simul.	<0.001
		simul.	0.22 ± 0.07		no - head	<0.001
		head	0.13 ± 0.04		simul. - head	0.409
Turbidity [NTU]	early	no	2.81 ± 1.68	<0.001	no - simul.	<0.001
		simul.	1.15 ± 0.59		no - head	0.014
		head	1.84 ± 0.32		simul. - head	0.078
	late	no	7.35 ± 2.87	<0.001	no - simul.	0.001
		simul.	1.43 ± 0.98		no - head	0.002
		head	1.77 ± 0.21		simul. - head	0.796

3.5 Discussion

3.5.1 Density vs. diversity

The results of our study showed that oviposition as well as the abundance and development of *Cx. pipiens* larvae were both substantially inhibited in microcosms that included crustaceans compared with microcosms that lacked crustaceans. There are two mechanisms driving this negative relationship, namely predation and competition. The negative impact of predatory crustaceans, particularly Cyclopoida and Ostracoda, on mosquito larval populations has been frequently reported (Lardeux et al., 1992; Rey et al., 2004; Rossi et al., 2011). Filtrating crustaceans like Caldocera limit food resources (see below) and were found to reduce mosquito larval abundances in both mesocosm and field settings (Knight et al., 2004; Duquesne et al., 2011; Kroeger et al., 2013). Female mosquitoes select breeding sites for oviposition carefully, avoiding those ponds already colonized by predators or competitors (Blaustein and Kotler, 1993; Munga et al., 2006) but preferring ponds with high nutrient levels (Reiskind and Wilson, 2004). In our study, microcosm containing crustacean communities of multiple taxa were characterised by presence of predators and reduced nutrient level caused by competitors; hence those ponds were less attractive for female mosquitoes and consequently oviposition rate was low.

We found that the diversity of crustacean taxa, and not crustacean abundance, was the most important factor driving the negative impact of crustacean communities on *Cx. pipiens* oviposition and larval abundance. Some researchers have suggested that an increase in biodiversity increases the impact of competition by differentiation between the spatial niches shared by competitors, which limits the resources available for each competitor (Menge and Sutherland, 1976; Nascimento et al., 2011). As discussed below, analysis of the species composition of crustacean communities suggests that similar effects can be assumed.

3.5.2 Feeding behaviour of crustacean species

Scapholeberis spp. mainly feed on the water surface (Thorp and Covich, 2010), whereas larvae of *Cx. pipiens* mainly feed in the water column (Merritt et al., 1992). Hence, competition between these species is limited by spatial constraints. *Daphnia* spp. also feed in the water column, and have a strong impact on mosquito larvae, causing an increase in the time to metamorphosis and a decrease in size at metamorphosis (Knight et al., 2004; Stav et al., 2005; Duquesne et al., 2011). However, larvae of *Cx. pipiens* are able to avoid competition with one competitor species by shifting their feeding behaviour, for example, from filter feeding to periphyton grazing (Yee et al., 2004). This strategy is not feasible in the presence of Ostracoda, which are mainly peiphyton grazers (Roca et al., 1993) and have also been reported to feed on mosquito larvae (Rossi et al., 2011).

The presence of an additional predator, e.g. Cyclopoida, enhances the negative effect of interspecific competition on mosquito larvae (Knight et al., 2004). Hence, a balanced community of diverse crustacean species was more effective in limiting mosquito larvae than a crustacean community dominated by a single species because all food niches were already occupied and mosquito larvae were additionally stressed by predation. The reduction of food resources by crustaceans was also confirmed by the analyses of physiochemical water parameters in the present study. Indeed, our results showed more pronounced declines in chlorophyll levels (associated with declines in phosphate, dissolved oxygen and turbidity) in microcosms that included crustacean populations than in those that lacked crustaceans. Chlorophyll was thereby negatively correlated with the diversity of crustacean taxa but not with crustacean abundance, which indicated that a diverse community of crustaceans was more efficient in filtering the water column compared with a single species.

3.5.3 Combined effect of crustaceans and Bti treatment on populations of mosquito larvae

We found that both the sensitivity of *Cx. pipi*ens larvae to Bti and the time span during which Bti was effective increased with increasing competition and predation pressure caused by crustaceans. Increased effects of toxicants in the presence of competitive pressure have been demonstrated frequently. Examples include the combined effect of competition and a toxicant in a simple two-species system (Foit et al., 2012), as well as in multi-species outdoor systems (Liess, 2002; Knillmann et al., 2012). The presence of interspecific competitors limits the amount of food resources, resulting in delayed development and decreased survival of mosquito larvae. However, at our study crustacean communities included not only competitors but also predators. Several studies demonstrated, that mosquito larvae reduce foraging behaviour, resulting in lower feeding rates, when cues of predators were present (Juliano and Reminger, 1992; Juliano and Gravel, 2002; KESAVARAJU and JULIANO, 2004). The additive stress caused by predators and competitors result in higher vulnerability of mosquito larvae to toxicant exposure (Beketov and Liess, 2007). In addition, inhibition of recolonization by *Cx. pipiens* larvae after Bti treatment in the presence of crustaceans was also observed in our study. This result is consistent with other studies that demonstrated that competition delays the recovery of population structure after exposure to toxicants (Liess and Foit, 2010; Foit et al., 2012). However, in the present study, the effect of Bti on *Culex* was hardly detectable in the long term owing to the strong impact of crustaceans. Indeed, the impact of Bti was restricted to a short-term decrease in the abundance of *Cx. pipiens* larvae, while interspecific competition due to highly diverse crustacean communities reduced the abundance of *Cx. pipiens* in the long term and also inhibited both oviposition and larval development of *Cx. pipiens*.

Our findings highlight the importance of biodiversity for mosquito control, and show for the first time that crustaceans are more sustainably effective against mosquito larval populations than Bti treatment.

3.6 Conclusions

The presence of a diverse crustacean community prevents the colonization of ponds by mosquito larvae in a more efficient and sustainable manner than insecticide treatment. However, further investigations are necessary to identify the interactions between insecticide treatment and natural antagonists of mosquitoes, in order to take advantage of this combination within integrated control strategies.

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4 Sustainable control of mosquito larvae in the field by the combined actions of the biological insecticide Bti and natural competitors

Kroeger, I.[1], *Liess, M.*[1] , *Dziock, F.*[3], *Duquesne, S.*[1,2]

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[1] Department of System Ecotoxicology, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany
[2] Department of Biology Conservation, UFZ, Helmholtz Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany
[3] Department of Agriculture and Landscape Management, HTW Dresden, University of Applied Sciences, Professorship in Animal Ecology/Applied Ecology, Pillnitzer Platz 2, D-01326 Dresden, Germany

4.1 Abstract

Integrated management of mosquitoes is becoming increasingly important, particularly in relation to avoiding recolonisation of ponds after larvicide treatment.
We conducted for the first time field experiments that involved exposing natural populations of the mosquito species *Culex pipiens* to: a) application of the biological insecticide *Bacillus thuringiensis israelensis* (Bti), b) the introduction of natural competitors (a crustacean community composed mainly of *Daphnia spp*.), or c) a combined treatment that involved both introduction of a crustacean community and the application of Bti. The treatment that involved only the introduction of crustaceans had no significant effect on mosquito larval populations, while treatment with Bti alone caused only a significant reduction in the abundance of mosquito larvae on the short-term (within 3–10 days after treatment). In contrast, the combined treatment rapidly reduced the abundance of mosquito larvae, which remained low throughout the entire observation period of 28 days. Growth of the introduced crustacean communities was favoured by the immediate reduction in the abundance of mosquito larvae following Bti administration, thus preventing recolonization of ponds by mosquito larvae at the late period (days 14-28 after treatment).
Both competition and the temporal order of establishment of different species are hence important mechanisms for efficient and sustainable mosquito control.

Keywords: biological control, Cladocera, colonisation, intraspecific competition, insecticide, priority effect

4.2 Introduction

The control of mosquitoes is becoming increasingly challenging because climate change and global trade favour the spread of invasive mosquito species (Roiz et al., 2008; Schäfer and Lundström, 2009) and strongly increase the associated risk of vector-borne diseases (Weaver and Reisen, 2010). Most strategies for mosquito control are based on the use of insecticides. However, intensive use of insecticides has unwanted effects on non-target species (Suma et al., 2009; Mommaerts et al., 2010), and increases the risk of target species developing resistance (Akiner et al., 2009; Melo-Santos et al., 2010); furthermore, treated populations can recover after application of the insecticide (Seleena et al., 1999). As a consequence, integrated pest management, using biological antagonists either alone or in combination with insecticides, has drawn increasing interest (Gurr et al., 2003; van den Berg and Takken, 2009; Gentz et al., 2010).

Strategies for mosquito control that are based on biological methods have been used since the early 20th century (Legner and Sjogren, 1984). In particular, the use of predators (e.g., *Gambusia affinis,* Dystiscidae, Odonata) has been investigated intensively (Bence, 1988; Blaustein et al., 1995, 1995; Fincke et al., 1997; Kumar and Hwang, 2006; Beketov and Liess, 2007). However, difficulties in colonisation and the management of predators, as well as a lack of synchrony between the life cycles of predator and prey, have impeded their deployment (Bay, 1974; Kindlmann and Dixon, 2001; Kumar and Hwang, 2006). As a consequence, we have changed our focus to the use of natural competitors (i.e., crustaceans) to control mosquito larvae. Many crustacean species show similar biotope preferences (i.e., early colonisation of temporary ponds) and similar feeding behaviour (i.e., filter feeding) to that shown by mosquito larvae (Williams, 2006). Given that crustaceans are found in nearly all types of pond, they could be used as agents for mosquito control without disturbing the natural biotope fauna (Liess and Duquesne, 2009). Previous field investigations found that competing communities of crustaceans had a negative impact on populations of mosquito larvae (Chase and Knight, 2003; Beketov et al., 2010; Meyabeme Elono et al., 2010). In semi-field experiments, Cladocerans (e.g., *Daphnia magna*) negatively affected mosquitoes (i.e., *Culex pipiens* or *Anopheles quadrimaculatus*) by reducing the amount of oviposition (Duquesne et al., 2011), increasing time to pupation (Knight et al., 2004; Stav et al., 2005), and reducing total abundances of mosquito larvae (Knight et al., 2004; Duquesne et al., 2011). However, the negative effect of Cladocerans was only found for well-established populations, that is, Cladoceran populations that had developed for more than one week before colonisation by mosquito larvae (Knight et al., 2004; Stav et al., 2005; Duquesne et al., 2011). Hence, under field conditions, control of mosquito larvae by crustaceans will be limited during the initial phase of community development, when abundances of crustaceans are still low. Thus, the use of insecticides may still be required to control mosquito larvae until populations of crustaceans have become established. In this respect, the use of the biological insecticide *Bacillus thuringiensis israelensis* (Bti) in combination with the introduction of a crustacean community might be an appropriate integrated approach to mosquito management. Indeed, Bti has no negative effect on crustacean populations (Rivière et al., 1987; Becker et al., 1992). However, its ability to eliminate mosquito larvae is only temporary (Boisvert and Boisvert, 2000).

On the basis of the findings mentioned above, we hypothesized that the long-term effects of crustaceans will strengthen the impact of the insecticide, owing to additive and complementary effects of these two stressors on larval populations of mosquitoes (Liess and Duquesne, 2009). In the present study, this hypothesis was evaluated under field conditions by attempting to eliminate mosquito larvae that had colonised ephemeral ponds by either introducing only natural competitors, applying Bti alone, or applying Bti in combination with introduced natural competitors. The aim of the study was to investigate whether the integrated control approach was more effective and sustainable than the common strategy for mosquito control, which involves Bti treatment alone.

4.3 Material and Methods

4.3.1 Location

Field investigations were conducted in a forested area in Leipzig (51°18’15.60” N 12°21’44.39” E) during the period from 16 June, 2008 until 14 July, 2008. We monitored

18 natural ponds with surface areas that varied between 1 m^2 and 8 m^2 and a water depth that varied between 3 cm and 25 cm. No vegetation was present inside the ponds, but trees and bushes surrounded them. A natural community of mosquito larvae and other insects had colonised the ponds before treatment. However, these insect populations consisted mainly of mosquito larvae (Culicidae). Other insects, such as members of Ephemeroptera, Chironomoidae, and Notonectidae, were found only sporadically, and thus were excluded from the analysis. The results obtained from ponds that provided less suitable breeding conditions for mosquitoes as indicated by less than 50 mosquito larvae per litre on the day of treatment (N = 3) are not reported.

4.3.2 Treatment

Three different treatments were tested. For the first treatment, which is referred to as "Competition", a natural community of crustaceans was introduced. The organisms that formed these crustacean communities were collected from a lake near Rosslau (51°53'08" N 12°19'11" E) using a plankton net (mesh size, 500 µm). Organisms were introduced such that their final density in the treated ponds was approximately five individuals per litre. The introduced community comprised *Ceriodaphnia* spp. (74.7%), *Simocephalus* spp. (7.5%), *Daphnia* spp. (3.6%), *Scapheloberis* spp. (2.7%), Ostracoda (9.7%), and Cyclopoida (1.7%).

For the second treatment, which is referred to as "Bti", ponds were treated with 1,000 µg Bti/L, which is the concentration used routinely in mosquito control programmes (Becker et al. 2003). The liquid stock solution used (Vectobac 12 AS, Valent BioScience Corporation, Lyon, France) had an activity of 1,200 International Toxin Units (ITU) per milligram.

For the third treatment, which is referred to as "Bti+Competition", Bti was applied at a concentration of 1,000 µg Bti/L, and a community of natural crustaceans was introduced as in the treatment "Competition".

For the control condition, no treatment was applied.

Four replicates were considered in the data analysis for the treatments "Bti", "Bti+Competition", and control, and three replicates for the treatment "Competition".

4.3.3 Sampling

A water sample with a total volume of 0.5–2 L (2 L for ponds with a surface area >5 m^2, 1 L for ponds with a surface area 1–5 m^2, 0.5 L for ponds with a surface area <1 m^2) was collected twice a week from each pond. Each water sample consisted of several 300-mL subsamples, which were collected with a scoop from different parts of the pond. The samples were filtered through a plankton net (55-µm mesh size), and preserved in 70% ethanol (approximately 30 mL). The abundances and composition of the zooplankton were then analysed using a binocular Leica S6D microscope (Wetzlar, Germany). Mosquito larvae were characterised to the species level using the determination key of (Becker et al., 2010). All other in vertebrates were characterized to suborder or family level using the following determination keys (Brauer, 1909; Stresemann, 1957; Einsele, 1993; Klausnitzer, 2009).

The concentration of dissolved oxygen, temperature, pH, and conductivity of water samples were measured twice a week, between 9 a.m. and 2 p.m., using an Oxi340 oxygen meter (WTW, Weilheim, Germany) and a pH/EC/TDS Combo testing meter (Hanna Instruments, Kehl am Rhein, Germany). Water parameters did not differ significantly among treatments (data not shown), and thus were not included in the subsequent analysis.

4.3.4 Statistics

All data were log transformed before all data analyses, which were performed using three steps.

- First, data on abundances were analysed for variance between experimental (“Competition”, “Bti”, “Bti+Competition”) and control treatments at each single sampling day using Student’s t-test. Significant differences were denoted by asterisks in graphical representations of the data. Data were tested for a normal distribution (using the Shapiro–Wilk Normality Test) and homogeneity (F-test) to verify that underlying statistical assumptions were not violated.
- Second, the changes in population size (i.e. slope) over a period of time of mosquito larvae and crustaceans following the different treatments were compared with the changes in population size under control conditions. The periods of time were separated in two periods, i.e. the early (days 3–14 after treatment) and late (days 14–28 after treatment) time periods. The separate datasets collected for each time period were analysed using a generalized least squares (GLS) mixed model approach. Our response variable was “mosquito larval abundance” or “crustacean abundance”. The predictor variables were “day of development” and the “type of treatment” (“Competition”, “Bti” or “Bti+Competition”). The analysis used multiple measures over time in multiple ponds, thus violating the statistical assumption of independence of observations for standard testing (Pinheiro and Bates, 2000; West et al., 2006). As a consequence, we used the factorial variable “pond” as a random effect for a first model. Given that temporal autocorrelation of subsequent measures in the same pond was to be expected, we calculated a second additional model using an autocorrelation structure (AR1: autoregressive model of order 1) (Zuur et al., 2009). We then compared these two models using Akaike’s information criterion (AIC), and chose the model structure with the lowest AIC (Zuur et al., 2009). As a result, the first model was used to analyse crustacean development, whereas the second model was considered to be appropriate for analysing mosquito larval development. The final models were presented using the restricted maximum likelihood (REML) calculation. All models were validated by plotting theoretical quantiles versus standardized residuals (Q–Q plots) to assess the normality of residuals. Homogeneity of variance was evaluated by plotting residuals versus fitted values, and influential data points were identified using Cook’s distance method (Quinn and Keough, 2002).
- Third, the impacts of antagonists (Cladocera, Ostracoda, and Cyclopoida) on the abundance of mosquito larvae were investigated on the last day of the observation period (day 28 after treatment) using multiple linear regression. The abundances of antagonists with a significant impact were plotted against the abundances of mosquito larvae and the linear regression line was added.

Analyses were performed using the ‘R’ statistical and programming environment (R Development Core Team 2010) and the ‘nlme’ (Pinheiro et al., 2009) and ‘lattice’ (Sarkar, 2009) packages.

4.4 Results

Invertebrate communities were dominated by mosquito larvae (i.e. Culicidae) and crustaceans (i.e. Crustacea). Other taxa (i.e. Notonectidae and larvae of Chaoberidae, Chironomidae, Ephemeroptera, Megaloptera) were only sporadically observed and therefore excluded from analyses.

The pattern of development of the introduced natural community of crustaceans over time indicated the existence of two distinct periods: an early time period (days 3–14 after treatment), which was characterised by increasing abundances of crustaceans, and a late time period (days 14–28 after treatment), which was characterised by stable crustacean populations (see below). Hence the following analyses were performed separately for these two time periods.

4.4.1 Effect of treatments on abundances of mosquito larvae

Populations of mosquito larvae consisted mainly of *Culex pipiens* (>99%) and initial mosquito abundances were similar in all treatments (238 ± 96 individuals/L).

The “Competition” treatment failed to decrease the number of mosquito larvae significantly. Indeed, although both the abundance of mosquito larvae at each sampling time and the overall increase in the population size of mosquito larvae during the early (days 3–14) and late (days 14–28) time periods were slightly lower for the “Competition” treatment than the control treatment (Fig.4.1, Table 4.1), the differences were rarely statistically significant.

The “Bti” treatment significantly reduced the number of mosquito larvae on the short-term, but not on the long-term. Indeed, during the early time period, at 3 days, 7 days, and 10 days after treatment, the “Bti” treatment resulted in significantly lower abundances of mosquito larvae than the control treatment (Fig. 4.1). However, after day 3, when the lowest value was observed, the abundance of mosquito larvae increased significantly (days 3–14, Table 4.1), and by day 14 had reached a similar abundance to that recorded in the control (Fig. 4.1). In the longer term (days 14–28), there were no significant differences between the control and “Bti” treatments in terms of either the abundance at specific days or changes in population size over that period of time (Fig. 4.1, Table 4.1).

The “Bti+Competition” treatment caused a significant decrease in the abundance of mosquito larvae in the short term, with significant differences in relation to the control treatment evident on days 3, 7, and 10 after treatment (Fig. 4.1). In contrast to the “Bti” treatment, mosquito larval population size did not increase significantly under the combined treatment at the end of the early time period, but rather decreased further during the late time period (Table 4.1). Consequently, under the “Bti+Competition” treatment, the abundance of mosquito larvae was significantly lower than for the control treatment on almost all sampling days (Fig. 4.1).

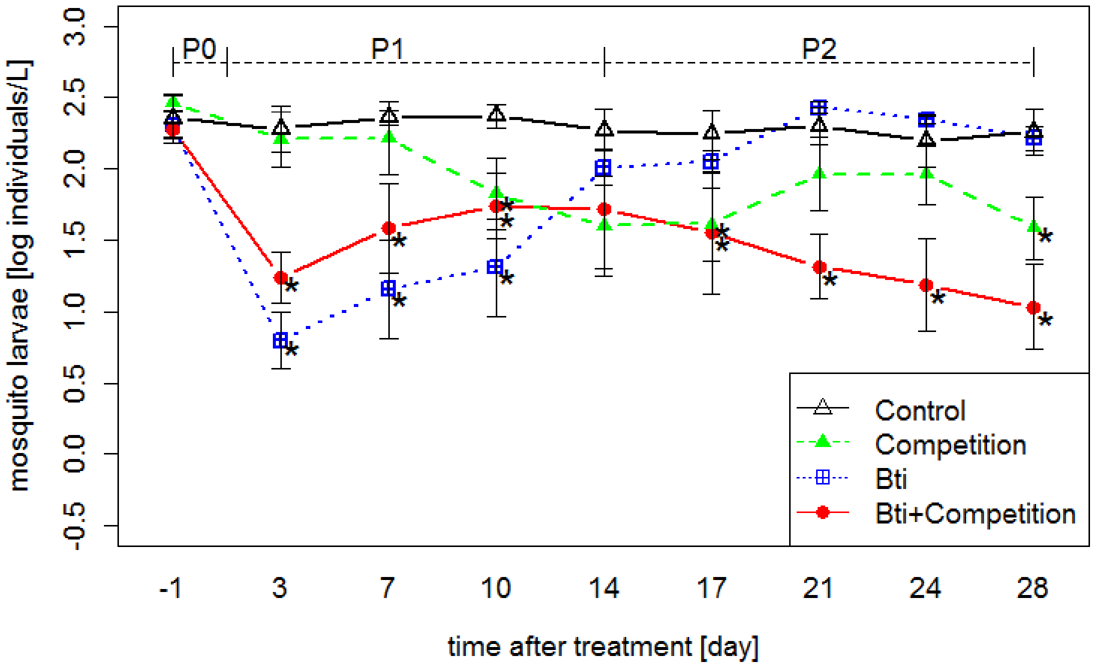

Figure 4.1: Changes in the abundance of mosquito larvae (mean ± SE) in different treatment groups [Control (N=4) = no treatment; Competition (N=3) = treatment with introduction of a crustacean community; Bti (N=4) = treatment with Bti (1,000 µg/L); Bti+competition (N=4) = treatment with Bti (1,000 µg/L) and the introduction of a crustacean community] over time [P0 = before treatment; P1 = 3 – 14 days after treatment; P2 = 14 – 28 days after treatment].

*** Significant differences compared to control (t-test, $p < 0.05$)**

Table 4.1: Effects of treatments [Competition (N=3) = introduction of crustacean community; Bti (N=4) = treatment with Bti (1,000 µg/L); Bti+competition (N=4) = treatment with Bti (1,000 µg/L) and the introduction of a crustacean community] as compared with control conditions (N=4) on the changes in population size (i.e. slope) of zooplankton (mosquito larvae and crustaceans) over different time periods (early = 3–14 days after treatment; late = 14–28 days after treatment).

Organisms	Treatment	Period	SE	t-value	p-value	statistics
Mosquito larvae	Competition	early	0.442	-1.099	0.283	AR-1
		late	0.340	-0.186	0.854	AR-1
	Bti	early	0.281	2.452	0.021	AR-1
		late	0.259	0.935	0.356	AR-1
	Bti+competition	early	0.336	1.307	0.202	AR-1
		late	0.024	-2.019	0.047	AR-1
Cladocera	Competition	early	0.446	0.413	0.683	Random
		late	0.340	-0.257	0.798	Random
	Bti	early	0.135	-1.065	0.296	Random
		late	0.246	-0.029	0.977	Random
	Bti+competition	early	0.302	2.699	0.012	Random
		late	0.317	-0.429	0.671	Random
Ostracoda	Competition	early	0.052	-1.233	0.229	Random
		late	0.011	-0.778	0.442	Random
	Bti	early	0.033	0.198	0.844	Random
		late	0.005	1.407	0.168	Random
	Bti+competition	early	0.069	0.246	0.807	Random
		late	0.026	2.253	0.030	Random
Cyclopoida	Competition	early	0.028	-1.335	0.195	Random
		late	0.026	-0.455	0.652	Random
	Bti	early	0.266	-0.232	0.818	Random
		late	0.082	-2.125	0.041	Random
	Bti+competition	early	0.048	0.018	0.986	Random
		late	0.027	-0.405	0.688	Random

4.4.2 Effect of treatments on crustacean populations (abundances and composition)

For all treatments, the crustacean communities comprised organisms of three orders: Cladocera (77.1% ± 33.6%), Cyclopoida (14.7% ± 31.1%), and Ostracoda (8.25% ± 19.0%). Organisms within each of the three orders were analysed separately. Cladocera were dominated by *Daphnia* spp. (82.8% ± 25.6%), with all other species each accounting for less than 3% of the total population of Cladocera. Abundances of crustaceans were initially very low (Cladocera at 7.20 ± 19.2 individuals/L, Cyclopoida at 1.80 ± 6.69 individuals/L, and Ostracoda at 0.13 ± 0.52 individuals/L), and were similar in all treatments before the competitive crustacean communities were introduced (Fig. 4.2).

The abundances of Cladocera did increase in the control during the early time period (days 3–14), but stabilised at a higher level during the late time period (days 14–28) (Fig. 4.2a). A similar pattern was observed for the “Bti” and “Competition” treatments (Table 4.1). Although in the “Competition” treatment, the abundances of Cladocera increased over time (Fig. 4.2a), there was no significant difference as compared with the control (Fig. 4.2a). In contrast, following the “Bti+Competition” treatment, the abundance of Cladocera increased significantly during the early time period (Table 4.1), and remained significantly higher than that of the control during the late time period (Fig. 4.2a).

The abundance of Ostracoda increased slightly in the control during the early time period, but these species had apparently disappeared completely by the end of the late time period (Fig. 4.2b). A similar observation was made following the “Bti” and “Competition” treatments (Table 4.1), with some significant differences in abundance evident between the “Competition” and control treatments at few time points (Fig. 4.2b). Following the “Bti+Competition” treatment, changes in the abundance of Ostracoda showed similar trends to those seen for the control treatment during the early time period. However, the abundance of Ostracoda increased significantly during the late time period (Table 4.1), and reached significantly higher values than those seen in the control at 21 days after treatment (Fig. 4.2b).

Abundances of Cyclopoida remained stable over time for the control, “Competition”, and “Bti+Competition” treatments (Fig. 4.2c). For the “Bti” treatment, the abundance increased slightly during the early time period (Fig. 4.2c) and decreased significantly during the late time period (Table 4.1).

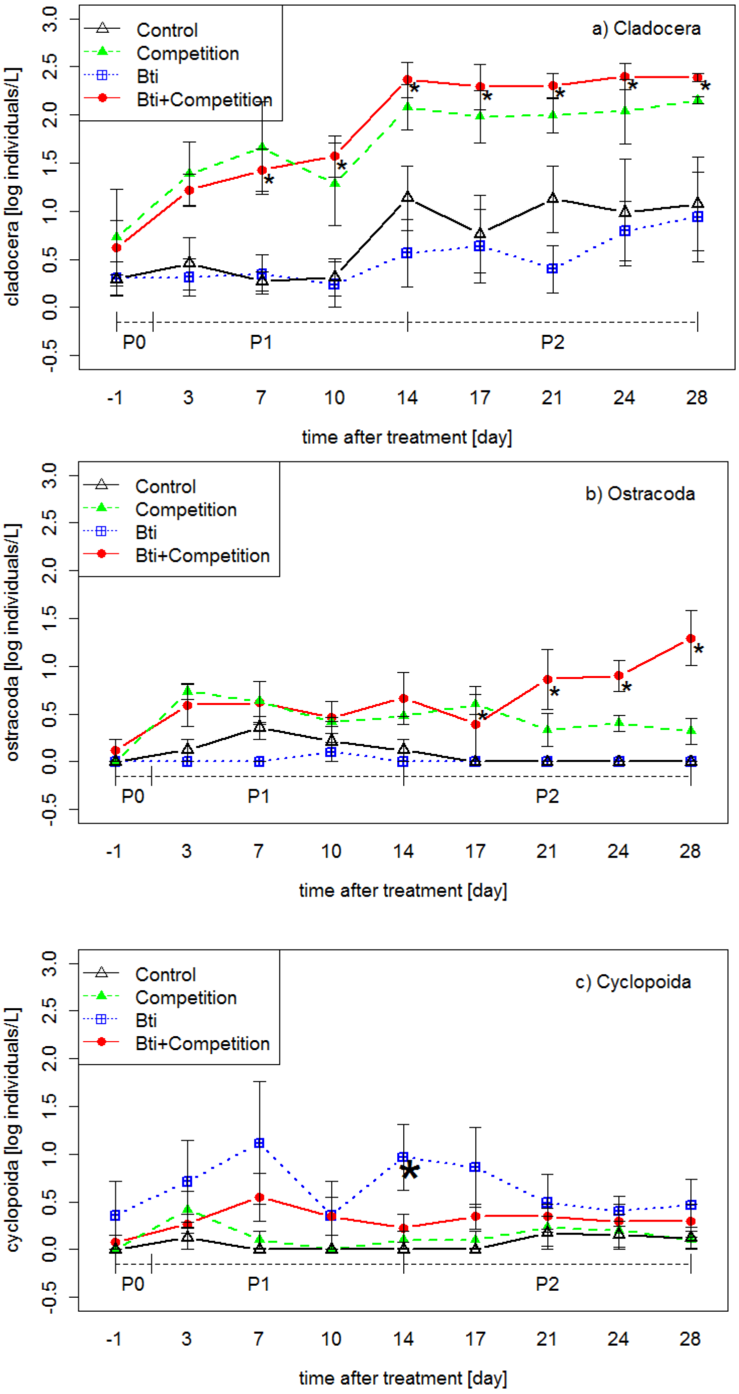

Figure 4.2: Changes in the abundances of (a) Cladocera, (b) Ostracoda, and (c) Cyclopoida. Abundances (mean ± SE) in different treatment groups [Control (N=4) = no treatment; Competition (N=3) = treatment with the introduction of a crustacean community; Bti (N=4) = treatment with Bti (1,000 µg/L); Bti+competition (N=4) = treatment with Bti (1,000 µg/L) and the introduction of a crustacean community] over time [P0 = before treatment; P1 = 3 – 14 days after treatment; P2 = 14 – 28 days after treatment].
*** significant differences compared to control (t-test, $p < 0.05$).**

4.4.3 Correlations between changes in the abundances of crustaceans and mosquito larvae

The separate analysis of abundances of mosquito larvae and crustaceans described in sections 4.4.1 and 4.4.2. showed that during the late time period (days 14–28 after treatment), a low abundance of mosquito larvae was associated with a high abundance of crustaceans (Fig. 4.1 and Fig. 4.2). The direct relationship between mosquito larvae and crustaceans was analysed subsequently using the data from the last day of the observation period (day 28) to minimise the effect of the Bti treatment. The impact of crustaceans from the orders Cladocera, Ostracoda, and Cyclopoida on the abundance of mosquito larvae was analysed using multiple linear regression. The results showed that only members of Cladocera, which was by far the best represented order in the crustacean population, had a significant impact on the number of mosquito larvae (ANOVA of multiple linear regression model, $p_{\text{Cladocera}} = 0.004$, $p_{\text{Ostracoda}} = 0.374$, $p_{\text{Cyclopoida}} = 0.377$) (Fig. 4.3).

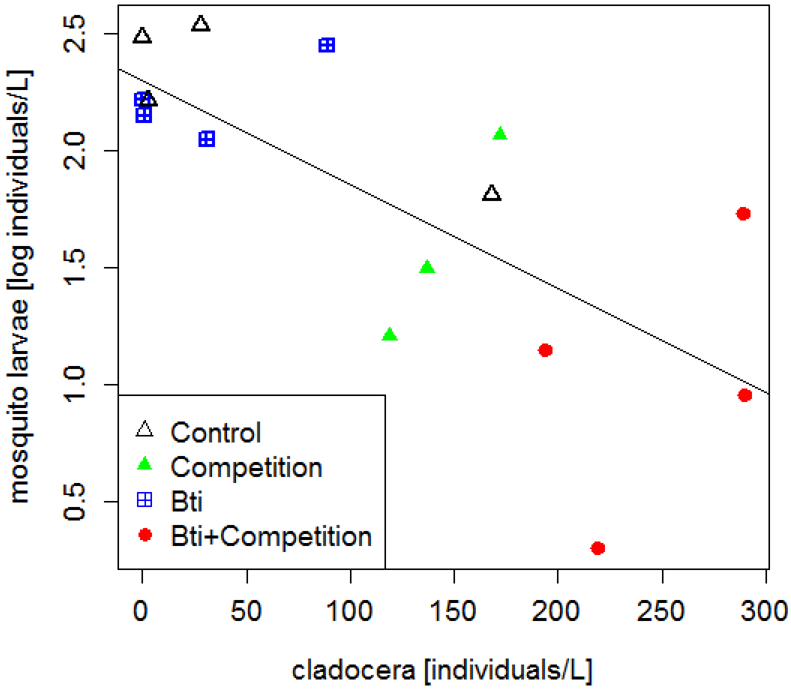

Figure 4.3: Correlation between the abundances of mosquito larvae and Cladocera on day 28 for all treatment groups [Control (N=4) = no treatment; Competition (N=3) = treatment with the introduction of a crustacean community; Bti (N=4) = treatment with Bti (1,000 µg/L); Bti+competition (N=4) = treatment with Bti (1,000 µg/L) and the introduction of a crustacean community]. Regression line added ($y = 2.30 – 0,004x$, $p = 0.003$, $R^2 = 0.512$)

4.5 Discussion

The results of the present field study, which was performed in temporary ponds in forested areas in Saxony (Germany), clearly show that the combined treatment of natural ponds with the biological insecticide Bti and the introduction of natural crustacean communities reduced the abundance of mosquito larvae more sustainably than single treatments that involved either Bti treatment or the introduction of crustaceans alone. This result is consistent with our earlier hypothesis that simultaneous application of Bti and introduction of crustaceans prolongs the effect of Bti application (Liess and Duquesne, 2009). The present study demonstrates the effectiveness of this approach under field conditions and revealed those mechanisms driving the positive effect of the combined approach.

The dominant mosquito species in all ponds analysed was *Cx. pipiens*, which is a mosquito species that is found commonly in urban areas of Germany during the summer (Becker et al., 2010). Treatment of ponds with Bti alone almost completely eliminated the populations of *Cx.pipiens* larvae (as much as a 96% reduction in their sizes) within three days. However, these populations recovered after recolonisation, and had reached sizes similar to those of the control group within two weeks. This finding is consistent with other studies, which showed that Bti is active against mosquito larvae for only a few days (Karch et al., 1991; Aldemir, 2009) and that repeated treatment is needed to ensure long-term reductions in the sizes of mosquito populations (Becker, 2003).

Crustacean communities were dominated largely by Cladocera (mainly *Daphnia* spp.), which are common species in all types of freshwater pond (Williams, 2006). Besides members of Cladocera, members of Cyclopoida and Ostracoda were also present, although only Cladocera affected the size of the population of mosquito larvae significantly. The dominant role of Cladocera in this regard is consistent with other field and outdoor mesocosm studies, which have also demonstrated the negative effect of Cladocera on the establishment of populations of larvae of *Cx. pipiens* and *Aedes* spp. (Chase and Knight, 2003; Meyabeme Elono et al., 2010; Duquesne et al., 2011). A correlation between the abundances of mosquito larvae and Cladocera spp. at the end of the observation period (day 28 after treatment) demonstrated that competition between these antagonists was density dependent (Fig. 3). This is consistent with findings from studies that focused only on mosquitoes, which showed that under both laboratory and field conditions, an increasing density of competitors (i.e., intra- or interspecific mosquito competitors) is linked to increased mortality of the mosquito species of concern, delayed maturity, reduced adult size, and reduced adult longevity (Renshaw et al., 1993; Teng and Apperson, 2000; Agnew et al., 2002; Braks et al., 2004; Reiskind and Lounibos, 2009). In studies of outdoor pond mesocosms, both oviposition and the development of *Cx. pipiens* larvae were reduced more in the presence of high densities of Cladocera than in the presence of low densities (Duquesne et al. 2011). Hence, together with previous studies, the present study demonstrates that only large numbers of competitors can control populations of mosquito larvae.

However, competition is not a one-way road, and interspecific competition works in both directions. Indeed , the abundance of Cladocerans increased less following the introduction of a crustacean population alone than following the combined treatment. One explanation for this is that the reduced number of replicates used for the treatment that involved the introduction of crustaceans alone reduced the statistical power of the results for this treatment compared with the results obtained for the combined treatment. Another explanation is that competition alters according to sequence of introduction in a way that the competitor arriving first gains advantages of the competitor arriving later (Lawler and Morin, 1993; Blaustein and Margalit, 1996; Stokes et al., 2009). Indeed, (Foit et al., 2012) showed that larvae of *Cx. pipiens* delay the development of offspring of *Daphnia magna* when the sizes of *D. magna* populations have already been suppressed by application of a chemical compound. In contrast, established populations of *D. magna* affect both oviposition and the larval development of *Cx. pipiens* negatively (Duquesne et al., 2011), as well as time to metamorphosis and the size of larvae at the time of metamorphosis (Stav et al., 2005).

The timing of the succession of different populations influenced the outcome of the competition between mosquitoes and crustaceans. Simultaneous administration of Bti and introduction of a crustacean community disturbed the normal competitive interaction as a result of the ability of Bti to cause an initial reduction in the number of prior colonisers (i.e., mosquito larvae). In fact, from an ecological perspective, the Bti insecticide acts as a stressor that alters the interactions between competing groups of species, in this case, weakening the population of mosquito larvae (Griswold and Lounibos, 2005; Juliano, 2007). The decline in the size of the population of mosquito larvae promoted propagation of the introduced communities of crustaceans, enabling them to become the dominant group within two weeks and thus to prevent recolonisation of ponds by additional mosquito larvae. However, when established populations of mosquito larvae were not eliminated by Bti, as in the case of the control treatment or the treatment that involved introduction of a crustacean population alone, the development of a natural crustacean community was largely inhibited owing to the increased abundance of mosquito larvae.

Our results showed that competition is an important determinant of the community structures of ephemeral ponds (Blaustein and Chase, 2007; Juliano, 2009). Furthermore, given that competitors can be affected by each other, the temporal order in which species enter a system is of major importance, because it can affect competitive processes.

4.6 Conclusion

Early establishment of crustacean communities can be highly effective in the prevention of outbreaks of mosquitoes (and hence potential outbreaks of mosquito-borne diseases). In cases in which larval populations of mosquitoes are already established, combined treatment that involves the administration of a biological pesticide, such as Bti, and introduction of a crustacean community ensures sustainable control of the sizes of mosquito populations.

Acknowledgments

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5 Discussion

This thesis investigated the impact of crustaceans in combination with Bti-treatment for an integrated mosquito control strategy. In particular we investigated a) the **relevance** of crustaceans for mosquito larval control under field conditions, b) the **mechanisms** driving the negative impact of crustacean communities on mosquito larval populations during the process of pond colonization and after treatment with Bti and c) the **effect** of a combined approach using Bti in combination with crustaceans for sustainable mosquito larval control in the field.

The main results of the three investigations are presented in the following.

5.1 Summary of results

5.1.1 Summary of study 1

In a first step, evaluating the relevance of crustaceans as mosquito control agent, we investigated the biological niche overlap between different crustacean taxa and larvae of different mosquito species in the field (chapter **Fehler! Verweisquelle konnte nicht gefunden werden.**). A field monitoring of temporary ponds (i.e. hydroperiod < 7 weeks) was conducted at three different geographical regions in Germany (Leipzig, Rosslau, Spreewald) and at three different biotopes (i.e. grassland, reed-covered wetland and forest) during the summer season (June – September 2007). Mosquito larval populations were dominated by the two species *Aedes vexans* and *Culex pipiens*, while crustacean communities consisted of Copepoda (Cyclopoida and Harpacticidae), Cladocera (Chydoriidae and Daphniidae) and Ostracoda.

In a first step we identified those species sharing similar biological niches. Therefore the impact of physiochemical parameters (e.g. water temperature, pH), spatial parameters (geographical location and biotope structure) and temporal parameters (duration of hydroperiod and date) on species abundances were analysed.

In summary, niche overlap was identified for larvae of *Ae.vexans* and Cylcopoida, which indeed occurred during the same time window (i.e. first 2 weeks of hydroperiod) at the same locations (i.e. mainly grassland biotopes). Both taxa were significantly and negatively correlated. Niche overlap was also identified for larvae of *Cx.pipiens* and Cladocera, which occurred during the later stage of hydroperiod (i.e. > 2 weeks) at various biotopes. Both taxa were significantly and negatively correlated. However, a closer look on temporal occurrence of both taxa showed, that Cladocera abundances increased slower with time compared with larval abundances of *Cx.pipiens*, hence their impact on *Cx.pipiens* populations is relevant only during an advanced stage of hydroperiod (≥ 3 weeks).

There was no spatial niche overlap identified for Ostracoda and any mosquito species. Ostracoda occurred preferably at one location (i.e. Leipzig) only at biotopes of reed-covered wetlands, which were not colonized by any mosquito species. High abundances of Ostracoda from the beginning of hydroperiod onwards prevented mosquito larval colonization. In general we found that biotopes of reed-covered wetland provided better conditions of initial development of crustaceans, and hence mosquito larval abundances were generally low. The opposite scenario was found for grassland biotopes, which favoured early development of mosquito larvae while crustacean communities established later.

5.1.2 Summary of study 2

In the second study (chapter 3) we investigated the effect of Bti-treatment in combination with altering crustacean community structures on mosquito oviposition, larval abundances and larval development. Therefore a multi-species outdoor microcosm experiment was designed. Populations of the mosquito *Cx. pipiens* colonised artificial ponds that contained crustacean communities at different time points of colonization by crustaceans: 1) “no colonization” (no crustaceans), 2) “simultaneous colonization” by crustaceans and mosquitoes, and 3) “head-start colonization” by crustaceans (preceding colonisation by mosquitoes). All types of pond were treated with three concentrations of Bti (10, 100, or 1,000 $\mu g\ L^{-1}$).

Results showed, that both oviposition, larval abundances and larval development was most efficiently reduced by crustacean communities of high taxa diversity. High crustacean abundances caused by population overshoot of one crustacean species (which was characteristic for early crustacean development at simultaneous colonization set-ups) had a lower impact on mosquito larval populations. After 2 weeks of propagation, crustacean taxa composition became more diverse at simultaneous colonization set-ups. Consequently the negative impact on mosquito larval populations increased and became similar to those of head-start colonization set-ups. A combination of different crustacean taxa characterised by different feeding behaviour (i.e. filter feeders at the surface and within the water column, as well as bottom feeders and predators) reduced food resources more efficiently compared with crustacean communities dominated by a single taxa.

After treatment with Bti we observed increased sensitivity of mosquito larvae towards Bti as well as delayed recolonization when communities of crustaceans were present. Crustacean communities of both simultaneous and head-start colonization set-ups were characterised by elevated taxa diversity at the time point of Bti-treatement, hence both conditions had a strong impact on mosquito larval populations. Indeed, presence of crustaceans caused a strong decline of both oviposition and mosquito larval abundances even in non-Bti treated setups. Hence an additional effect of Bti-treatment was hardly visible on the long-term.

5.1.3 Summary of study 3

Previous field and mesocosm studies showed, that development of mosquito larval populations was supressed at presence of crustacean communities. However, the adverse effect of crustacean communities is limited during the early stage of establishment, but increases with time. In the third step (chapter 4), we combined the short-term effect of Bti with the long-term effect of crustaceans. Therefore the effect of joint treatment, applying Bti and crustaceans at the same time, was compared with the effect of single treatment under field conditions. Temporary ponds at a forested area at Leipzig, which were already colonized by mosquito larvae of the species *Cx.pipiens*, were treated with either Bti, crustaceans or a combination of both.

We found that mosquito larval populations were nearly extinguished in joint treatment over the whole observation period (i.e., 5 weeks). Single contamination with crustaceans caused no significant effect, while single treatment with Bti reduced mosquito larval abundances only within the first 2 weeks after treatment. Crustacean abundances reached a much higher level in joint treatment compared to single crustacean treatment.

5.2 Discussion of results

The studies presented in chapter 2-4 investigated the impact of crustaceans for biological control of mosquito larval populations with special emphasises on a combined approach including the insecticide Bti. We found, that crustaceans are a powerful and sustainable mosquito control agent, increasing the sensitivity of mosquito larvae to Bti and delaying recolonization after treatment with Bti – but only if populations of crustacean can establish fast enough.

5.2.1 A matter of time

The most important factor to consider, when using crustaceans for mosquito control, is the time gap between mosquito larval colonization and crustacean establishment. Both field and outdoor microcosm studies presented in this thesis showed, that larval populations of the most abundant mosquito species (i.e. *Aedes vexans* and *Culex pipiens*) are able to colonize newly established ponds rapidly within the first 2 weeks of hydroperiod. On the contrary, a high temporal variability in establishment of crustacean communities was observed, including rapid establishment during the first week of hydroperiod, delayed establishment or even no establishment. There are three factors influencing time of colonization by mosquito larvae and crustaceans: 1) reproduction traits of species, 2) environmental conditions and 3) previous colonization by antagonistic species

Reproduction traits of species

Female imagines of *Cx.pipiens* oviposit directly on the water surface, where larvae hatch within a few days. Female imagines of *Aedes vexans* lay their eggs in the soil, so that larvae are able to hatch immediately after ponds are refilled (Becker et al., 2010a). Both levels of dissolved oxygen and water temperature (Becker et al., 2010a) were major factors inducing hatch (Becker *et al.* 2010a) and larvae of *Ae.vexans* are reported to occur within 24h after conditions became suitable (Horsfall, 1956; Williams, 1998; Becker et al., 2010b). The ability of adult mosquitoes to leave their aquatic biotopes and search actively for new breeding sites give them a temporal advantage compared with crustaceans, which are restricted to their original biotopes or depend on passive dispersal. Vectors for such passive dispersal are flying insects like notonecta as well as vertebrates like ducks or wind (Meutter et al., 2008; Lindholm et al., 2009). However crustaceans dispersed by those pathways will arrive at new biotopes not necessarily at the beginning of hydroperiod. Hence colonization will take a while.

Another method of crustacean colonization is based on seed banks. Most crustacean taxa colonizing temporary ponds (that dry out more or less regluary) produce dormant stages. Resting eggs or encapsulated individuals survive desiccation and form new populations when ponds are refilled (Dahms, 1995; Gleason et al., 2004; Rossi et al., 2012). Previous studies concluded, that seed banks in the soil are the major sources of crustacean occurrence after flooding events (Lindholm et al., 2009; Vanickova et al., 2011). However, dormant stages from different crustacean species do not revivify simultaneously. Some dormant stages of Cyclopoida (i.e. copepodit stage without encystment) revived after 4h (Dahms, 1995), while resting eggs of many Cladoceran species needed to be incubated for about 2 weeks before offspring hatch (Vandekerkhove et al., 2005). Ostracoda are reported to produce either resting eggs or survive desiccation in torpor (Horne, 1993). Torpid individuals are able to revive rapidly within few days (Delorme and Donald, 1969). Variation in crustacean dormant stages were reflected by results of our field monitoring, where Cyclopoida were found to colonize ponds most rapidly, while Cladocera establishment was delayed by two weeks. The variety in crustacean temporal occurrence, however, was not only influenced by life-history traits but also by environmental conditions.

Environmental conditions

Environmental conditions like physiochemical parameters (e.g. water temperature, dissolved oxygen) or seasonal patterns are known to induce crustacean dormancy as well as time point of revival (Williams, 1998). The field monitoring presented within this thesis documented initial higher crustacean abundances during the first week of hydroperiod at ponds located at reed covered wetlands compared with ponds located at grassland biotopes. Ponds of different biotopes differed in terms of physiochemical parameters (e.g. pH) but also provided different conditions regarding water capacity. Layers of detritus at ponds of reed-covered wetlands probably prevented full drying of the ground during desiccation. Crustaceans (mainly Ostracoda) were able to survive at those biotopes, hence recolonizing ponds rapidly after flooding. Ponds at grassland biotopes did not contain detritus and exposed a higher risk of full drying. This was indicated by in general shorter hydroperiod compared with ponds of reed-covered wetlands. Results showed that biotope structure altered time of species occurrence. The earlier colonizer thereby does not only benefit from undisturbed development (due to absence of predators and competitors), but could even prevent colonization of antagonists.

Priority effect

Time of arrival within new habitats plays a major role affecting species interactions. Several studies investigating competition between mosquito larvae and tadpoles demonstrated, that time of entrance alters competition impact, with the species arriving first gaining advantage over the species arriving later (Sredl and Collins, 1991; Lawler and Morin, 1993; Blaustein and Margalit, 1996). Studies on competition between mosquito larvae of Culex sp. and the crustacean species *Daphnia magna* showed that prior establishment of Culex larval populations delayed development of *Daphnia magna* (Foit et al., 2012), while prior establishment of *Daphnia magna* inhibited both Culex oviposition and larval development (Duquesne et al., 2011). All three studies presented within this thesis also showed that mosquito larval colonization occurred only if crustacean populations were not yet established. The third study did additionally demonstrate that high abundances of prior established populations of *Culex pipiens* inhibited colonization by Daphniidae. The mechanisms of antagonistic interactions between crustaceans and mosquito larvae are discussed in the following.

5.2.2 Mechanisms driving the adverse effect of crustaceans on mosquito larvae

Effects on mosquito oviposition

The second study presented within this thesis demonstrated that crustacean communities inhibited oviposition, larval abundances and larval development of *Culex pipiens*. This is in line with other studies investigating the impact of antagonistic species on mosquito oviposition. Adult females of many mosquito species, e.g. *Culiseta longiareolata* or *Anopheles gambiae* are known to select oviposition sites carefully to avoid those ponds already colonized by predators (e.g. Notonecta, Odonata) or competitors (Tadpoles, Cladocera) (Stav et al., 2000; Kiflawi et al., 2003; Munga et al., 2006). Female mosquitoes are able to detect chemical cues from predators (Beketov and Liess, 2007), but they can also assess food availability (Blaustein and Kotler, 1993; Reiskind and Wilson, 2004). Those ponds are preferred for oviposition, where conspecific larvae had already developed successfully and hence contained volatile pheromones (emitted by conspecific larvae) and certain bacteria cultures (Dhileepan, 1997; Sumba et al., 2008). On the other hand, oviposition was inhibited, if conspecific larvae were highly abundant and hence intraspecific competition was high (Sumba et al., 2008).

Results of our second study showed, that crustacean communities consisting of several taxa reduced oviposition of *Culex pipiens* more effectively compared with crustacean communities dominated by a single crustacean taxa (i.e. Scapholeberis). Diverse communities of crustacean consisted both of filtrating and predatory crustaceans. Hence both reduction of nutrients as well as presence of chemical predator cues inhibited oviposition, while such predatory cues were missing in non-diverse communities.

Oviposition was not investigated in the present field studies (i.e. first and third study), because egg clutches were hardly visible at natural ponds partly covered by vegetation. However, reduced abundances of mosquito larvae at ponds containing crustaceans did most likely result both from reduced oviposition as well as from reduced larval development.

Effects on mosquito larval development

Results of our second study showed that larval development of *Cx.pipiens* was inhibited by crustacean communities. This means that early instar larvae (1st and 2nd instar) were not able to develop into late instar larvae (3rd and 4th instar). This is in line with other studies investigating the density-dependant effect of competitors on mosquito larval development. Populations of *Daphnia magna* prolonged time to metamorphosis and decreased size at metamorphosis of *Culex pipiens* (Stav et al., 2005). Cladocerans also reduced larval survival of *Anopheles quadrimaculatus* (Knight et al., 2004), *Culiseta longiareolata* (Stav et al., 2005) and *Culex pipiens* (Duquesne et al., 2011). Cladoceran such as *Daphnia magna* are filter feeders and their feeding behaviour is similar compared with that of *Cx.pipiens* larvae (Stav et al., 2005). The strong impact of Daphnia on *Cx.pipiens* larvae was also demonstrated in our third study, where high abundances of Daphnia sp. were sufficient to fully prevent colonization of *Cx.pipiens*.

Beside the density dependent effect of crustaceans on mosquito larval development, the second study of this thesis additionally demonstrated a diversity dependent effect of crustacean communities. Those communities with high taxa diversity had a stronger effect on *Cx.pipiens* larval development compared with communities of low taxa diversity, although both crustacean communities were of similar quantities. There are two possible reasons for this phenomenon. On the one hand food resources are probably more efficiently reduced when several species of different feeding behaviour are present. Diverse communities present in the second study consisted of surface feeding Scapholeberis, bottom feeding Cyclopoida and Ostracoda as well as of Daphniidae feeding in the water column. Hence food resources were reduced within all sections of the water body, while communities dominated by Scapholeberis reduced food resources mainly at the surface. Larvae of *Cx.pipiens* are able to shift their feeding behaviour in order to avoid spatial competition (Yee et al., 2004). Hence they were able to seek for unoccupied feeding grounds in presence of low diverse crustacean communities and therefore had more access to food resources than in presence of high diverse crustacean communities.

On the other hand, crustacean communities of high taxa diversity contained not only competitors but also predators of mosquito larvae. Both Cyclopoida and Ostracoda are reported to feed on mosquito larvae (Rey et al., 2004; Rossi et al., 2011). Presence of these predators did probably not only reduce mosquito larval abundances by direct predation, but also caused divergence in prey behaviour resulting in lethal and sublethal effects. Indeed, chemical cues of predators were found to reduce filtering and foraging behaviour of larval *Aedes triseriatus* and *Culex pipiens* (Juliano and Reminger, 1992; Juliano and Gravel, 2002; Beketov and Liess, 2007). This is especially affecting those mosquito larvae living under low food conditions, which requires in fact increased foraging behaviour (Beketov and Liess, 2007). Hence a combination of competitors (limiting food resources) and predators (limiting foraging behaviour) are most efficient for mosquito larval control.

In conclusion we found that presence of established crustacean communities have a strong negative impact on mosquito oviposition and larval development (see 5.2.2). However, according to environmental factors and colonization traits of crustacean taxa, there meight be a gap in time between mosquito larval occurrence and establishment of crustacean populations (see 5.2.1). However, early establishment of mosquito larvae could even inhibit crustacean propagation (see 5.2.1). Hence an additional component is needed, which reduce mosquito larval abundances during the early stage of pond colonization without negatively affecting crustacean propagation. Therefore we tested the use of the insecticide *Bacillus thuringiensis israelensis* (Bti) within a combined approach.

5.2.3 Combined effect of Bti and crustacean communities

The insecticide Bti is reported to act specifically on larvae of Culicidae, without negatively affecting non-target species. Results of our studies showed that crustacean populations were not negatively affected by Bti neither under artificial conditions (study 2) or field conditions (study 3). In addition we found, that Bti did not influence *Cx.pipiens* oviposition. This means, that female imagines of *Cx.pipiens* were either not able to detect the presence of Bti in the water, or they did not classify the presence of Bti as harmful component.

The adverse effect of Bti on investigated mosquitoes was therefore restricted on larval populations. Results of our second and third study showed that single application with Bti caused rapid reduction of *Cx.pipiens* larval abundances within 24h at concentrations of 1000µg/L, which is equivalent to concentration commonly used for field application in Germany by the KABS, (verbal communication). However, larvae of *Cx.pipiens* recolonized ponds treated with Bti immediately, reaching similar abundances compared with non-treated set-ups within 2 weeks after treatment. This is in line with previous results, demonstrating rapid effect of Bti (Becker, 1997), but short period of activity due to sedimentation and natural degradation of toxic components (Sheeran and Fisher, 1992).

The combination of Bti and crustacean communities increased the effect of Bti in two ways, 1) increasing the sensitivity of Cx.pipiens larvae towards Bti and 2) delaying larval recolonization after treatment.

Effects on mosquito larval sensitivity towards Bti

Results of the second study showed that the sensitivity of *Cx.pipiens* larvae increased in the presence of crustacean communities by a factor of 10. Increased effects of toxicants in the presence of competitive pressure have been demonstrated frequently. Examples include the combined effect of competition and a toxicant in a simple two-species system (Foit et al., 2012), as well as in multi-species outdoor systems (Liess, 2002; Knillmann et al., 2012). The presence of interspecific competitors limits the amount of food resources, resulting in delayed development and decreased survival of mosquito larvae (Duquesne et al., 2011).

However, in our study crustacean communities included not only competitors but also predators. Schulz and Dabrowski, 2001, demonstrated synergistically increased adverse effects of a sublethal concentration of a pesticide on mayfly nymphs when combined with predatory stress. The authors have argued that mayflies were weakened by sublethal pesticide stress and therefore were not able to perform usual predator-avoidance behaviour. Similar effects may be responsible for the increased sensitivity of mosquito larvae in our study. Another explanation could be that presence of predators reduced foraging behaviour of mosquito larvae, while competitors reduced total amount of food resources (see 5.2.2.). Hence mosquito larvae are weakened by food scarcity and hence are therefore more vulnerable to sublethal insecticide treatment (Beketov and Liess, 2007).

Effects on mosquito larval recolonization after Bti-treatment

In addition to increased sensitivity of *Cx.pipiens* larvae towards Bti also inhibition of recolonization by *Cx. pipiens* larvae after Bti treatment in the presence of crustaceans was observed both in the second and third study. This result is consistent with other studies that demonstrated that competition delays the recovery of population structure after exposure to toxicants (Liess and Foit, 2010; Foit et al., 2012). Combined application of Bti and crustaceans resulted in the reduction of mosquito larval populations, while crustacean communities were able to proliferate. When activity of Bti declined, crustacean communities had reached sufficient abundances to prevent *Cx.pipiens* oviposition as well as larval development (see 5.2.2).

Our results showed that Bti and crustacean communities did not only complement each other in terms of the effective time window, but also reciprocally increased the impact of their mode of action.

5.3 Relevance of the findings in terms of mosquito control management

5.3.1 The relevance of species identity

All investigations presented above showed the strong effect of food competitors on mosquito larval populations. However, this effect was mainly detected for the mosquito species *Cx.pipiens*, which was by far the most abundant species at all investigated sites. *Cx.pipiens* is known to be a weak competitor in comparison to other mosquito species, e.g. *Aedes albopictus* (Costanzo et al. 2005, Carrieri et al. 2003). Therefore it is not surprising that *Cx.pipiens* is also sensitive towards competition with crustaceans. This means in terms of mosquito control that crustaceans will be most efficient against mosquito species with weak competition abilities. Further investigations are necessary to clarify, if crustacean can also affect other mosquito species, which have stronger competition abilities.

In terms of crustacean taxa we found that all antagonistic crustacean classes (i.e. competitors or predators) were able to affect mosquito larval populations. Crustacean composition varied between the biotopes and locations; however all kinds of crustacean communities caused strong adverse effects on mosquito larval populations. For mosquito management those crustacean species should be applied, which are best adapted to local environmental conditions. Consequently different areas might need different crustacean communities for an optimised mosquito control.

5.3.2 Advantages of a combined approach for mosquito control

Our results showed that the combination of Bti and crustaceans was most efficient against mosquito larval populations. The combined application could improve mosquito control management in several ways. Because of the prolonged effect of the combined approach, single application of the control agents during one flooding period would be sufficient. In consequence less disturbance of the environment due to application methods (spraying by men, application via helicopter etc) would occur, which is particularly important in nature protection areas with sensitive flora and fauna (e.g. nesting birds). Secondly, the reduction of application times would also reduce the costs of mosquito control, because the amount of expensive Bti- formulations can be reduced and the application costs would be lower. This is particularly interesting for poor countries, where mosquitoes play the most important role as vectors for diseases, but budgets for mosquito management are limited. Thirdly, reduced application of Bti would also reduce the risk of resistance development. The presence of crustaceans could even strengthen this argument. Resistance is always costly for an organism, often resulting in lower fitness indicated by lower fecundity or longer development time (Baker et al. 2007). However, mosquito larvae with reduced fitness would have even less chances to compete against crustaceans and would therefore most likely not survive. However, this hyothesis needs examination.

5.3.3 Outlook

The mechanism of competition is highly relevant in terms of mosquito control and can be used in integrated management strategies. However, some work still needs to be done, to make this mechanism useful for commercial practice. The application of living crustaceans, as it was done within the present studies, is most likely not feasible for large-scale applications. Transport of the organisms would be too complex and too stressful for the crustaceans; application via spraying or helicopter is impossible. However, there meight be other ways to use that strategy. One possibility could be the use of permanent eggs. Many crustaceans produce such eggs, which can survive long periods of droughts (Williams 2006). Permanent eggs could be transported easily and they are suitable for several application methods. However, more investigations are needed to find out wether populations of crustaceans are able to develop fast enough from permanent eggs. Another way of using crustaceans for mosquito control could be to establish permanent ponds containing crustacean communities within target areas. If those areas are flooded, crustaceans could be washed out from those ponds, immediately propagating within the new created biotopes. Again, this method has not been investigated yet.

Mosquito management is a complex issue, which needs to consider the effectiveness, practicability and costs as well as potential side effects on the environment of different control agents. In addition, mosquito control strategies should be carefully selected according to the needs of the involved human populations. In case of disease outbreaks it is necessary to decrease vector mosquito species as much as possible. However, if mosquitoes are “only” nuisance, it could be sufficient to limit mosquito populations to a certain extent and within certain areas. In this case communication with the local population could be as important as mosquito control. Information about mosquito life cycles and their importance within the food chain could increase acceptance of mosquitoes within human populations and hence lower the needs for mosquito management.

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Curriculum vitae

Personal Information

Birth 31.03.1979 in Munich
Nationality: German
Personal status: married
Children: Timo (28.03.09), Ronja (11.10.10)

Professional career

08/2013 – 12/2013	Leibniz Centre for Agricultural Landscape Research (ZALF), Scientific employee
03/2013 – 07/2013	Leibniz Centre for Agricultural Landscape Research (ZALF), Trainee
05/2012 – 12/2012	Helmholtz Centre for Environmental Research (UFZ), Leipzig Scientific employee and project leader
06/2006 – 04/2012	Helmholtz Centre for Environmental Research (UFZ), Leipzig PhD student
11/2005 – 06/2006	Max-Planck-Institute for Evolutionary Anthropology, Leipzig & German Primate Centre, Göttingen (DPZ) Freelancer

Education

10/1999 – 10/2005	Technical University of Munich
09/1989 – 09/1999	Bertolt- Brecht- Secondary School, Munich
09/1985 – 09/1989	Primary school, Munich

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Schreiben des Manuskripts	KI 100%
Korrektur und Kommentierung	DS 100%

Kroeger, I. (KI), Duquesne, S. (DS), Liess, M. (LM). 2013. Crustacena biodiversity as an important factor for mosquito larval control. Journal of Vector Ecology, 38: 390-400

Konzept	LM 80%, DS 20%
Feldarbeit	KI 100%
Probenaufbereitung (v.a. taxonomische Bestimmung)	KI 100%
Datenaufbereitung und statistische Analyse	KI 80%, LM 20%
Interpretation der Daten	KI 50%, LM 40%, DS 10%
Schreiben des Manuskripts	KI 100%
Korrektur und Kommentierung	LM 70%, DS 30%

Kroeger, I. (KI), Liess, M. (LM), Dziock, F. (DF), Duquesne, S. (DS). 2013. Sustainable control of mosquito larvae by the combined actions of the biological insecticide Bti and natural competitors. Journal of Vector Ecology 38: 82-89.

Konzept	LM 35%, DS 35%, KI 30%
Feldarbeit	KI 100%
Probenaufbereitung (v.a. taxonomische Bestimmung)	KI 100%
Datenaufbereitung und statistische Analyse	KI 70%, DF 30%
Interpretation der Daten	KI 70%, LM 30%,
Schreiben des Manuskripts	KI 100%
Korrektur und Kommentierung	DS 90%, LM 10%

Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende
Arbeit selbstständig und ohne unerlaubte Hilfsmittel verfasst
Und keine anderen als die angegebenen Quellen verwendet habe.

Iris Angela Kröger
Leipzig, den 21.10.2013