The effect of pond dyes on mosquitoes and other freshwater invertebrates

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Freshwater habitats are important because they represent two percent of Earth's water resources, are highly diverse in aquatic organisms and are the most productive and threatened ecosystem worldwide. Pollution, urbanization and climatic changes are responsible for drastic changes in these ecosystems. The creation of new ponds offers an opportunity to increase biodiversity, landscape connectivity and provide new habitat for organisms. However, new ponds might be a good habitat for mosquitoes to lay eggs.

Mosquitoes have worldwide distribution and are responsible for most of the vector-borne diseases, affecting thousands of people and causing millions of deaths. British mosquitoes currently do not carry human diseases, but they are a biting nuisance. Their distribution, abundance, species composition and potential for mosquito disease transmission are intimately linked to the physical environment. *Culex pipiens* is commonly found in UK gardens and is a potential vector of viruses including the West Nile Virus. However, any environmental factors that significantly change the distribution and population of *Cx. pipiens* could impact future risks of disease transmission.

Pond dyes are a cosmetic product for garden ponds and lakes; they inhibit algal growth and improve the overall appearance of the water body reflecting surrounding planting. The dyes block red light from entering the water, interrupting the process of photosynthesis and therefore inhibiting the growth of certain aquatic plants such as algae. Although these dyes are non-toxic to fish and invertebrates, their use in urban gardens raises questions linked to mosquito oviposition, since coloured water can be an attractant.

This research focussed on the impact of pond dyes on mosquitoes and freshwater macroinvertebrates. Gravid female *Culex* mosquitoes preferred to lay eggs in black dyed water under laboratory conditions and in a semi-field choice test but there was no evidence that these results translated into a difference in a fully natural ecosystem. Mosquito survivorship in black dyed water was significantly reduced; Dyes of other colours had no impact on oviposition but did impact survival in both field and laboratory conditions.

The impact of black pond dye on small artificial ponds found effects depending on the experimental method. In ponds that were cleared of all animals but they reseeded with known species of macroinvertebrates dyes impacted negatively on the biodiversity, evenness and abundance of species. By contrast, in the second pond study, where dye was applied to the existing macroinvertebrate communities, no differences were detected between treated and untreated ponds in respect to biodiversity or abundance.

A final study on the impact of black pond dye on mosquito larval predation by *Chaoborus flavicans* and *Gammarus pulex* found no difference in predation.

Considering the concerns over potential future spread of disease in urban environments and the properties of these dyes, more studies are needed to understand how aquatic animals and interactions between species respond in presence of colour dyes.

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LIST OF ABREVIATIONS

µl : Microlitre
ml : Millilitre
L : Litre
cm : Centimetres
m : Metres
mm : Millimetre
µm : Micrometre
lbs : Pounds
Kg : Kilogram
g : Grams
mg : Milligram
WNV : West Nile Virus
N : North
W : West
L : D : Light/Dark
Log : Logarithm
df : Degrees of freedom
SEM : Standard Error of the mean
nm : Nanometres
ppm : Parts per million
GLM : Generalized linear model
h : Hours

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INTRODUCTION

Freshwater habitats are important to support a diverse range of aquatic organisms. In the UK, rivers and streams (running waters) and ponds and lakes (standing waters) form a landscape of freshwater habitats. Many suffer from anthropogenic interference including pollution, urbanization and climatic changes. Ponds are important habitats that support a high diversity of plants and animals, including amphibians, reptiles and insects. In Great Britain, pond creation has been on the increase, with around 2, 000 excavated in the lowlands (Williams *et al*., 2008). Pond creation promotes biodiversity, increases landscape connectivity and provides rare animals such as great crested newt with new habitat in which to thrive (Williams et al., 2008; Pond-Conservation, 2017). Whilst pond creation is good news for many animals that rely on this habitat, ponds and other freshwater (such as water butts) offer mosquitoes an excellent habitat in which to lay eggs. In urban areas, this may present a potential mosquito biting nuisance (Townroe and Callaghan, 2014).

Since the 1990s, DyoFix products have been used to improve the appearance of ponds and lakes through light reflection. Pond dyes limit algal growth by restricting light absorption thereby interfering with photosynthesis (DyoFix, 2015b). The manufacturers claim that there is no evidence of toxicity or negative effects on fishes, wildlife or plants (DyoFix, 2015b). There is no published work to provide any evidence for this claim but also the impact of the dye may not be restricted to toxic effects. A reduction in algae would logically be linked to a reduction in the populations of macroinvertebrates that rely on this food. Therefore, one possible impact of pond dyes on wildlife would be either a reduction in the abundance of macroinvertebrates and/or a change in the community structure.

One of the Dyofix dyes is black, a popular choice among garden designers. However, it is well known that dark or coloured waters are attractive to female mosquitoes seeking sites for oviposition (Beckel, 1955; Harrington *et al*., 2008; Derraik, 2005; Torrisi and Hoback, 2013; Hilburn *et al*., 1983; Panigrahi *et al*., 2014). A possible consequence of pond dye use might be an increase in mosquito use of garden ponds for breeding, bringing mosquitoes close to humans.

Global climate change and shifts in arthropod-vector diseases are a concern for the future. A recent study on urban mosquitoes showed that both *Culex pipiens, Anopheles plumbeus* and *Anopheles claviger* successfully breed in the urban landscape (Townroe and Callaghan, 2014; Townroe and Callaghan, 2015). Alteration to the distribution, density and phenology of *An. plumbeus* is of human health importance and should be monitored since this species is a day active human biter, a candidate bridge vector of WNV and has confirmed competence for malaria, *P. vivax* and *P. falciparum* (Schaffner et al., 2012b).

Cx pipiens is the most common British mosquito and a possible future UK vector of West Nile Virus (WNV). To date, there is no evidence for the presence of WNV in the UK, but studies have shown that native birds can contain antibodies for WNV which is indicative of exposure (Buckley *et al*., 2003). British mosquitoes are currently controlled in coastal areas such as Hayling Island and Epping Forest where they are a biting nuisance (Lockwood, 1986; Marshall, 1938) but they are not seen as a pest elsewhere. The use of black dyes in garden ponds might raise the problem of an increase in nuisance mosquito in urban areas and a future linked to vector-borne diseases like WNV.

CHAPTER 1. LITERATURE REVIEW

1.1. Freshwater environments

Freshwater can be broadly divided into wetlands, open waters and floodplains (Rees, 2008). Such water bodies cover 0.8% of the Earth's surface and are considered to be the most diverse in habitats and species (Collen *et al*., 2014). Almost 35% of all vertebrates and 10% of animal species are present in freshwater ecosystems (Stendera *et al*., 2012; Dudgeon, 2014) and approximately 126,000 species of plant and animals have been described in freshwater habitats (Balian *et al*., 2008). In Great Britain, it has been reported that at least two-thirds of the British freshwater plant and animal species are present in pond habitats (Williams *et al*., 2010; Williams *et al*., 1997).

Many freshwater ecosystems are vulnerable to degradation and ecological change, putting their biodiversity at serious risk of extinction (Thornhill *et al*., 2017; Paul and Meyer, 2001; Walsh *et al*., 2005; Hill *et al*., 2015; Revenga *et al*., 2005). Physical and chemical treatments and stressors have impacted and degraded the quality of inland water bodies worldwide (Revenga *et al*., 2005). Land use changes and deforestation, chemical and sewage pollution, flow alteration, invasive species and climate change are all anthropogenic in origin leading to alteration in freshwater ecosystems (Stendera *et al*., 2012; Vörösmarty *et al*., 2010; Staley *et al*., 2015; Dudgeon, 2014; Søndergaard and Jeppesen, 2007; Ebenman and Jonsson, 2005). Agricultural activity, for example, has led to extensive pond drainage (Heath and Whitehead, 1992) and the eutrophication of ponds, lakes and streams have been a direct consequence of run-off from the large-scale application of organic fertilizers containing nitrates and phosphates (FAO, 1996; Khan *et al.*, 2014). Intensive farming in the 20th century has seen the development and application of hundreds of pesticides such as insecticides, fungicides and herbicides, for the control of insect pests, plant diseases and water weeds (Helfrich *et al.*, 2009; Staley *et al.*, 2015). Generally non-specific in their target, these pesticides have entered water bodies, either intentionally or through runoff from land, where they have had long-term effects on the flora and fauna of freshwater ecosystems (Hurlbert *et al.*, 1972; Cope, 1966; Schäfer *et al.*, 2011).

It has been suggested that waterbodies and wetlands are more likely to be degraded if they are close to urban areas. In the early 20th century, only about 10 % of the world's population was living in urban areas but by 2070 this will have risen to about 70% (Grimm *et al.*, 2008). In the United Kingdom, the urban landscape has increased by 141,000 hectares between 2000 and 2010 (Khan *et al.*, 2013) and approximately 60% of the population lives in urban areas (Pateman, 2011). Hydrosystems including rivers, streams, canals and ponds have been modified in urban areas due to changes such as new buildings and roads or other changes in land use (Grimm *et al.*, 2008). Such changes can alter the topographic alignment, timing, quantity and composition of runoff of freshwater ecosystems, and consequently can impact biodiversity, in respect of both richness and abundance of species, as well as species composition and the interaction between communities and individual species abundance (Smith *et al.*, 2006a; Dudgeon, 2014; Smith *et al.*, 2006b). As a consequence of increased urbanisation, many freshwater habitats have been reduced and others have been lost entirely (McKinney, 2002).

1.1.1. Ponds

Ponds are a type of standing (i.e. not flowing) open water which includes reservoirs, lakes and gravel pits (Khan and Din, 2015) (Figure 1.1).

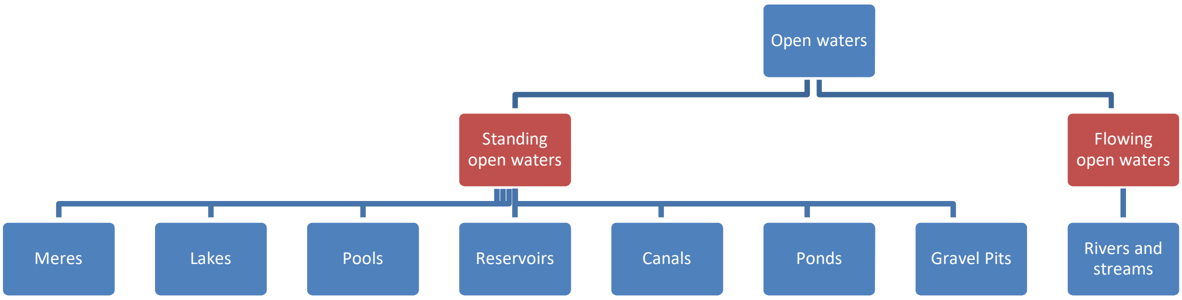

Figure 1.1 Types of open water (from Khan and Din, 2015).

Ponds can be defined as small bodies of water between approximately 1 m^2 and 2 ha in area (Biggs et al., 2005). They are found naturally in almost all types of landscape, including moorland, grassland, woodland, mountainous and coasts (Wood *et al*., 2003; Céréghino *et al*., 2014). They are present in both rural and urban anthropogenic habitats (Hill *et al*., 2017), where they have been created for a variety of uses, including commerce, recreation, and conservation (Figure 1.2) (Grimm *et al*., 2008; Moggridge *et al*., 2014; Hassall, 2014; Williams *et al*., 2003; Wood *et al*., 2001; Wood *et al*., 2003; Hill *et al*., 2015; Hill and Wood, 2014; Gledhill *et al*., 2008; Hassall and Anderson, 2015).

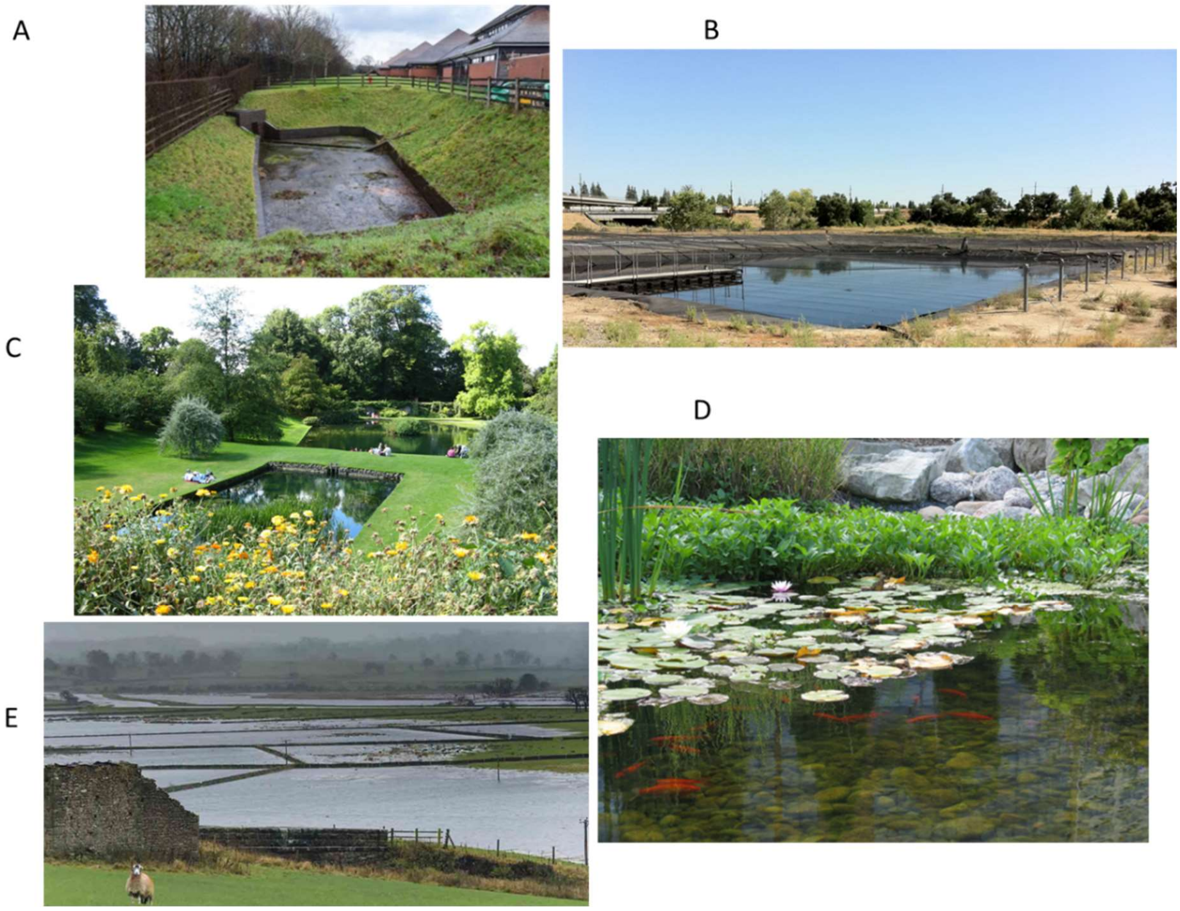

Figure 1. 2. Different pond uses. **A** - Sustainable urban drainage system. **B** - Industrial ponds. **C** - Garden ponds. **D** - Ornamental ponds. **E** - Flood water storage.

Although garden ponds can provide a variety of abiotic and biotic conditions that are beneficial to freshwater organisms, in the last century, many ponds have been lost and those that remain are often in poor condition. In Great Britain, between 1998 and 2007, approximately 80% of the ponds were reported to be in a “poor” or “very poor” condition (Wildlife-Trusts, 2017) and nearly 18,000, representing 4% of the total, were lost (Williams *et al*., 2010). For example, Boothby (1997) reported that 210 ha of agricultural land will be used in a project at Manchester International airport, destroying 43 small water bodies and significantly affecting the natural habitat of the internationally protected Great Crested Newt (*Triturus cristatus).*

In the UK, a number of organizations have implemented projects to create and restore ponds in urban and rural areas (Pond-Conservation, 2011; Wildlife-Trusts, 2017). For example, the Wildlife Trusts has projects that help to restore natural water levels, improving bankside vegetation, re-wetting floodplains and provide habitats for rare species (Wildlife-Trusts, 2017). The Pond Conservation has estimated that nation's garden have been created between 2-3 million garden ponds (Pond-Conservation, 2011). New ponds and constructed wetlands are important to increase habitat for freshwater wildlife, link fragmented wildlife and communities, restore habitat lost and preservation of biodiversity; however, at the same time, they can increase water supply, floodwater retention, nutrient removal (Pond-Conservation, 2011; Sartori *et al*., 2014).

In the UK, it has been estimated that at least 22.7 million homes (87% of households) have contact with a domestic garden, and there are 2.5 to 3.5 million ponds in domestic gardens, representing about 349 ha of standing water (Davies *et al*., 2009). However, few studies have described and quantified the biodiversity in urban ponds (Hassall, 2014). A recent UK wide study found that urban versus rural ponds have similar alpha diversities (i.e. the diversity of each pond) with respect to aquatic macro-invertebrates at a family and species level, and also similar gamma diversities (the diversity of the landscape) at the family level. However, higher estimated gamma diversity on the species scale were found in rural ponds (Hill *et al*., 2017).

Urban ponds are often man-made or man-managed, but are important for several reasons. They are essential habitats for amphibians, fishes, plants and invertebrate biodiversity (Rees, 2008; Wood *et al*., 2001; Hill *et al*., 2017; Biggs *et al*., 2005; Hill and Wood, 2014; Smith *et al*., 2005) and they provide a source of nutrients for aquatic and non-aquatic mammals and birds (widely reported). Hence they are one of the biggest contributors to regional biodiversity generally, and in comparison to larger freshwater habitats such as lakes and rivers (Céréghino *et al*., 2008; Gledhill *et al*., 2008; Williams *et al*., 2003). Increasingly, therefore, they play an important role in the conservation of biodiversity (widely reported). (Dudgeon *et al*., 2006).

Management of urban and rural ponds is therefore vital if urban ponds are to play an increased role in conserving macroinvertebrate diversity. Unfortunately, from this perspective, domestic gardens are often managed for aesthetic purposes and difficulties can, therefore, arise in maintaining normal ecosystem function (Hunter and Hunter, 2008).

1.1.1.1. ***Algae in ponds***

Algae generate oxygen through photosynthesis which is vital for aquatic organisms to survive (Addy and Green, 1996; McCormick and Cairns, 1994). They play an important role in ponds, lakes and streams because they are the most important primary producers in both freshwater and marine habitats and are crucial to the reproduction of some aquatic invertebrates (Addy and Green, 1996; McCormick and Cairns, 1994; Lukešová and Frouz, 2007; Stevenson, 1996). Algae have been used as bioindicators, to evaluate the quality and status of aquatic environments (McCormick and Cairns, 1994).

Whilst algae are important in food webs and aquatic ecosystems, if growth is not maintained in balance by predatory grazing, and a balanced nutrient content, they can grow out of control forming “algal blooms” (Mitra and Flynn, 2006; Chislock *et al*., 2013). Algal blooms form in response to variation in the environment, such as an influx of agricultural fertiliser, and a few species can dominate and quickly develop to form large assemblages (Egerton *et al*., 2014). Severe algal blooms can cause economic damage to fisheries, and can seriously affect human health from the toxic compounds they produce (Jonsson et al., 2009). They also affect aquatic ecosystems directly through toxin production and indirectly by limiting light penetrating to the water column below. Dissolved oxygen can also become depleted from the amount of decomposition taking place, killing fish and other organisms (Chislock *et al*., 2013).

Many methods to control algae have been employed, from the mechanical removal of algae by dredging, cellular disruption by ultrasound, application of chemical agents such as clay flocculants and copper-based algaecides (Wang et al., 2012). These methods can be expensive, often ineffective in the case of ultrasound and can cause unwanted environmental damage. They are also unsuitable for use in domestic ponds, therefore alternative methods for algal prevention and removal are needed.

1.1.1.2. *Freshwater Colour Dyes*

In the last few decades, alternative products to control algae growth have been developed. Lake and pond dyes function by reducing light penetration to the freshwater habitats, thereby controlling phytoplankton snow and aquatic plants (Tew, 2003). Daylight (white light) is composed of a series of colours (e.g. red, orange, yellow, green, blue, indigo and violet) named as a spectrum (DyoFix, 2015b). These dyes act as red light filters to restrict photosynthesis, blocking peak absorption by chlorophyll a at 650nm (Simis *et al*., 2012).

A number of manufacturers of these dyes exist. For example, Sanco Industries (Fort Wayne, IN, USA) sells a light-attenuating dye called Crystal Blue-Ocean to control benthic algae (Tew, 2003). The dye contains a blend of blue and yellow dyes, and they are designed to control plant photosynthesis by absorbing specific wavelengths of the light (Aquashade, 2017). Rose bengal, an analogue of fluorescein and methylene blue (thiazine dye) are chemicals used in medicine and biochemical research; however, they have also been tested to see if they control algal growth (Martin *et al*., 1987). Studies have shown that photosynthesis rates were reduced, decreasing primary productivity and controlling plant growth (Buglewicz and Hergenrader, 1977; Spencer, 1984), but also it was observed that dissolved oxygen concentration (DO) decreased (Martin *et al*., 1987; Tew, 2003).

In the UK pond dyes can be purchased from DyoFix as a commercial product for garden ponds and lakes to improve their appearance (Figure 1.4). According to their manufacturer, Dyofix products are not dangerous to wildlife, insects, pets and aquatic plants and are basically food dyes (DyoFix, 2015b). Dyofix claims “the extremely dense growths of filamentous algae and submerged weeds that also cause serious problems to fish, as a result of night time oxygen depletion, will also be controlled” and “Pond Blue dye also negates the need for chemicals, algaecides, herbicides which, if not used in precise doses or under strict supervision by experts, can cause an imbalance of the natural nutrients in the pond that can have a negative effect on fish, plants and other species”. Pond and lake dyes are available in a variety of colours, including Blue, Lake Shadow (red), and black (DyoFix, 2015b). Black dyes are marketed specifically to garden designers to “create an obsidian black mirror reflection for a stunning garden pond design. Sunlight, moonlight or candlelight, the black mirror finish of the black pond dye looks amazing and puts the finishing touch to your water feature” (DyoFix, 2018).

Figure 1.3. DyoFix products for lake and ponds.

No research has been conducted on these dyes. The manufacturers have stated that there have been concerns about the environmental impact of their chemicals, but that the Environment

Agency has not collected any data on this. They have posted a letter from Dr J. Newman, a former employee of the Centre for Ecology and Hydrology commenting on the use of dyes. In this letter Dr Newman states that no adverse effects on invertebrates have been found, although there is no evidence that he actually conducted any sampling. He goes on to suggest that most invertebrates are found in the shallow margins of the lake and that here the dye had little effect on vegetation if the depth is less than 30cm. He states an impact on algae "altering behaviour to compensate for lower light levels by staying closer to the surface of the water" and suggests that this encourages filter feeding *Daphnia* etc to migrate to the surface where the algae are concentrated (DioFix, 2017).

1.1.2. Other standing water in urban habitats

Within the UK, many homeowners collect and store rainwater for domestic use in residential gardens; driven in part by weather patterns that are increasingly putting pressure on water resources. Water butts collect rain from roof or greenhouse guttering and often accumulate moss, leaves, animal detritus and heterotrophic microorganisms (including bacteria, algae, fungi and protozoans), and therefore provide both a habitat and food resource for a broad range of organisms. Artificial habitats such as these can support diverse species of macroinvertebrates including *Daphnia* spp., copepods, *Cyclops* spp., mosquitoes and *Chaoborus* spp. can all colonise these habitats (personal observation). Within the urban landscape, these artificial containers, combined with garden ponds, can form a network of freshwater habitats also known as a pondscape (Boothby, 1997). However, they are also easily accessible prime mosquito larval habitat (Vezzani, 2007a). This, in combination with the Urban Heat Island effect, which can increase the temperature in large UK cities by as much as 8.9 °C compared to surrounding rural areas (Kolokotroni and Giridharan, 2008), may favour increased mosquito larval production in urban habitats.

In the UK, several mosquito species breed in water butt habitats, including *Culex pipiens* L 1758.*, Culex torrentium* Martini 1925*, Culiseta annulata* Schrank 1776 and *Anopheles plumbeus* Stephens 1828 (Snow and Medlock, 2006a; Snow and Medlock, 2006b). Mosquito species which are a human biting nuisance, or which may potentially be vectors of human or wildlife diseases such as West Nile Virus (WNV) or Avian Pox Virus (Poxviridae), given current climate change projections, are potentially going to thrive in urban environments where they will be in close contact with humans and birds. To date, mosquito communities have been impacted by the creation of the new breeding sites in urban areas and mosquito communities have thrived in container habitats and man-made breeding sites in urban areas (Muturi *et al*., 2017), affecting their overall abundance and geographic distribution (Landau and van Leeuwen, 2012; Akram *et al*., 2009). However, Ferraguti (2016) and Townroe and Callaghan (2014) found that mosquito abundance and species richness were lower in urban areas than in rural habitats and decreases in both as human populations rise. Invasive non-native mosquitoes (*Aedes* spp.) have been reported in Europe (Medlock *et al*., 2012b) and in Great Britain (Medlock *et al*., 2017). It is, therefore, increasingly important to identify the ecological factors that influence mosquito abundance, diversity and communities.

1.2. Introduction to Mosquitoes

Worldwide, there are approximately 3500 described species of mosquito, grouped into 41 genera (CDC, 2015; Wallace, 2008; Harbach, 2008). All mosquitoes belong to the order Diptera, suborder Nematocera and family Culicidae (Snow, 1990). Adult mosquitoes can be recognised by their long legs, long proboscis, wing venation and scales on the legs, thorax and wings and for being a slender and small insect (4-10mm in length) (Harbach, 2008; Snow, 1990). There are two important subfamilies, notable morphological and behavioural differences are shown in Table 1.1, Anophelinae (*Anopheles* spp.) and Culicinae (*Aedes* spp., *Culex* spp*., Culiseta* spp.). Species included in this study are all of the Culicinae, the current taxonomic arrangement of the family is shown in Table 1.2.

Mosquitoes are fairly ubiquitous, being found in both temperate and tropical regions and surviving in diverse ecological environments (Service, 1993). Adults have been encountered in semi-arid areas, cultivated lands, urbanised areas, and equatorial rain forest (Service, 1993) and have an extraordinary capacity to adapt to new environments, allowing climate changes to affect their global dispersal (Goddard, 2008).

All Culicidae undergo complete metamorphosis, with four developmental stages spanning both aquatic and terrestrial habitats: egg, larvae and pupae are aquatic, adults are terrestrial flying insects (Figure 1.4) (Becker *et al*., 2010; Wallace, 2008). Eggs are laid singly, as in *Anopheles*, *Ochlerotatus* and *Aedes,* or attached together to form *"*batches*" or "*rafts*"* as in *Culex* and *Culiseta* (Becker *et al*., 2010). Larvae, also known as "wrigglers", go through four instars before they pupate (Goddard, 2008; Harbach, 2008); pupae are active but do not feed (Goddard, 2008). Depending on water temperature, most of the eggs will hatch into larvae within 48 hours, larvae will take about two weeks to develop into pupae and pupae will change into an adult between 1-4 days later (Becker *et al.*, 2010; AMCA, 2017).

Table 1.1 Morphological/physiological and behavioural differences between the Anophelinae and Culicinae families of mosquitoes (Clements, 1992; Clements, 1999; Becker et al., 2010).

Category of Difference	**Anophelinae**	**Culicinae**
Morphological / Physiological		
Eggs may be drought resistant and overwinter for up to several years	NO	YES
Larvae lack a respiratory siphon	YES	NO
Abdominal terga and sterna are densely covered by scales	NO	YES
Adults have longer legs	YES	NO
Palps of both sexes are approximately the same length as the proboscis (adults)	YES	NO
Ejaculatory duct is a short slender tube	NO	YES
Behaviour		
Eggs batches clumped together, "batches" or "rafts"	NO	YES
Eggs laid just above the water line	NO	YES
Larvae resting on the water surface	YES	NO
Larvae inhabit moving water (rivers, streams)	YES	Few species
Larvae exhibit thigmotaxis (body tends to keep contact with solid objects)	YES	NO
Larvae and pupae have an alarm response (escape behaviour)	Few species	YES
Larvae heads rotate 180°	YES	NO
Oviposition in polluted waters	NO	YES

Table 1.2. Native species of mosquito present and possible invaders in Great Britain (Snow, 1998; Vaux and Medlock, 2015; Medlock et al., 2012b; Medlock et al., 2017).

Family	**Subfamily**	**Genus**	**Specie**	
Culicidae	Anophelinae	*Anopheles*	*An. atroparvus*®	*An. maculipennis*
			An. messeae®	
			An. daciae	
			An. plumbeus	
			An. algeriensis	
			An. claviger	
	Culicinae	*Aedes*	*Ae. aegypti**	
			*Ae. albopictus**	
			Ae. cinereus	
			Ae. punctor	
			Ae. vexans	
		Culex	*Cx. pipiens pipiens*®	*Cx. pipiens*
			Cx. molestus®	
			Cx. quinquefasciatus	
			Cx. territans	
			Cx. torrentium	
		Culiseta	*Cs. annulata*	
			Cs. morsitans	
			Cs. fumipennis	
			Cs. litorea	
			Cs. subochrea	

* Species not present and possible invaders in Great Britain. ®Species members of *Anopheles maculipennis* and *Culex pipiens* that are morphologically identical but considered different species.

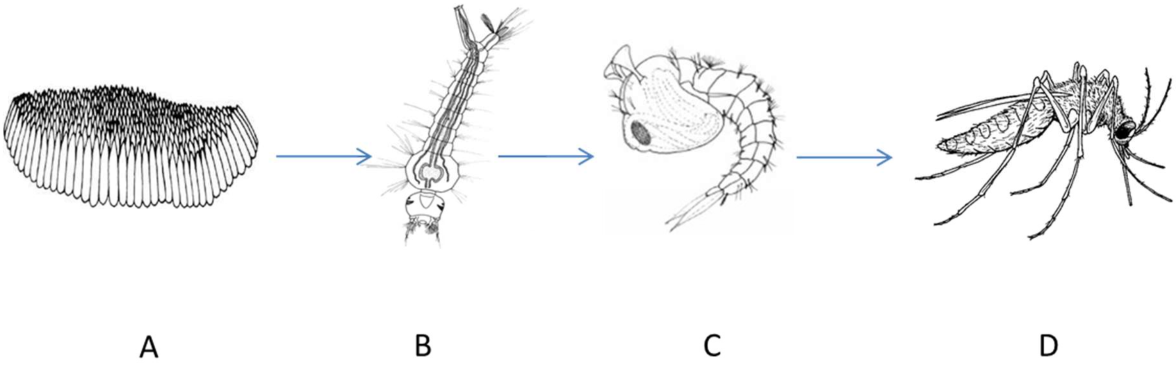

Figure 1.4. The four stages of development in Culicinae Mosquitoes. Mosquito life stages, not to scale. **A** - Egg batches. **B** - Larva. **C** - Pupae. **D** - Adult. Figure after https://extension.umaine.edu/ipm/ipddl/publications/5109e/.

In Culicidae family, adults of both sexes of *Culex pipiens* obtain nutrients from the nectar and juices of flowers and fruits (Snow, 1990; Goddard, 2008). However, most females require blood (anautogeny), which is sucked up with a specialized proboscis, for nutrition to facilitate egg development and maturation (oogenesis) (Goddard, 2008; Snow, 1990; Clements, 1992; Burdick, 1921; Wallace, 2008; Cranston *et al*., 1987; Vezzani, 2007b). In the case of *Culex pipiens* biotype *molestus*, females develop egg batches without a blood meal (autogeny). Egg development and maturation are dependent on food reserves accumulated in larval or adult stages (Snow, 1990).

Mosquitoes use a wide range of breeding sites that may be natural, such as leaf axes, habitats may or may not have vegetation, or artificial, many types of container that can accumulate water, such as buckets. Breeding sites may be temporary or permanent, clean as well as highly polluted and in small or large water bodies (Becker *et al*., 2010). Ponds, swamps, rice fields, car tyres, funerary vases, living structures of plants (such as bromeliad rosettes), holes in plants and trees, bottles, rock pools, cisterns, cans, water buckets, other artificial containers, and rain butts and barrels have all been found to provide a suitable habitat for the development of the immature stages of these insects (Bentley and Day, 1989; Becker *et al*., 2010; Okogun *et al*., 2005; Service, 1993; Burdick, 1921; Wallace, 2008).

However, based on the flight behaviour, visual and chemical clues, mosquito females will select a specific type of oviposition sites (Wallace, 2008; Clements, 1999; Day, 2016). For example, in West Africa, females of *Anopheles melas* deposit their eggs in brackish pools in mangrove swamps, and *Anopheles gambiae sensu stricto* lay eggs in shallow rain-filled pools (Clements, 1999). *An. claviger* and *Coquillettidia richiardii* prefer to lay eggs in clean and cold ponds or lakes (Becker *et al*., 2010; Medlock *et al*., 2012b). This selection of breeding site has caused a nuisance problem in Great Britain, especially from those species that lay eggs in rural, urban and coastal habitats (Medlock *et al*., 2005a; Snow, 1990).

Previous studies have shown that diversity of macroinvertebrate communities and densities of mosquitoes depend on the physical characteristics of the water bodies they inhabit. Low mosquito density and high diversity of macroinvertebrates are positively associated with open water bodies, steeply sloping margins and scarce vegetation (Hassall *et al*., 2011). In contrast, low diversities of macroinvertebrates and high diversities of mosquitoes are linked to wetlands with vegetation, shallow water, poor quality water and low predator presence (Sarneckis, 2002). However, healthy as well as degraded and damaged water bodies have been shown to provide a habitat for mosquitoes. *Cx. pipiens,* a vector of WNV will use all such habitats, but rarely lay eggs in healthy water bodies (USDA, 2008). Predators and competitors have an impact on mosquito populations in healthy wetlands (Medlock and

Vaux, 2011), their role can form part of integrated environmental management plans for controlling mosquito populations.

1.2.1. Mosquitoes as vectors of disease

Vector-borne diseases are those transmitted by suitable hosts (often arthropods such as insects) to humans or other animals. Transmission of the disease agent to a vertebrate host usually occurs through the vector feeding on blood (CDC, 2012). Between 1940 and 2004, 335 emerging infectious diseases (EIDs) had been reported globally (Jones *et al*., 2008), approximately 22.8% of which are vector-borne diseases (Jones *et al*., 2008). A wide range of arthropod species can act as vectors for the transmission of pathogens, including mosquitoes such as *Aedes*, *Culex* and *Anopheles*, sandflies (*Phlebotomus* spp*.),* ticks (Ixodidae and Argasidae), and bugs, such as the Reduviidae subfamily Triatominae (Farajollahi *et al*., 2011; Meyabeme Elono *et al*., 2010) Table 1.3 gives some examples of vector-borne diseases and their vectors.

Table 1.3. Some diseases transmitted by arthropod vectors (WHO, 2017; Asnis *et al*., 2000; Buckley *et al*., 2003; ECDC, 2010).

Disease	**Agent**	**Vector**	**Vector Group**
Malaria	*Plasmodium* spp. (Protozoa)	*Anopheles* spp.	Mosquito
Dengue Fever	*Flavivirus* (virus)	*Aedes* spp.	
Yellow fever	*Flavivirus* (virus)	*Aedes* spp.	
West Nile Virus	*Flavivirus* (virus)	*Culex* spp.	
Lymphatic Filariasis	Filarioidea (Nematoda, roundworms)	*Culex, Aedes* and *Anopheles* spp.	
Chagas disease	*Trypanosoma cruzi* (Protozoa)	Triatomine bugs	True bugs
Leishmaniasis	*Leishmania spp.* (Protozoa)	Phlebotominae	Sandflies
Lyme disease	*Borrelia burgdorferi* (Bacteria)	*Ixodes* spp.	Ticks

Mosquitoes are arguably the most important carrier of vector-borne diseases, affecting millions of people and causing thousands of deaths (WHO, 2015b; Dekoninck *et al*., 2010). Malaria infection is present in 97 countries on three different continents, mostly in Africa, Asia and Central and South America (WHO, 2015b) and is pandemic in several countries (Bueno-Marí and Jiménez-Peydró, 2011). Dengue, Japanese encephalitis, Chikungunya, West Nile and Yellow Fever are mosquito-borne viruses present in tropical and subtropical regions. Worldwide, Dengue virus is one of the most important viral diseases transmitted by mosquitoes after Malaria. The main vector of Dengue is *Aedes aegypti* (Linnaeus, 1762), yellow fever mosquito, which originated in West Africa and has rapidly spread around climatically suitable areas of the world (WHO, 2015a; Lounibos, 2002). A second main vector of Dengue, *Aedes albopictus* (Tiger mosquito), has an extreme capacity to adapt to new environments and habitats because of its strong competitive behaviour (ECDC, 2016; Tomasello and Schlagenhauf, 2013). Both vectors are spreading in parts of Europe, increasing their ranges and raising concerns of the possible transmission of infectious diseases (Gratz, 2004a). Another virus moving into Europe is Chikungunya virus (CHIKV). This arbovirus belongs to the family Togaviridae, genus *Alphavirus* (Tomasello and Schlagenhauf, 2013; Staples *et al.*, 2009; Kucharz and Cebula-Byrska, 2012) and is also transmitted by *Aedes* mosquito species. In Europe, Chikungunya was first reported in 2007 in the Emilia-Romagna province of Italy (ECDC, 2016). However, most of the dengue and chikungunya cases reported in Europe are imported by travellers returning from infected areas.

The West Nile Virus (WNV) (*Flaviviridae, Flavivirus*), a zoonotic pathogen, was initially isolated in Uganda in 1937 (Rizzoli *et al.*, 2015; Kilpatrick, 2011; Kilpatrick *et al.*, 2008; Reed *et al.*, 2003; Gratz, 2004b). The enzootic mosquito vectors transmit WNV from non-human animals (mostly birds) to humans or horses (considered as "dead-end hosts") (Kilpatrick, 2011; Asnis et al., 2000; ECDC, 2010). Worldwide, WNV has been isolated from at least 75 species of mosquito (Higgs *et al.*, 2004), but the main vectors of WNV belong to the genus *Culex*, in particular, *Culex pipiens* which is widespread in the UK (WHO, 2011; CDC, 2013). WNV swept through the USA in the late 20th century and approximately 1.8 million people were infected, of which 360,000 presented illnesses. 12,852 cases of encephalitis/meningitis were reported and 1308 deaths occurred between 1999 and 2010 (Kilpatrick, 2011). WNV is now considered as the most important non-native vector-borne disease in the USA (CDC, 2015; Kilpatrick, 2011; Reed *et al.*, 2003).

1.3. Infectious diseases in Europe

Old diseases are re-emerging and new infectious diseases are developing in both tropical and temperate areas (Smolinski *et al*., 2003). Some infectious diseases are changing their geographic distributions, and/or are increasing in incidence (Prasad, 2010; Medlock *et al*., 2012b). Globalisation, trade, human travel/immigration, shipping, aeroplanes, urbanisation, population density, and climatic and environmental changes are factors favouring the establishment of vectors and diseases in new regions (Van den Berg *et al*., 2013; Gratz, 2004b; Jones *et al*., 2008). *Culex* spp., *Aedes* spp., and *Anopheles* spp. are all implicated in the emerging transmission of infectious diseases worldwide.

In Europe, nine genera and a total of 103 mosquito species have been described (Schaffner *et al*., 2009; Linton *et al*., 2005; Snow and Ramsdale, 2003; Snow, 2010). Eighteen species of *Anopheles* have been recorded in Europe (Ramsdale and Snow, 2000) some of which have been recognised as possible malaria vectors in the UK (Snow, 1998). However, higher threats and risks of the establishment of emerging diseases arise from exotic, invasive species which can rapidly expand their distribution (ECDC, 2010; Medlock et al., 2012b). Six *Aedes* species have been introduced and established in Europe (Figure 1.5) (Van den Berg *et al*., 2013; Snow and Ramsdale, 2003). *Aedes albopictus* has been reported in at least in 12 European countries (Kucharz and Cebula-Byrska, 2012).

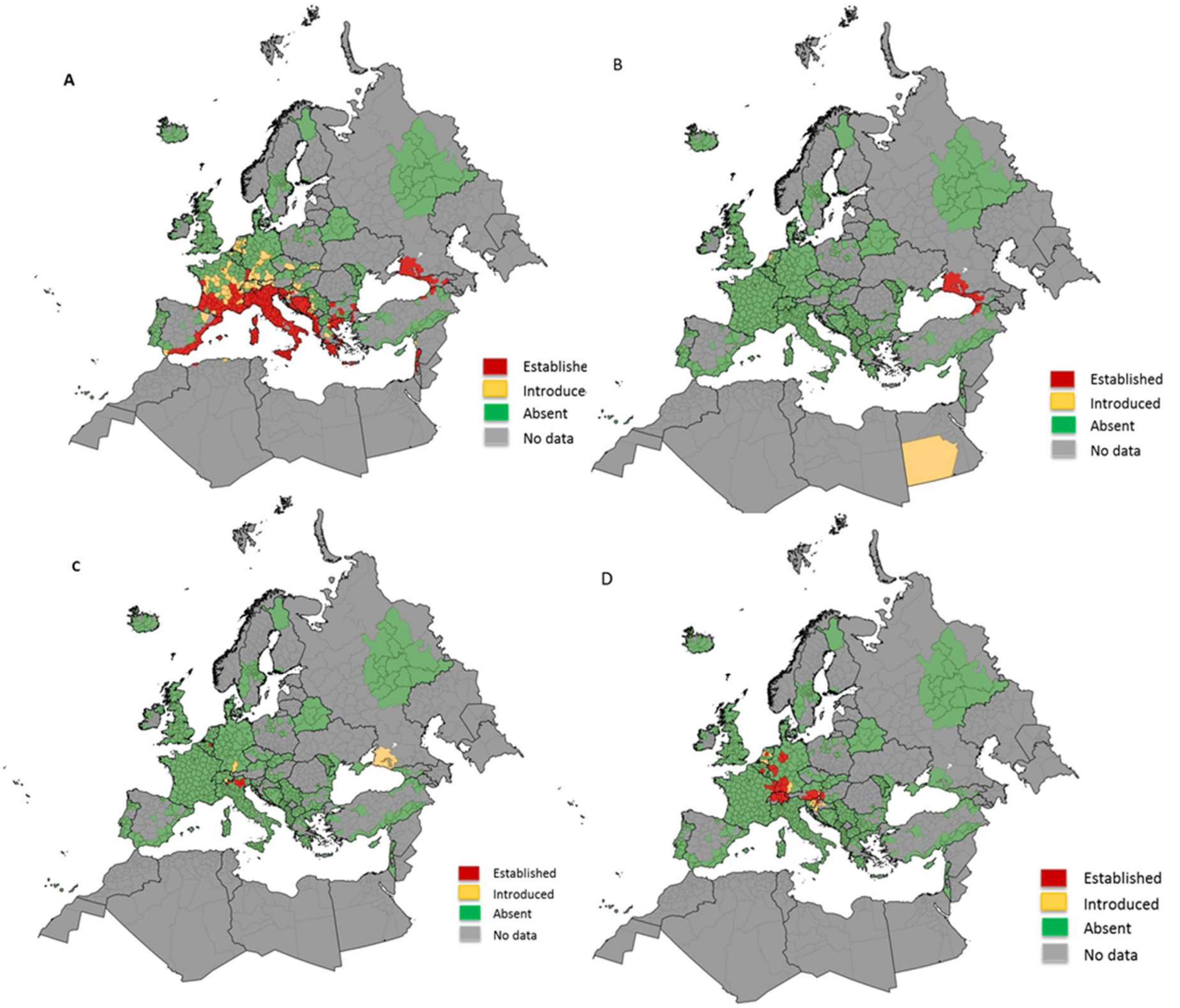

Figure 1.5. European Distribution of some invasive Aedes spp. mosquitoes, April 2017. **A** - *Aedes albopictus*. **B** - *Aedes aegypti*. **C** - *Aedes koreicus*. **D** - *Aedes japonicus*. Source: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-map.

In Europe, the incidence and number of vector-borne disease are lower than in tropical and developing countries, but the prevalence of these diseases has grown, and their distribution has increased as a consequence of ecological and environmental changes (Gratz, 2004b). The effect of global warming and changes in landscape use might lead the resurgence of some diseases and the establishment of others never before seen on the European continent (Gratz, 2004b; Patz and Olson, 2006).

Malaria was endemic in Europe until the Second World War (Bueno-Marí and Jiménez-Peydró, 2011; Gratz, 2004b). Most of the countries affected were those in Southern Europe, where morbidity and mortality were extremely high, due to suitable climatic conditions (Bueno-Marí and Jiménez-Peydró, 2011). However, malaria outbreaks and seasonal epidemics have also occurred in northern Europe (Scandinavia) during the 19th century (Bueno-Marí and Jiménez-Peydró, 2011). In Scandinavian countries, the best malaria vectors were those females that were semi-active and hibernating but not in complete diapause (anthropophilic and endophagic anophelines) (Bueno-Marí and Jiménez-Peydró, 2011). As a consequence, malaria transmission generally occurred indoors or in similar conditions. By 1970, malaria was eradicated on the European continent, following a number of control programmes (Gratz, 2004b), however, in the European Union between 10,000 and 12,000 cases of imported malaria are recorded every year (Gratz, 2004b).

During the first World war, malaria transmission was once endemic in the UK, wetlands of East Anglia, Kent, Essex and on the south coast of England (Snow, 1990; Chin and Welsby, 2004; Snow, 1998). The protozoan parasite *Plasmodium vivax* was the most likely species responsible (Chin and Welsby, 2004; Lindsay and Thomas, 2001), and *Anopheles atroparvus* was the most likely vector. "Fen ague", as it is locally known, started declining after the 18th century (James, 1929), as a consequence of swamp drainage, house improvements and anti-malarial drugs, the former two led to a reduction in the density of *An. atroparvus* mosquitoes (Snow, 1990; Chin and Welsby, 2004). The last cases reported in the UK were in 1983, where two people living close to Gatwick airport were bitten by *Anopheles* mosquitoes

(Snow, 1990). At present, there are no cases of malaria in the UK (Snow, 1990; Medlock *et al.*, 2007), unless imported as described above.

1.4. Mosquitoes in Great Britain.

In the UK, more than 30 mosquito species have been recorded, including six species of *Anopheles,* and 27 species of Culicinae: *Aedes* (3), *Coquillettidia* (1)*, Culex* (4)*, Culiseta* (7), *Dahliana* (1), *Ochlerotatus* (11) *and Orthopodomyia* (1) (Medlock *et al.*, 2007; Medlock and Vaux, 2009; Medlock *et al.*, 2012a). *Anopheles daciae* was identified in the early 2000's in Somerset South-West England (Linton *et al.*, 2005). *An. atroparvus, An. algeriensis, An. maculipennis, An. claviger and An. plumbeus* have all been reported in the UK recently (Figure 1.6), of these, *Anopheles atroparvus* is the most efficient malaria vector (Snow, 1990; Snow, 1998; Lindsay and Thomas, 2001).

The availability of breeding sites determines the distribution of British mosquitoes. In England, Government departments and agencies have developed a Wetland Vision, which consists of new strategies in urban areas for the extension of existing wetlands and creation of new ones (Medlock and Vaux, 2015). However, concerns have been raised over the impact that this might have on the abundance and distribution of mosquitoes and in consequence on vector-borne diseases (Zimmerman, 2001).

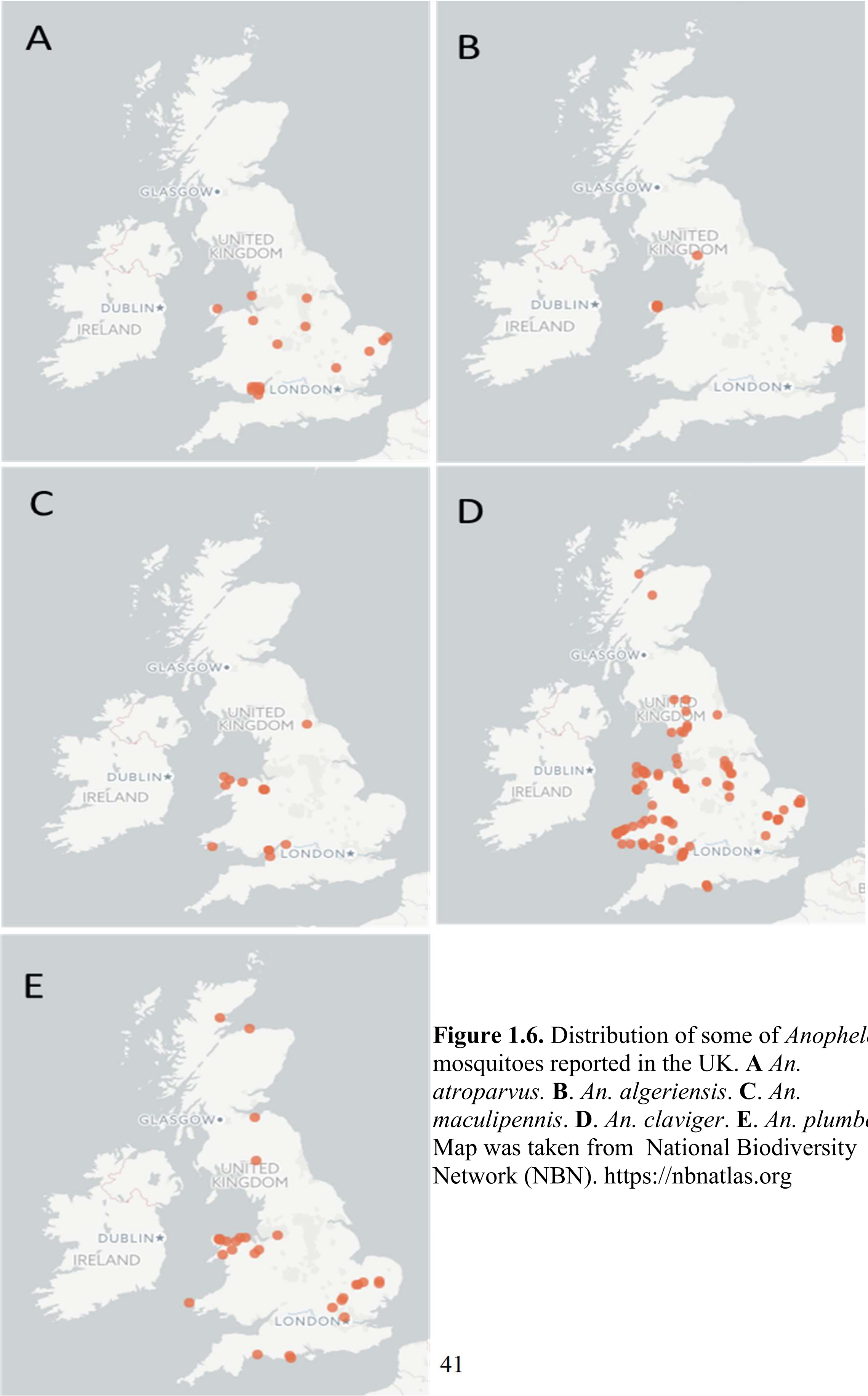

Figure 1.6. Distribution of some of *Anopheles* mosquitoes reported in the UK. **A** *An. atroparvus*. **B**. *An. algeriensis*. **C**. *An. maculipennis*. **D**. *An. claviger*. **E**. *An. plumbeus*. Map was taken from National Biodiversity Network (NBN). https://nbnatlas.org

Neighbouring towns and villages could experience an increase in adult mosquito numbers as a result of the increase in larval habitats (Medlock and Vaux, 2011). Most British mosquitoes are associated with nuisance biting in rural and urban areas and the scale of nuisance is linked to the proximity of aquatic habitats (Medlock *et al*., 2005a; Snow, 1990; Malcolm, 2009; Snow, 1987; Medlock *et al*., 2012a).

Mosquito hosts include a wild range of mammals (rodents and cattle), birds, amphibians and reptiles (Brugman *et al*., 2017; Medlock *et al*., 2012a). From the approximately 34 species of mosquito that occur in Britain, six feed on birds and humans, eleven more feed on humans but rarely feed on birds (Medlock *et al*., 2005a).

In Great Britain, mosquitoes have seasonal activity patterns and overwintering mechanisms depending on environmental conditions (Cranston *et al*., 1987; Marshall, 1938; Medlock and Vaux, 2015; Snow, 1990). Mosquitoes can produce one (univoltine), two (bivoltine) or multiple generations per year (multivoltine) (Table 1.4). For example, *Coquillettidia richiardii* Ficalbi 1889, *Ae. cantans, Ae. rusticus, Ae. punctor,* and *Ae. flavescens* have a generation per year which adults start to emerge during spring; *An. plumbeus* presents two peaks of adult abundance, the first in May-June and the second in August-September. Finally, *Cs. annulata, An. atroparvus* and *Cx. pipiens* present multiple generations per year which the peaks of abundance of adults overlap during the year (Snow, 1990; Medlock *et al*., 2015).

Labarthe *et al*. (1998) explained that multivoltine species might be better vectors than univoltine species. A possible reason is that multivoltine species will be frequent during the year. Adults will present different peaks in abundance through the year, so the transmission of the disease might increase when the highest abundance of adults are.

Table 1.4. UK occurring mosquito species, feeding and overwintering habits. Voltinism: U- Univoltine; **B –** Bivoltine; **M** – Multivoltine. After (Cranston *et al*., 1987; Medlock *et al*., 2012a; Medlock *et al*., 2007; Medlock *et al*., 2005; Schaffner *et al*., 2012; Snow, 1990; Snow and Medlock, 2008; Rees and Snow, 1992; Marshall, 1938).

Mosquito Species	**Habitat**	**Voltinism**	**Overwintering stage**	**Feeding behaviour**
*Aedes cinereus***	Flooded habitats	Univoltine	Eggs	Cattle, humans and birds
*Anopheles plumbeus**ǂ	Tree-hole	Bivoltine	larvae (4th instar)	mammals, humans and birds
Anopheles atroparvus®	Coastal waters	Multivoltine	-	humans, infrequent birds
Anopheles claviger®	Permanent water	Multivoltine	-	humans, infrequent birds
Anopheles messeae®	Permanent water	Multivoltine	-	humans, infrequent birds
*Culex europaeus***	-	-	-	humans and birds
*Culex modestus***	Permanent water	-	-	humans and birds
*Culex pipiens***	Permanent water	Multivoltine	Adults	birds
Culex torrentium±	Permanent water	Multivoltine	Adults	Humans and birds
Culex molestus	Underground	Multivoltine	All stages	
*Coquillettidia richiardii***	Permanent water	Univoltine	All stages	Cattle, humans and birds
*Ochlerotatus cantans***	Woodland pools	Univoltine	Eggs	Cattle, humans and birds
*Ochlerotatus caspius***	-	Multivoltine	-	humans and birds
*Ochlerotatus detritus**ǂ	Coastal waters	Multivoltine	larvae (4th instar)	Cattle, humans and birds
*Ochlerotatus punctor**ǂ	Woodland pools	Univoltine	Eggs and larvae	Cattle, humans and birds
Ochlerotatus annulipes®	Temporary pools	Univoltine	-	humans, infrequent birds
Ochlerotatus dorsalis®	Coastal waters	-	-	humans, infrequent birds
Ochlerotatus flavescens®	-	Univoltine	-	humans, infrequent birds
Ochlerotatus geniculatus®	-	-	-	humans, infrequent birds
Ochlerotatus rusticus®	Flooded habitats	Univoltine	-	humans, infrequent birds

Table 1.4. Continued

Mosquito Species	**Habitat**	**Voltinism**	**Overwintering stage**	**Feeding behaviour**
Ochlerotatus leucomelas©	-	-	-	birds
Ochlerotatus sticticus©	-	-	-	birds
*Culiseta annulata**ǂ	Permanent water	Multivoltine	All stages	humans and birds
*Culiseta litorea**ǂ	Permanent water	Univoltine	larvae (4th instar)	humans and birds
*Culiseta morsitans**ǂ	Permanent water	Univoltine	larvae (4th instar)	humans and birds
Culiseta subochrea	-	-	-	humans, infrequent birds
Culiseta longiareolata©	-	-	-	birds
Culiseta fumipennis±	Permanent water	-	-	exclusively on birds

*	Mosquito species that could be considered as a potential vector in the UK.
**	Mosquito species linked to West Nile virus transmission in Europe and USA.
ǂ	Mosquito species that feed on birds and humans.
®	Mosquito species that feed on humans but rarely on birds.
©	Mosquito species rare in the UK.
±	Mosquito species that feed exclusively on birds

1.5. *Culex pipiens*

Culex pipiens was first described in Great Britain in 1934 and is the most common mosquito (Rees and Snow, 1992; Snow and Medlock, 2008; Marshall, 1938; Onyeka, 1983). Figure 1.7 shows their distribution. *Culex pipiens* is present in urbanised and semi-urban areas in North America and Europe (Almeida *et al*., 2008; Snow, 1990). To date, two forms of *Culex pipiens* have been reported in Britain: *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* (Rees and Snow, 1992; Smith and Fonseca, 2004). *Culex pipiens* is responsible for the transmission of St Louis Encephalitis virus, West Nile Virus, Bancroftian. Lymphatic filariasis *(Wuchereria bancrofti*), and Avian malaria (Barbosa *et al*., 2007; Fonseca *et al*., 2004). No human cases of St. Louis Encephalitis virus, WNV and Lymphatic filariasis *(Wuchereria bancrofti*) have been detected so far in the UK (Medlock *et al*., 2005a).

The number of studies reviewing the ecology, biology and potential risk of vector-borne diseases has increased as a result of the recent outbreaks of WNV in Europe and the USA. Townroe and Callaghan (2014) conducted a long-term study in the South East of England to compare rural and urban areas in the distribution of species of mosquitoes. They found that urban containers were less species-rich but significantly higher densities of *Culex pipiens*. However, *An.plumbeus* was abundant in urban containers, indicating that this specie is expanding to artificial containers.

Figure 1.7. Distribution of *Culex pipiens* reported in the UK. Source: National Biodiversity Network (NBN). **https://nbnatlas.org**

A survey was carried out in eleven airports and sea-ports in the United Kingdom to identify suitable mosquito habitats (Vaux *et al*., 2011). *Culex pipiens* and *Culiseta annulata* were the most common species found during the two years of sampling. No invasive species were detected. However, in another survey, in late September 2016, 37 eggs of *Aedes albopictus* were detected in suitable habitat close to the Eurotunnel near Kent (Medlock *et al*., 2017), identification was positively confirmed with larval, adult and egg morphology. The authors concluded that the infestation might arise from a single female introduced to the UK by a car arriving from Europe and that there is no risk of it leading to the establishment.

1.6. Mosquito predators in Great Britain

A wide diversity of invertebrate taxa share the same aquatic habitats (E Campos et al., 2007; Meyabeme Elono et al., 2010), hence competition is common, increasing when the resources become insufficient or limited (Stav *et al*., 2005). Recently, several studies have focused on competition between conspecific larvae present in the same trophic level (Blaustein and Chase, 2007). Predators potentially control the larval and pupal stages of mosquitoes, as oviposition, numbers of eggs and larval densities are all reduced or inclusively inhibited (Duquesne *et al*., 2011). Predators, however, often have a varied diet; not feeding on larvae exclusively perhaps because larvae are not always co-located (Duquesne *et al*., 2011). Mosquitoes nevertheless are prey to a wide variety of predators. Many arthropods breed both in artificial containers and permanent groundwater and may be potential predators of mosquito larval and pupal stages (Onyeka, 1983). In the UK, at least six insect orders and thirteen families of arachnids as well as crustaceans, amphibians, birds, fish and mammals may predate mosquito larvae (Medlock and Snow, 2008) (Table 1.5).

Previous studies used precipitin test to identify potential predators of *Ochlerotatus cantans* in four localities in South England (Service, 1973). Service (1973) observed that the most important predator observed was the diving beetle Dytiscidae. Predation on larval stages produced a small decrease in the density of the population of pre-adults, however, the reduction of the population depends on the number of predators and the number of larvae consumed (Service, 1973). Similar studies showed that in woodlands near Monks wood in Huntingdonshire, trichoptera and Isopoda larvae and the amphipod *Gammarus pulex* (L.) were the most common predators associated with larval stages of *Aedes cantans* (Service, 1977). In a second study, two permanent ground ponds at Silwood Park in Ascot, southern

England were sampled to detect *Culex pipiens s.l.* Linnaeus and *Culex torrentium* Martini predators (Onyeka, 1983). Onyeka (1983) found that Odonata (dragonflies and damselflies), Coleoptera (Dytiscidae and Halipidae), Hemiptera (Gerridae and Notonectidae) and amphibians were related as possible predators of mosquito larvae. Gut smear was performed to analyse predation of the amphibian *Triturus vulgaris* on mosquito larvae. The results showed high levels of larvae consumption (30 consumed/40 total) and approximately 75% individuals were positive in the reaction (Onyeka, 1983).

Table 1.5. Predators of mosquito larvae, pupae and adults. Sources: Medlock and Snow, 2008; Onyeka, 1983; Roberts, 1995; Service, 1973; Service, 1977; Lockwood, 1986).

Stage Predated	**Mosquito habitat**	**Predators**	
		Groups	**Species/Genus**
Eggs	Temporary and Permanent habitats	Acari (mites)	*Macrocheles* spp.
			Peragamasus spp.
			Gepholapsis spp.
		Coleoptera (beetles)	Carabidae
			Dytiscidae
			Hydrophilidae
			Scydmaenidae
			Staphylinidae
			Cryptophagidae
			Lathridiidae
			Anthicidae
		Chilopoda	
		Lumbricidae (earthworms)	Earthworms
Larvae and pupae	Permanent freshwater	Odonata (dragonflies and damselflies)	*Sympetrum striolatum*
			Libellula depressa
			Coenagrion mercuriale
			Ischnura elegans
			Pyrrhosoma nymphula
		Coleoptera (beetles)	Dytiscidae
			Halipidae
			Gerridae
		Hemiptera (bugs)	Notonectidae
		Diptera (flies)	*Chaoborus* spp.
		Amphibians	*Trituris vulgaris*
			Trituris cristatus
			Trituris vulgaris
			Rana temporaria
			Bufo bufo
		Fish	*Phoxinus phoxinus*
			Gambusia affinis
			Alburnus alburnus
			Carassius auratus
			Cyprinus carpio,
			Tinca tinca
			Gasterosteus aculeatus
			Gobius microps
			Scardinius erythrophthalmus

Table 1.5. Continued.

Stage	Mosquito habitat	Predators	
		Groups	Species/Genus
Larvae and pupae	Temporary woodlands	Trichoptera	*Trichostegia minor* *Glyphotaelius pellucides*
		Coleoptera	*Agabus bipustulatus* *Dytiscus semisulcatus* *Agabus sturmii* *Dytiscus marginalis* *Gyrinus natator*
		Hemiptera	*Gerris lacustris* *Gerris gibbifer* *Hydrometra stagnorum*
		Plecoptera	*Nemoura cinerea*
		Ephemeroptera	*Cloeon dipterum*
		Tricladida	
		Isopoda	*Asellus aquaticus*
		Amphipoda	*Gammarus pulex*
		Diptera	*Chaoborus*
	Brackish water salt-marsh habitat	Amphipoda	*Gammarus dubenei* *Gammarus pulex*
		Decapoda	*Palaemonetes varians*
		Isopoda	*Sphaeroma rugicauda*
	Artificial containers	Coleoptera	*Agabus bipustulatus* *Dytiscus marginalis* *Hydroporus sp.*
		Diptera	*Chaoborus spp*
	Tree holes	Chironomid	*Metriocneumus martini*
		Coleoptera	*Helodes* sp. *Prionocyphon serricornis*
Adult mosquitoes	Flying mosquitoes	Birds	*Apus apus* *Delichon urbica* *Hirundo rustica* *Parus caeruleus*

1.7. Aims and objectives of the study

Whilst a number of studies have been conducted to investigate the use of dye colourings in aquatic environments (Buglewicz and Hergenrader, 1977; Martin *et al.*, 1987; Spencer, 1984; Tew, 2003), none shows how the application of the dyes might affect animal community compositions in ponds. This study begins to redress this important gap in our knowledge, which, as shown, might have potential implications for biodiversity on national and international scales as well as for human health and well-being, by considering the effects of pond dyes on some macroinvertebrate species and macroinvertebrate communities, in laboratory and semi-field study conditions. We focus on the effects of pond dyes on mosquito populations and common, naturally developed macroinvertebrate populations.

This project aimed to collect ecological data and study the impact on macroinvertebrates and British mosquitoes in freshwater ecosystems and artificial containers of a cosmetic product (DyoFix). The study was undertaken at the campus of the University of Reading with a combination of laboratory and field experiments under mesocosms conditions.

Chapter 2- Pond dyes are *Culex* mosquito oviposition attractants.

An investigation of the impacts of black pond dye on wild *Culex pipiens* mosquito breeding behaviour in laboratory and semi-field conditions (tent) and on mosquito species in water butts in different habitats (glasshouse and woodland areas); success on survival rates and emergence of mosquito larvae in water butts in field conditions.

Chapter 3- The effect of pond dyes on oviposition and survival in wild *Culex* mosquitoes. An investigation of the impacts of blue and shadow (red) coloured dyes on *Culex pipiens* mosquito breeding behaviour in laboratory and semi-field conditions; natural colonisation of containers with and without shadow in field conditions and the success on survival rates and emergence in water butts in the glasshouse habitat.

Chapter 4- The effect of pond dyes on macroinvertebrate communities.
An investigation of the impacts of black pond dye on communities in freshwater habitats, abundance and biodiversity of the aquatic invertebrates in mesocosms which previously were cleaned and placed a set number of macroinvertebrates into the ponds. A second dye application was added at the middle of the experiment (in the 11 weeks). To observe impacts on freshwater communities, abundance levels, total biodiversity and species-level effects were recorded weekly after the reintroduction of lesser water boatmen (*Corixa punctata*), non-biting midge larvae (*Chironomus plumosus*), mosquito larvae (*Culex pipiens*), dragonfly larvae (*Anisoptera* sp.) and *Daphnia pulex*.

Chapter 5- Do pond dyes have an impact on communities in freshwater habitats?
An investigation of the effects of the use of black pond dye on macroinvertebrates biodiversity and evenness, individuals and mosquitoes in field conditions. A difference from the previous chapter; the ponds were not cleaned and no macroinvertebrates were reintroduced. A second dye re-application was added to ponds treated for the first time in 2014. Abundance levels, total of biodiversity, and species-level effect were recorded once a week to observe the impact on freshwater communities.

Chapter 6- Predation of *Cx. quinquefasciatus* and wild mosquito larvae and functional response of *Chaoborus flavicans* and *Gammarus pule***x** in presence of black dyes.

An investigation of the predation potential of *Chaoborus flavicans* and *Gammarus pulex* on wild *Culex* sp. and *Culex quinquefasciatus* mosquito larvae, and effects on functional responses of predators, on larvae of wild *Culex* sp and *Culex quinquefasciatus*, in laboratory conditions.

CHAPTER 2: Pond dyes are *Culex* mosquito oviposition attractants

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Declaration

The Oviposition preferences of wild mosquitoes and emergence study: field conditions data were collected with company of Rebecca Gander, Oliver Abbey.

CHAPTER 2. Pond dyes are *Culex* mosquito oviposition attractants

2.1 Introduction

West Nile virus (WNV) is a positive-sense RNA virus belonging to the Flaviviridae family and is transmitted by mosquitoes, including *Cx. pipiens* complex mosquitoes. There have been several major outbreaks of WNV in Europe in recent years, affecting both humans and horses (Calistri *et al*., 2010; Di Sabatino *et al*., 2014; Hernández-Triana *et al*., 2014). A laboratory test of the vectorial competence of European *Cx. pipiens,* including the phenotypic and physiological variant *Cx. molestus*, demonstrated that both the molestus form and a hybrid between *Cx. pipiens* and *Cx. molestus* were capable of transmitting WNV (Brustolin *et al*., 2016). Both of these variants are found in Britain, raising the possibility that outbreaks of human or animal viral diseases could occur in Britain if conditions and climate permitted. Whilst the threat is likely to come from invasive species, more than 30 species of mosquito, including putative vectors of arboviruses, are native to the UK (Blagrove *et al*., 2016). To date, there is no evidence of mosquito-borne virus transmission of public health concern in the UK (Blagrove *et al*., 2016). However, we know that mosquitoes are established in both rural and urban habitats and are often found in gardens (Townroe and Callaghan, 2014). Understanding and mitigating future threats requires detailed ecological knowledge of the putative vector species and prediction of how mosquito populations are influenced by anthropogenic activity.

In England, 80% of the human population lives in towns and cities which cover more than 7% of the land area (Wilby and Perry, 2006). Urbanisation changes the physical environment in a way which is known to alter habitat types, species numbers and the community composition of ecosystems (McKinney, 2006; Sala *et al*., 2000). These changes are likely to impact British mosquito populations and influence distributions, abundances, species composition, mosquito-host interactions, biting nuisance and the potential for mosquito-borne disease to occur in the UK.

Gardens make up a large proportion of the urban area and provide a significant contribution to the green spaces within many UK cities providing areas of ecological value which may support diverse wildlife populations including mosquitoes (Smith *et al*., 2005). The creation of ponds is encouraged as a means of enhancing the biodiversity value of gardens, particularly in the face of a widespread decline of ponds in the wider rural landscape (Gaston *et al*., 2005). Although individually small (~2.5 m^2) and fragmented into small patches, urban ponds are distributed widely across the urban landscape and are likely to contain water all year round (Gaston *et al*., 2005). Where fish are not present, they are likely to provide a valuable breeding site for mosquitoes. Water butts also provide an ideal habitat for mosquitoes with a recent study recording five British mosquito species; *Anopheles claviger*, *An. plumbeus*, *Culiseta annulata*, *Cx. pipiens* and *Cx. torrentium* (Townroe and Callaghan, 2014). Predicted future changes to the climate, with increased summer temperatures and more frequent heavy rainfall in winter, will continue to place pressure on water supplies and encourage domestic water storage (Snow and Medlock, 2006b). This in turn is likely to increase populations of the most common species, *Cx. pipiens*, particularly in urban gardens. *Cx. pipiens* is a potential enzootic (primary) vector of West Nile Virus WNV (Medlock and Leach, 2015) and the species most likely to be directly affected by changes in water storage and pond formation (Townroe and Callaghan, 2014). The current risk of WNV transmission in the UK is considered low because the abundance of enzootic and bridge (non-primary) vectors is too low for sustained transmission (Medlock and Leach, 2015). Changes in climate, migration of mosquito species and longer flight seasons in dense urban areas creates conditions more conducive to high levels of human host biting and an increased risk of disease transmission. Therefore, any factors that significantly change the distribution and population of *Cx. pipiens* are likely to impact subsequent risk of disease transmission.

Pond dyes are a relatively new cosmetic product for garden ponds and lakes. They have proved to be popular at recent high profile garden shows such as Chelsea and Hampton Court. They stop the growth of algae by blocking the red end of the visible light spectrum (of wavelength 620 x 740 nm) from penetrating the water. The red end of the light spectrum is needed for photosynthesis, as peak absorption for photosynthetic pigments is approximately 650 nm (Douglas *et al*., 2003). Although there is no evidence to suggest that these dyes are toxic to fish and invertebrates, the impact on invertebrate communities may well be behavioural. In this study, we investigate the impact that pond dyes have on oviposition and survival in *Cx. pipiens* mosquitoes. Previous studies have shown that the cues for oviposition are often visual and have demonstrated a preference for oviposition in dark containers and dark waters (Beckel, 1955; Collins and Blackwell, 2000; Hilburn *et al*., 1983; Hoel *et al*., 2011; Panigrahi *et al*., 2014). We therefore predict that pond dyes will act as an attractant for mosquito oviposition, with a potential impact to increase mosquito population densities in garden ponds. This is the first study to specifically look at pond dyes to see if they impact on mosquito breeding behaviour and success.

2.2 Materials and Methods

2.2.1 Trapping wild gravid female mosquitoes

Wild gravid female Culicinae mosquitoes were trapped using modified oviposition traps (Reiter, 1987; Townroe and Callaghan, 2015). A total of 10 traps were placed on the Whiteknights campus at the University of Reading, Berkshire, England (51.4419° N, 0.9456° W) (Figure 2.1). Approximately 1,000 gravid female mosquitoes were caught in July and August 2014 and 2015. Most of the mosquitoes sampled belonged to the *Culex* genus although a few *Anopheles plumbeus* (<5) and *Culiseta annulata* (<5) were also trapped.

2.2.2 Oviposition preferences of wild mosquitoes

An oviposition choice experiment was performed by releasing 200 of the trapped gravid mosquitoes into a tent (245 x 145 x 95 cm) placed outdoors on campus (51.4419° N, 0.9456 °W) (Figure 2.1). Mosquitoes were allowed to freely oviposit in one of 14 2 L plastic containers placed randomly in the tent: seven with 1.2 L tap water and seven with 1.2 L tap water treated with pond dye at the concentrations recommended by the manufacturer (DyoFix, 2015b). After seven days, the containers were taken to the laboratory to count egg batches laid in each container. The experiment was performed three times with freshly trapped females and a choice between tap water and black colour dye. The choice experiment was repeated with wild-caught gravid females under laboratory conditions (25° C, 16:8 light:dark). Five groups of 20 gravid females were chosen randomly and each group transferred into a cotton net cage 25 x 25 x 25 cm per treatment set.

In each cage, two 200 ml plastic bowls were filled with 150 ml of either tap water or dye water. The choice experiments were repeated in normal rearing conditions (16:8 light:dark) and also in the absence of light (black bags were used as a cover in each cage during the experiment).

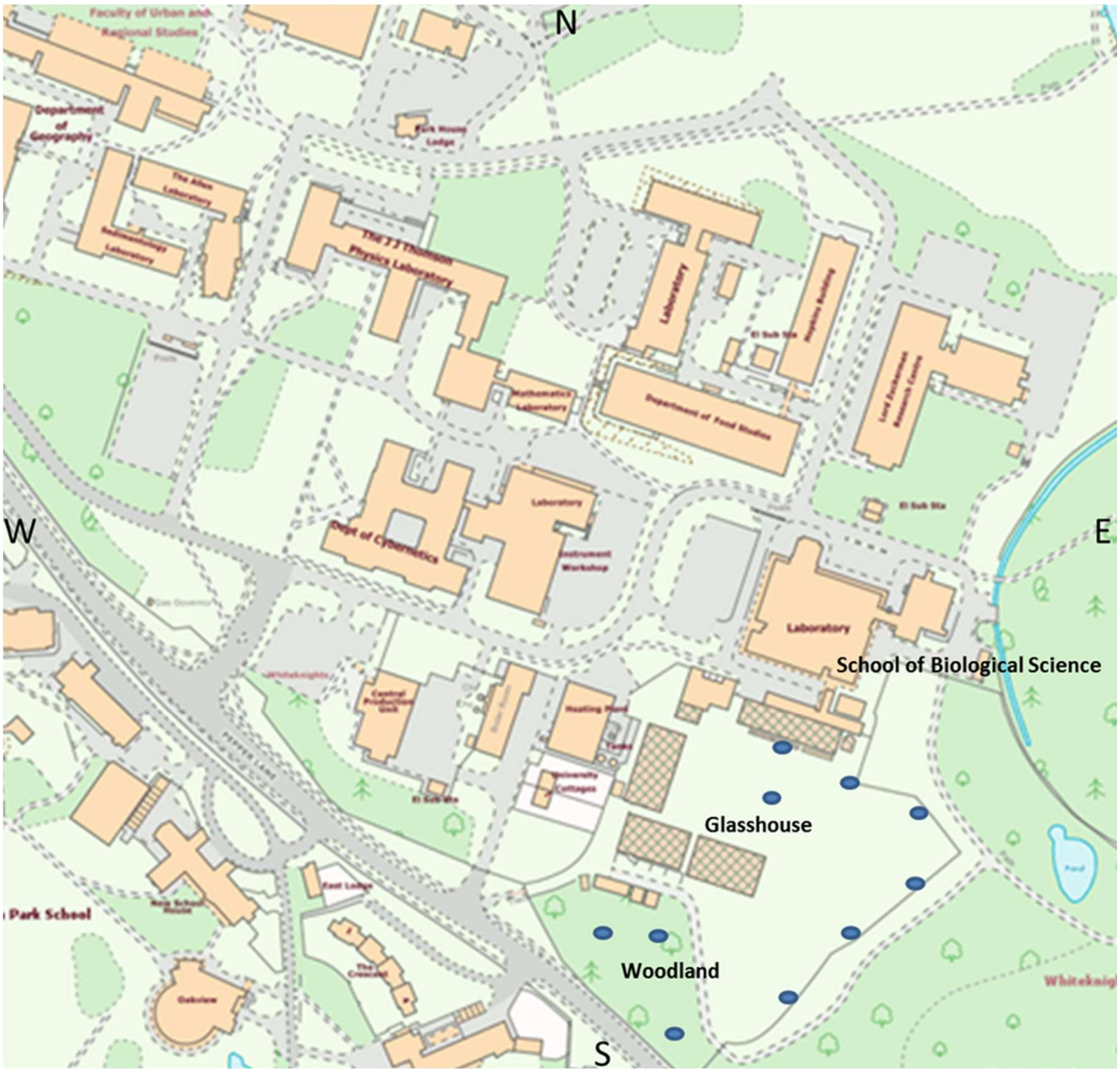

Figure 2.1. Location of the 10 oviposition traps in experimental grounds at the University of Reading. The blue circles are the oviposition traps and the blue circle is the tent. The map was taken from Digimap: http://digimap.edina.ac.uk.

2.2.3 Emergence study

A modified emergence trap (Hamer *et al.*, 2011) was used to measure the impact dye had on mosquito survival. Eggs from the oviposition experiment were hatched in the laboratory (25° C, 16:8 L:D) and reared in tap water through to 2nd instar, fed with pelleted rabbit food. One hundred were then transferred to each of 18 11 L plastic bins (23 x 28 cm) containing 10 L tap water or 10 L tap water and dye (Dyofix, Leeds, UK). Food was added to each bin (1.2 g guinea pig food) which was capped with a conical fabric mesh to trap emerging adults. The bins were placed outdoors in the area used to trap the females. Traps were monitored daily for emerging adult mosquitoes. These were captured using a manual aspirator, transferred into small tubes and stored at -20° C for identification (Snow, 1990).

2.2.4 Wild population numbers in dye treated and untreated artificial containers

Thirty two 80 L water butts (44.5 cm x 58.5 cm), (Figure 2.2) (Townroe and Callaghan, 2014) were placed around the secure area behind the School of Biological Sciences Harborne building on Whiteknights campus in the summer of 2014. Each container was filled with 60 L of tap water and 8 g of ground oak leaves. Bins were placed in pairs with the second bin additionally containing black pond dye added according to manufacturer’s instructions (DyoFix, 2015b). For each treatment, eight replicates were organized in each habitat: woodland (51°26’012.8” N; 0°56’39.7” W) and glasshouses (51°26’13.2” N; 0°56’31.22” W). Bins pairs were several metres apart. Containers were sampled weekly for 26 weeks in

2014. Sampling was carried out using a device adapted from Onyeka (1980) and Townroe & Callaghan (2014). The device included three sections of drain pipe (4 cm high, 0.4 cm thick and 8 cm diameter) bolted together in line with fine mesh net glued to the bottom of each ring and a flexible wire handle attached to the outer edge of the furthest two rings (Figure 2.3). The device was lowered into the container and allowed to rest on the bottom for 5 minutes before being drawn swiftly up through the water to collect animals. This method was carried out once per container per sampling event. The number of larvae and pupae collected were recorded and larvae were replaced in the container. All pupae were taken to the laboratory for rearing to adult then frozen at -22° C. Adults were identified using a 10-4 x magnification microscope using the key of Cranston *et al.*, (1987).

Figure 2. 2. 80 L water butts placed behind the School of Biological Sciences Harborne building on Whiteknights campus with treated and untreated treatment.

Figure 2. 3. The three-section drain pipe used to collect the samples in the containers.

2.2.5 Statistics

All statistical analyses were performed using R version 3.2.2 (R Development Core, 2015). Oviposition preferences of wild and laboratory strain mosquito data were tested for normality using a Shapiro Wilk normality test. The oviposition choice experiment data in laboratory conditions were normally distributed, parametric statistics were used and a paired t-test was performed. Differences in the choice experiment in normal conditions (16:8 light:dark) and also in the absence of light, one-way analysis of variance (ANOVA) was used. Oviposition in the tent was not normally distributed and a nonparametric Mann Whitney U-test was performed. Differences in adult emergence between treatments were analysed using a generalised linear model using a binomial test. Abundance data in water butts were transformed and the relationship between mosquito abundance, treatment and location was analysed using ANOVA using a package (lme4) (Bates *et al*., 2014).

2.3 Results

2.1 Mosquito oviposition selection in laboratory conditions and oviposition selection in the tent

In the laboratory experiment, wild gravid females laid significantly more egg batches in water treated compared with water untreated (t = 5:4928; df = 8; $P < 0.001$) (Figure 2.4 A). Similar results were observed in the tent; wild gravid females laid significantly more egg batches in the water treated compared to the water untreated (W = 250; $P < 0.001$) (Figure 2.4 B). Females laid significantly fewer eggs in the treated treatment when there was a reduction in light (t = 3.0358; df = 8; $P = 0.016$). Light had no significant effect on numbers of eggs laid in water untreated (t = 0.49237; df = 8; $P = 0.6357$). Even though females laid fewer eggs when light was reduced, they still preferred to lay in water treated rather than water untreated (t = 9; df = 8; $P \leq 0.001$).

2.2 Wild population numbers in dye treated and untreated artificial containers

Larval and pupal numbers were analysed by season; summer (June-August) and autumn (September-November), treatment and habitat (Figure 2.5). No significant differences were observed in larval or pupal densities between treated and untreated water in the summer (larval $F_{1,24} = 0.062$; $P = 0.8048$; pupal $F_{1,24} = 0.034$; $P = 0.856$) or in the autumn (larval $F_{1,24} = 0.162$; $P = 0.691$; pupal $F_{1,24} = 0.002$; $P = 0.962$). Habitat impacted on larval numbers, with higher numbers in the glasshouse in the summer ($F_{1,24} = 4.488$; $P = 0.045$) and higher numbers of pupae in the woodland in the autumn ($F_{1,24} = 4.240$; $P = 0.049$). However, there were no habitat differences in larval densities in the autumn ($F_{1,24} = 0.130$; $P = 0.722$) or in pupal densities in the summer ($F_{1,24} = 0.002$; $P = 0.969$). In this experiment was also observed *Anopheles plumbeus* in the woodland area and in water butts cover by vegetation in 2014. In contrast, *Culiseta annulata* was sampled during both years 2014-2015 in both areas glasshouse and woodland.

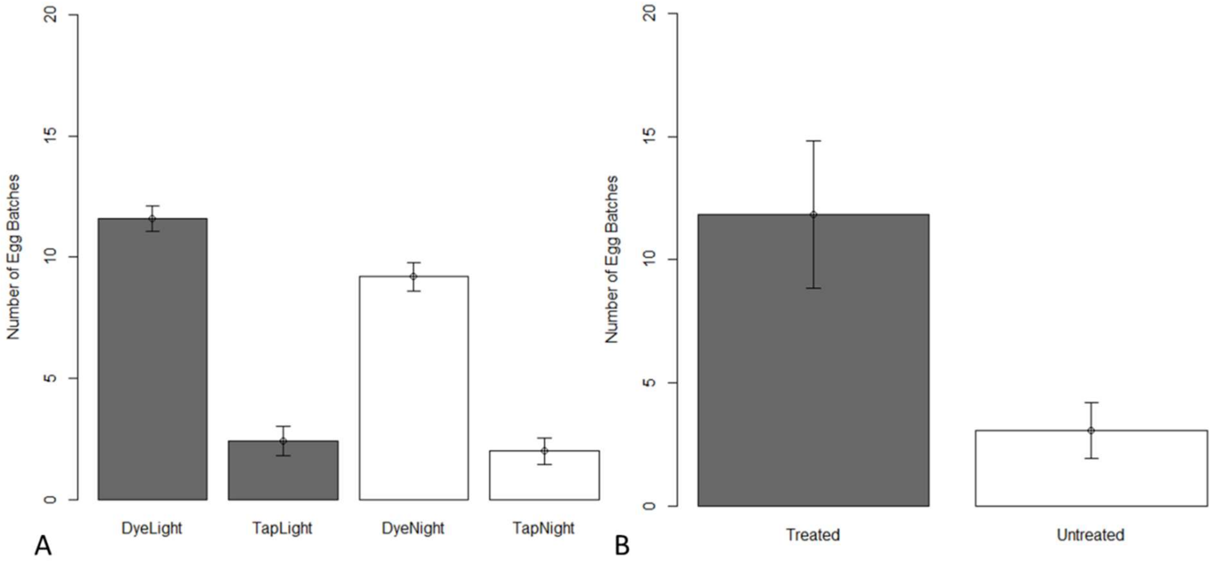

Figure 2. 4. Mean number of egg batches (± SE) laid by wild-caught *Cx. pipiens* in paired choice tests in (**A**) the laboratory with a 16:8 Light/Dark plus or minus a blackout cover and (**B**) semi-field conditions (tent).

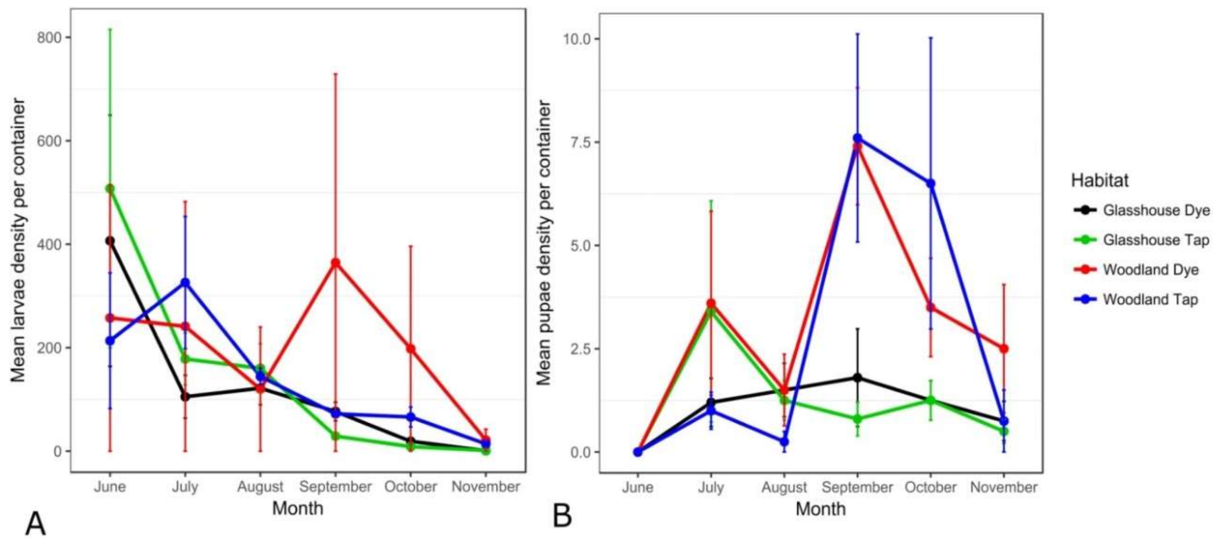

Figure 2. 5. Mean (± SE) number of *Cx. pipiens* larvae (**A**) and pupae (**B**) sampled in treated and untreated water in woodland and glasshouse habitats.

2.3 Emergence study: field conditions

The total number of adults emerging from different treatments varied significantly in both summer (Z = - 11.800, $P < 0.001$) and autumn (Z = - 9.172; $P < 0.001$) (Figure 2.6). In each season, fewer adults emerged from the black dye treatment.

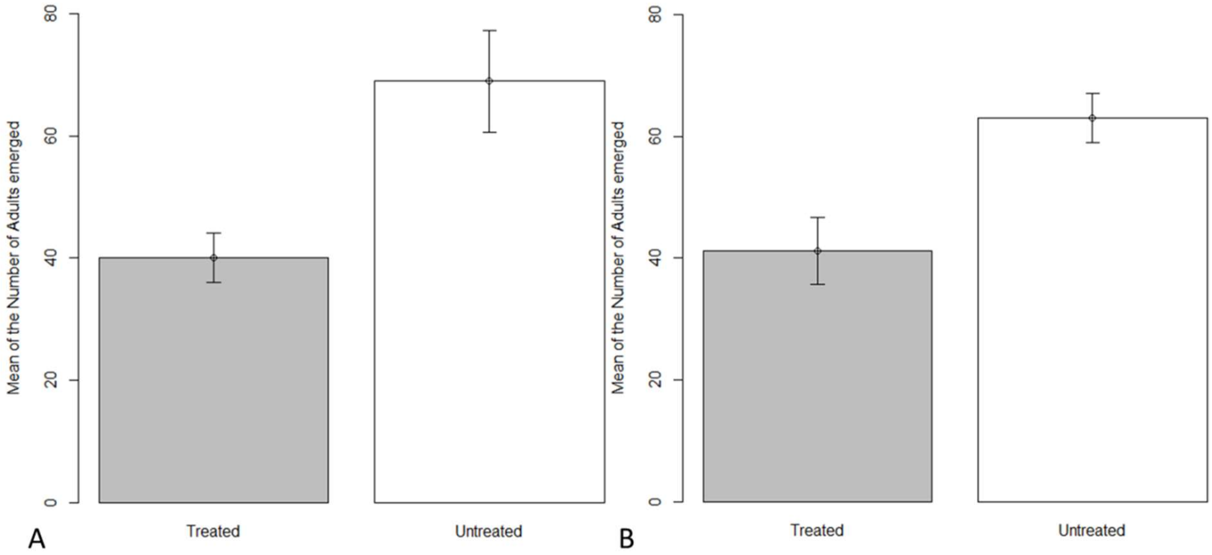

Figure 2. 6. Mean number (±SE) of *Cx. pipiens* adults emerging from bins containing tap water or tap water and dye in (**A**) summer and (**B**) autumn.

2.4 Discussion

Urban garden ponds represent an abundant and reliable network of aquatic resources within which juvenile mosquitoes may develop and adults move across the landscape. Adding pond dyes might influence the attractiveness of ponds as breeding sites to mosquitoes. This is important since the exploitation of human domestic habitats has facilitated mosquito-borne human disease outbreaks in other parts of the world, such as the WNV outbreaks in North America (Patz *et al*., 2004).

It was not unexpected to find that *Cx. pipiens* females prefer to lay eggs in water with the black dye. It is well known that female mosquitoes have preferences for oviposition in containers of different colours and previous studies have demonstrated oviposition choice in dyed water although no work has been undertaken on pond dyes (Beehler and DeFoliart, 1990; Beehler *et al*., 1993; Isoe *et al*., 1995; Collins and Blackwell, 2000; Li *et al*., 2009; Oliva *et al*., 2014). A possible explanation for this is that mosquitoes choose to oviposit in dark containers as it indicates depth and therefore a lower threat of desiccation before juveniles develop. It might also indicate a higher concentration of organic matter providing nutrition (Williams, 1962; Hoel *et al*., 2011). Another suggestion is that the dark water mimics shading of the water body (Vezzani *et al*., 2005).

Visual cues seem to have some importance. Covering the adult cages with black plastic in the laboratory oviposition experiment significantly reduced oviposition in black dye containers, although oviposition remained significantly higher than in the control suggesting that either some light was leaking in or that other factors were in play.

Although the black dye was an oviposition attractant, it had a significant negative impact on the survival of mosquitoes through to adults. Adult mosquitoes still emerged from the dye-treated water but the breeding success of the female was almost halved by the low survivorship. The results of the breeding experiment were repeated with the same significant reduction in emergence in dyed water. Laboratory tests have found no evidence of acute toxicity of dyes to *Culex* larvae over 48 h that would explain this result. The poor survival of mosquitoes is therefore unlikely to be related to dye toxicity. It is also unrelated to the algal-killing property of the dye. If mosquitoes were in a natural environment where algae were a significant part of their diet, we might hypothesise that dye would impact survival by killing the algae. However, in this artificial system larvae were given a supply of food and no algae were present in either treatment.

It is known that mosquito larvae and pupae dive in the water column in response to threat, relying on visual or mechanical cues (Awasthi *et al*., 2012). This requires considerable amounts of energy and constant or deep diving is associated with increased mortality (Lucas and Romoseri, 2001). It is pure speculation to suggest that the dye changes the behaviour of *Cx. pipiens* but in fourth-instar *Anopheles gambiae* growing in murky water columns deep diving increased significantly compared to clear water columns (Tuno *et al*., 2004).

Monitoring of wild population numbers in dye treated and untreated artificial containers were undertaken in two habitat types. The greenhouse habitat represented one in full sun where undyed water would reflect light presenting a large contrast between water treatments and the woodland habitat would have potentially less of a contrast since there was a lower light level.

Habitat type was found to be far more important than dye in determining the number of larvae and pupae, with the darker woodland habitat producing significantly more pupae compared to those in the brighter greenhouse area and the greenhouse habitat producing significantly more larvae. There is little information on the impact of shade on British mosquitoes but this result agrees with that of Fischer & Schweigmann (2004), who found that seasonal patterns of abundance of *Cx. pipiens* in urban Argentina showed positive relationships with vegetation cover. A further study on container breeding mosquitoes in an Argentinian cemetery found that the numbers of both *Cx. pipiens* and *Aedes aegypti* immatures were higher in shaded containers than in containers in full sun (Vezzani *et al*., 2005; Vezzani and Albicocco, 2009). Clearly it is likely that temperatures were higher in Argentina and shaded, cooler, containers have higher adult mosquito production rates because of a negative effect of high temperatures (Vezzani *et al*., 2005). In our study the greenhouse habitat produced significantly more larvae, although this did not translate into more pupae, possibly indicating larval mortality.

The fact that pond dye treatment had no impact on wild mosquito numbers can be explained by two possibilities. One is a balancing of oviposition preference against survival. If more eggs are laid and yet fewer mosquitoes emerge because of the dye, the net effect could well be neutral in terms of numbers of mosquitoes produced by the habitat. The second is that although pond dye is an important factor for *Culex* female oviposition in artificial environments, there are many factors in play that will influence the success of mosquitoes in a natural habitat, including temperature and shading.

We undertook the wild population experiment using water butts rather than ponds for two reasons. The first was to limit the number of factors that might interfere with the experiment such as competing mosquito species and predators to allow us to determine whether dye was an important factor in mosquito breeding success in a more natural setting. The second is that water butts are an important urban habitat for *Cx. pipiens* mosquitoes and an estimated 60% of UK garden water butts are colonised (Townroe and Callaghan, 2014). Our results demonstrate that the dyes do influence both mosquito behaviour and survival but there is no evidence that this translates into a significant difference in mosquito numbers.

Populations of *Cx. pipiens* are expected to increase with future changes to the landscape and climate, and it has been suggested that towns and cities represent some of the highest risk areas for potential transmission of bird-related mosquito-borne disease (Snow and Medlock, 2006b). The ornithophagic habit of *Cx. pipiens* limits its potential as a bridge vector but seasonal abundance and other eco-behavioural characteristics predispose this species to serve as a potential enzootic vector of WNV, capable of maintaining cycles among bird populations, in the UK (Medlock and Leach, 2015). It is important to understand environmental factors that might impact on mosquito population success in urban habitats, particularly if these factors are anthropological in nature.

CHAPTER 3: The effect of pond dyes on oviposition and survival in wild UK *Culex* mosquitoes

This chapter was published as a paper on PLOS One:

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Declaration

The data of emergence study in field conditions was collected with company of Rebecca Gander, Oliver Abbey.

CHAPTER 3. The effect of pond dyes on oviposition and survival in wild UK *Culex* mosquitoes

3.1 Introduction

The last 15 years have seen an unprecedented change in the status of vector-borne disease in Europe as a result of multiple and complex environmental changes influencing mosquito populations (Becker *et al*., 2010; Reiter, 2008). There are many examples throughout history, in temperate and tropical countries worldwide, of how changes to human activities, e.g. deforestation, agricultural practices and urbanisation, alter the distribution, ecology or behaviour of a disease vector and create the environmental conditions conducive to disease transmission (Reiter, 2008). In the UK, changes to land use, climate change and human activities in adaption to that change, are likely to affect mosquito populations. This provides a compelling rationale to investigate how we impact mosquito ecology and behaviour, especially considering their potential as vectors of diseases. It is against this backdrop that I have been studying urban artificial containers (such as water butts) and small ponds which are ideal habitats for a number of mosquito species (Townroe and Callaghan, 2014).

In previous studies, I investigated mosquito populations in water butts in both urban and rural habitats (Townroe and Callaghan, 2014; Ortiz and Callaghan, 2017) where I found a marked difference in mosquito species composition and abundance, with *Culex pipiens* (*pipiens*) dominating urban habitats (Townroe and Callaghan, 2014). I concluded that the storage of water in domestic gardens was increasing urban populations of *Culex pipiens*, a potential vector of West Nile Virus (WNV). Water butts are not the only artificial water bodies in gardens; many gardens have ponds. In the UK, domestic gardens are estimated to contain 2.5-3.5 million ponds (Davies *et al*., 2009), forming important reservoirs for taxa and helping to sustain aquatic biodiversity (Hill and Wood, 2014). Where ponds are developed as wildlife refugia, the lack of voracious fish predators means that mosquitoes can reach high densities (Greig 2008). Understanding factors that will impact on mosquito numbers is important information in the bank for any future issues with mosquito control.

Despite their potential importance for wildlife, domestic ponds are often managed for aesthetic purposes and difficulties arise in maintaining normal ecosystem function whilst retaining desirable aesthetic qualities (Hunter and Hunter, 2008). An example is the current fashion for using pond dyes to improve reflection and reduce algal growth. Pond and lake dyes are a relatively new commercially available product, sold as an environmentally friendly way to stop the growth of algae through the disruption of photosynthesis (DyoFix, 2015c). They have proved to be popular at recent high profile garden shows such as Chelsea and Hampton Court. One such product on the market is produced by DyoFix who state that their pond dyes are a blend of European food approved colour dye. The mode of action explained by the manufacturer is that it acts as a light filter, stopping colours on the red end of the spectrum from penetrating the water. Since the plant pigment chlorophyll *a,* which is crucial to photosynthesis, absorbs red light at 662nm, the theory is that addition of a red dye filter will prevent red light from reaching algae below the surface of the water, thereby inhibiting photosynthesis.

The concept of using dyes to limit algal growth by surface inhibition has been around for many years with an early example being aniline dye (Buglewicz and Hergenrader, 1977). Whilst effective at reducing blue-green algal growth it was a particularly hazardous chemical and was never intended for practical use. Modern pond dyes, however, claim to be environmentally friendly with manufacturers stating that they can be used not only at a domestic level in residential ponds, but also have commercial application, being able to work on large bodies of water such as lakes. The manufacturers of pond dyes are confident that they are environmentally friendly since they meet European Food Additive regulations, although very little actual toxicity information is available (DyoFix, 2015c). A few studies exist that look at the impact of wavelength- blocking pond dyes on algal growth, with mixed results. One found no significant impact on phytoplankton growth, with no difference in chlorophyll a concentrations at the concentrations of dye used (Aquasure, 4ml/m3) (Boyd *et al.*, 1982) whilst another found no reduction in microalgae growth until dye was applied at a high concentration (Aquashade, 5ml/m3 (Spencer, 1984))

However there are even fewer studies investigating the use of the product on freshwater fauna, and no investigations into any secondary non-lethal effects the dye may have on organisms, with the exception of a PhD thesis (Tew, 2003) where a dye (Crystal Blue-Ocean) had no impact on catfish survival and yield. Incidentally, this study also failed to find any difference in algal growth between dye-treated and untreated water.

The use of pond dyes in domestic and ornamental gardens raises questions linked to mosquito oviposition, since coloured water can be an attractant (Ortiz and Callaghan, 2017). *Culex pipiens* is commonly found in UK gardens and is a potential vector of viruses including the West Nile Virus (Brustolin *et al.*, 2016; Rizzoli *et al.*, 2015). Whilst currently there is no evidence of disease transmission in the UK, any factors that significantly change the distribution and population of *Cx pipiens* could impact future risks of disease transmission. My previous work demonstrated that *Cx. pipiens* females prefer to lay eggs in black dye water compared with the control in the laboratory and semi-field conditions. It was also observed that survival of larvae through to adults was significantly reduced in dyed water, suggesting that there is some form of chronic toxicity (Ortiz and Callaghan, 2017). These results suggest that the dyes are in fact not as environmentally friendly as previously suggested. It also raises the possibility that pond dyes could attract mosquitoes to lay eggs in garden ponds. Studies have reported that *Culex* sp. females use water reflection, darkness, temperature, pheromones and kairomones as part of the cues to choose an oviposition site (Barbosa *et al*., 2007). There is also evidence that mosquito oviposition is influenced by water body or container colour, type and size (Oliva *et al*., 2014; Harrington *et al*., 2008; Collins and Blackwell, 2000; Torrisi and Hoback, 2013).

Blue pond and shadow lake dyes (red colour) are products similar to the black pond dye which blocks the red end of the visible light spectrum penetrating the water. These dyes were created to be more natural than the black colour pond when applied to the water and are less reflective (DyoFix, 2015a). Pond blue is the most popular dye used and the most economic although lake shadow is a popular product because it is a colourless dye in the water (DyoFix, 2015a).

My previous work demonstrated that Dyofix black dye was an attractant to gravid mosquitoes with a significant effect on survival (emergence) but no measurable impact on mosquito numbers in a semi-natural habitat (Townroe and Callaghan, 2014). The lack of an effect in a natural habitat was explained by a balance between higher oviposition but reduced survival in a black dye treated habitat. Based on this hypothesis, two further pond dyes were studied to determine whether the impact was one found generally or whether the impact of pond dyes on mosquito numbers varied depending on the dye.

3.2 Materials and Methods

3.2.1 Trapping wild gravid females

Wild gravid female *Culex* were collected using Reiter ovitraps (1987) modified by (Townroe and Callaghan, 2014) (Figure 3.1A). Attracted to the bait infusion, females are pulled into a duct connected to the collection chamber by use of a fan located in the upper portion of the trap. The fan is connected to a valve-regulated lead-acid battery that produces a negative air pressure inside the box, allowing for mosquito collection (Figure 3.1B). The ovitrap consisted of two parts; a lightweight upper portion (a modified toolbox containing a fan, battery and trap for the adults) and a lower portion (5 litre tray) which contained the attractant infusion. Infusions were prepared by fermenting 1 lb of freshly cut grass, 1 lb of hay, 5 g of brewer's yeast and 60 L of tap water. The mixture was fermented in an 80 L black waste bin (44.5cm x 58.5 cm) outdoors for 7 days at the University of Reading. Prior to use the infusion was filtrated using a metallic ring that at the bottom presents a net to remove the grass and the hay.

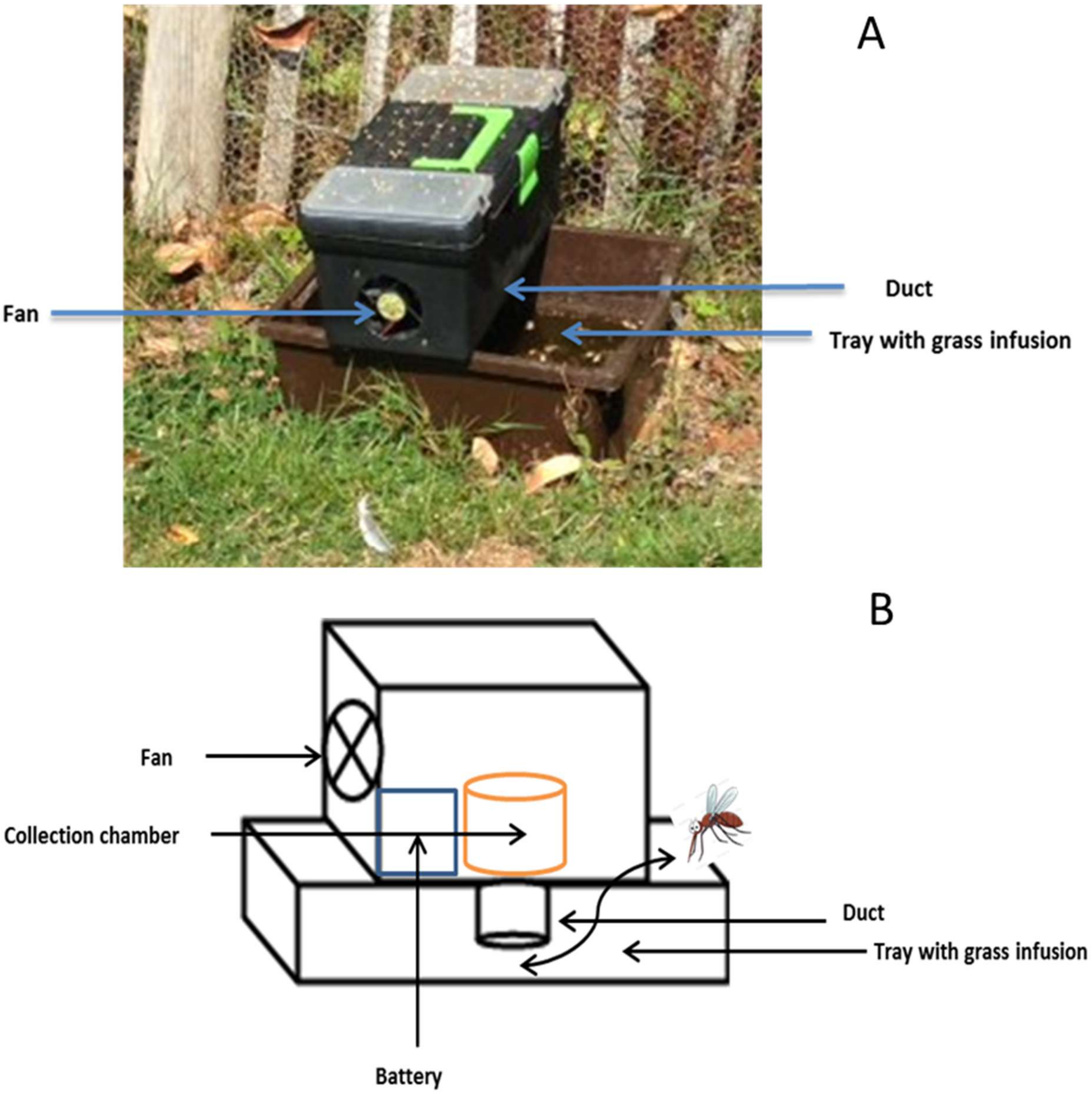

Figure 3.1. **A** modified emergence trap (Townroe and Callaghan, 2014) based on Reiter's gravid box trap design. **A**. A picture of the ovitrap. **B**. diagram of the ovitrap used during the experiments.

In total, 10 traps were used for this study, placed in the glasshouse area of the Whiteknights campus of the University of Reading, Berkshire, England (51.4419° N, 0.9456° W). Gravid female mosquitoes were collected in summer (July to August) in 2014 and 2015. Approximately 1000 gravid female mosquitoes were collected through the sampling period in 2014 and 2015. Most of the mosquitoes sampled belong to the *Culex* genus. However, a few numbers of *Anopheles plumbeus* (<5) and *Culiseta annulata* (<5) were also present throughout the trapping period.

3.2.2 Pond dyes

Two pond dyes (SGP Blue and SGP Shadow (DyoFix)) were used in this study, supplied as odourless solutions. Pond Blue has a pH of 5-6 at 10g/L water and a rat oral LD50 of 2g/Kg and fish LD50 of >100mg/L, and Lake Shadow has a pH of 7-8 at 10g/L water, a rat oral LD50 of 2g/Kg and fish LC50 of >100mg/L (DyoFix, 2015b).

3.2.3 Oviposition preferences of wild mosquitoes

A choice experiment was performed between July to September 2014 and again in 2015. A tent (245 x 145 x 95 cm) was placed adjacent to a wooded area in the same location as the gravid traps. Approximately, 200 gravid mosquitoes were collected from the modified traps and transferred into the tent 24 h post collection. Adult females were provided with a 10% sucrose solution. The tent contained 14 2 L plastic containers (14 length x 21width x 10 height cm): 7 with 1.5 L tap water and 7 with 1.5 L water treated with either blue or shadow pond dye (DyoFix, 2015c). A randomised block design was used to remove edge effects. After seven days, the containers were taken to the laboratory to count egg batches laid in each container. The experiment was repeated two times and with two types of dye: blue and shadow. Treatments were tap water, 1.5 µl of blue or 1.5 µl of shadow Dyofix pond dye/ 1.5L.

The choice experiment was repeated with wild-caught gravid females in laboratory conditions (25 ± 2°C) and normal light/dark photoperiod (16:8 h). Twenty gravid females were chosen randomly and transferred into each of 5 cotton net cages 30 x 30 x 30 cm per treatment set. Each cage contained a 10% sucrose solution and two 200 ml transparent plastic cups (12 x 5 cm) filled with 150 ml of either tap water or dye water (10 µ1 blue or 10 µl shadow). The plastic cups in each cage were rotated 90° daily to eliminate positional effect and collection of eggs was begun the day after the experiment set up. Eggs were removed from the plastic cups in each cage daily to eliminate oviposition effect. Treatments were tap water or blue and shadow DyoFix pond dye. 10 µl of blue or 10 µl of shadow were dissolved in 1L of tap water and then transferred to the 5 200 ml plastic cups. The choice experiments were repeated as above but in the absence of light; black bags were used as a cover in each cage during the experiment.

3.2.4 Emergence study

A modified emergence trap (Hamer *et al*., 2011) was used to measure the impact dye had on survival (Figure 2). Traps were made from lidded 11 litre cylindrical plastic bins (23 x 28 cm). The surface of each lid was removed, keeping the peripheral edges (ring) connected to the bin. Four holes were punched on each ring where two metallic cables were glued to create a conical structure. The conical structure was covered with a white net with an opening in the apex to remove adults. Each bin (9 replicates per treatment) was filled with 10 litres of tap water; a hundred wild larvae (above 2nd instar) and 1.2 g of guinea pig food (3mm pellets). The wild larvae were obtained from egg batches collected from wild mosquito females. Treatments were tap water, 10 µl of blue or 10 µl of shadow of liquid Dyofix pond dye, dyes colour were applied according to the manufacturer's instruction (DyoFix, 2015b). The bins were placed in 9 sites at the glasshouse area (51°26'13.2" N; 0°56'31.2" W).

Traps were monitored daily for adult mosquitoes. These were captured using a manual aspirator and transferred into small plastic tubes. The emergence experiment was repeated twice. The first experiment was performed on 27th August - 8 th September (Summer) and the second experiment was set up on 29th September - 16 th November 2015 (Autumn).

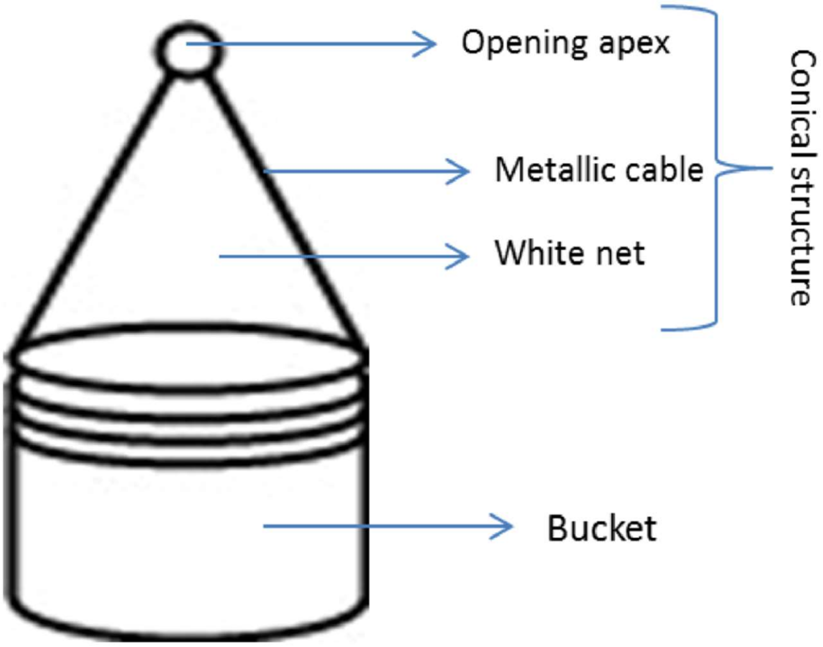

Figure 3. 2. A modified emergence trap.

3.2.5 Natural colonisation of containers with or without dyes.

Eighteen 10 L bins (26 cm x 26 cm) were filled with 8 L of water untreated or 8 L of tap water and shadow dye and 4 g of oak leaves collected from the Harris Garden at the University of Reading. For the dye treatment, 8 µl of dye was added to each container. The bins were placed in pairs in the experimental grounds of the School of Agriculture at the University of Reading (51.4419°N, 0.9456°W).

Containers were sampled weekly from August 11th 2014 until 12th November 2014. Sampling was carried out using an aquarium fish net (6 x 12 cm; 1 mm pore-net). The net was dropped into each container and moved in circles from the top to the bottom for 10 seconds before removal. The larvae and pupae collected were transferred to a tray where they were counted; the larvae were returned to the container. Pupae sampled with the net were placed in plastic tubes using plastic pipettes and taken to the laboratory to complete their development to adults. In addition to the net sampling, a visual search for pupae was performed to remove all pupae in each container.

3.2.6 Statistical analysis

All statistical analyses were performed using R version 3.2.2 (R Development Core, 2015). Oviposition preferences of wild and laboratory strain mosquito data were tested for normality using a Shapiro Wilk normality test. The oviposition choice experiment data in laboratory conditions were normally distributed, parametric statistics were used and a paired t-test was performed. Differences in the choice experiment in normal conditions (16:8 light:dark) and also in the absence of light, one-way analysis of variance (ANOVA) was used. Oviposition in the tent was not normally distributed and a nonparametric Mann Whitney U-test was performed. Differences in adult emergence between treatments were analysed using a generalised linear model using a binomial test. A Tukey post hoc test using package multcomp (Hothorn *et al.,* 2008) was used to compare the interaction between treatments. Abundance data in water butts were transformed Log (x+1) and the relationship between mosquito abundance, treatment and location were analysed using ANOVA using a package Linear, generalized linear, and nonlinear mixed models (lme4) (Bates *et al.,* 2014).

3.3 Results

3.3.1 Oviposition selection in a tent

The total number of egg batches laid by the ovipositing female mosquitoes were 593; 386 in (65.1%) treated treatments and 207 in (34.9%) in untreated treatment. Despite the difference in numbers, statistical analysis found no preference for either the blue or shadow dye compared to the water untreated (Shadow W = 126.5; *P* = 0.185 (Figure 3.3 A); Blue W = 135; P = 0.093 (Figure 3.3 B).

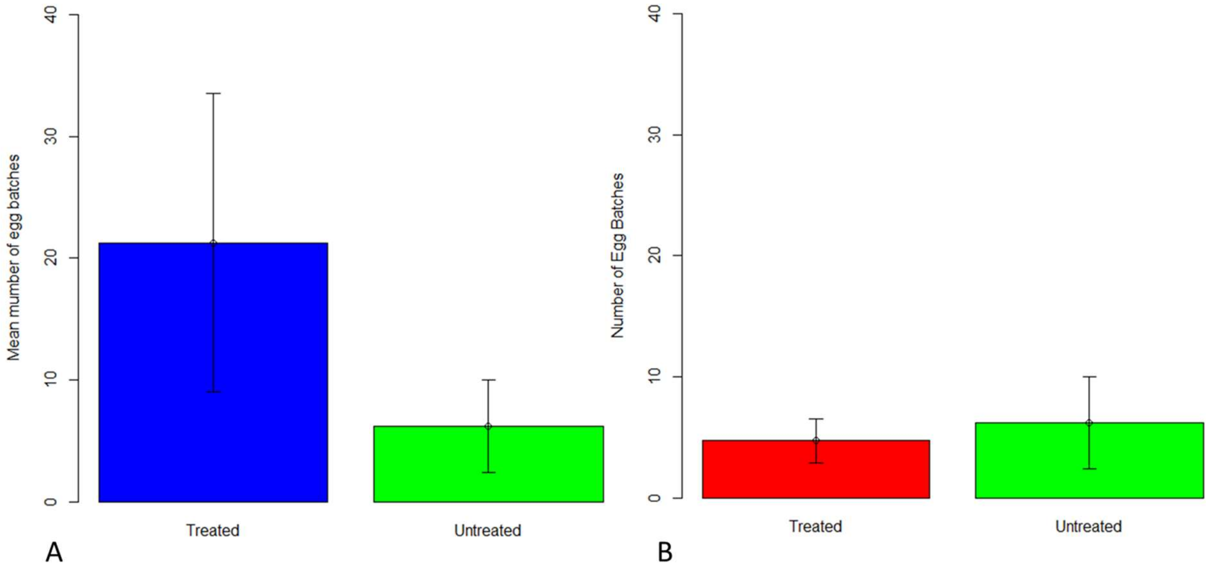

Figure 3. 3. Mean number of egg batches (±SE) laid by *Culex* spp. in semi-field conditions. **A.** Differences between blue colour dye and tap water. **B**. Differences between shadow colour dye and tap water.

Culex spp. showed no preference for oviposition in blue or shadow colour dye in normal light/dark (blue t = -1.776; df = 8; *P* = 0.114; shadow t = -0.919; df = 8; *P* = 0.385) or in darkened condition (blue t = 0.219; df = 8; *P* = 0.832 (Figure 3.4 A); shadow t = -0.888; df = 8; *P* = 0.400 (Figure 3.4 B)).

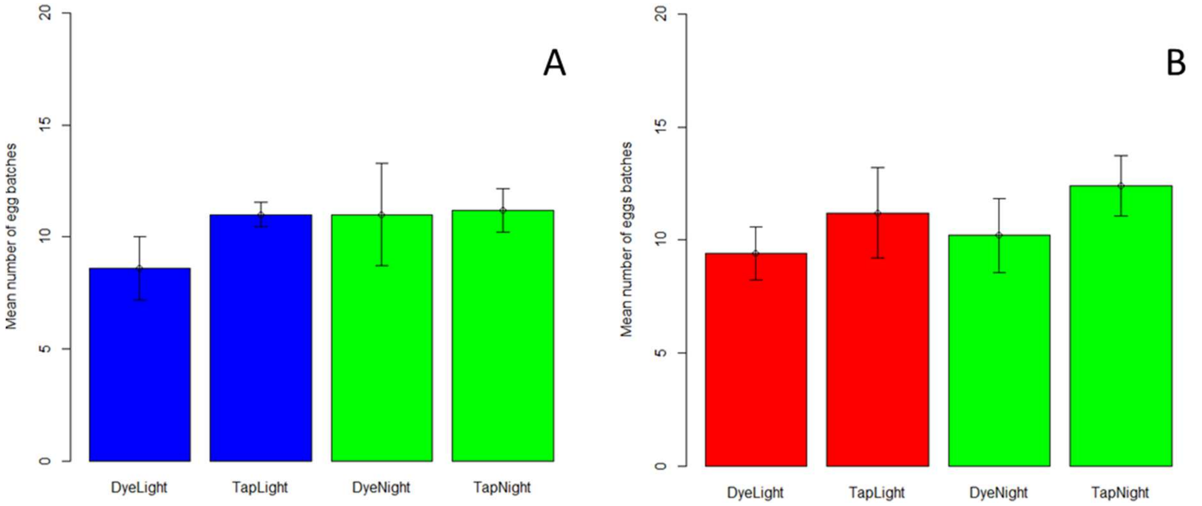

Figure 3.4. Mean number of eggs batches (±SE) laid by *Culex* wild mosquito in temperature control room in normal and darkened conditions. **A.** blue colour dye and tap water. **B**. shadow colour dye and tap water.

3.3.2 Emergence study: field conditions

The total number of adults emerging from different treatments was not significant in the summer (Z = -1.259, *P* = 0.208) but varied significantly in the autumn (Z = -4.049, *P* < 0.001) (Figure 3.5). A Tukey post-hoc analysis found significant differences between the shadow dye and untreated bins and between the blue dye and untreated bins in the autumn season (Table 3.1).

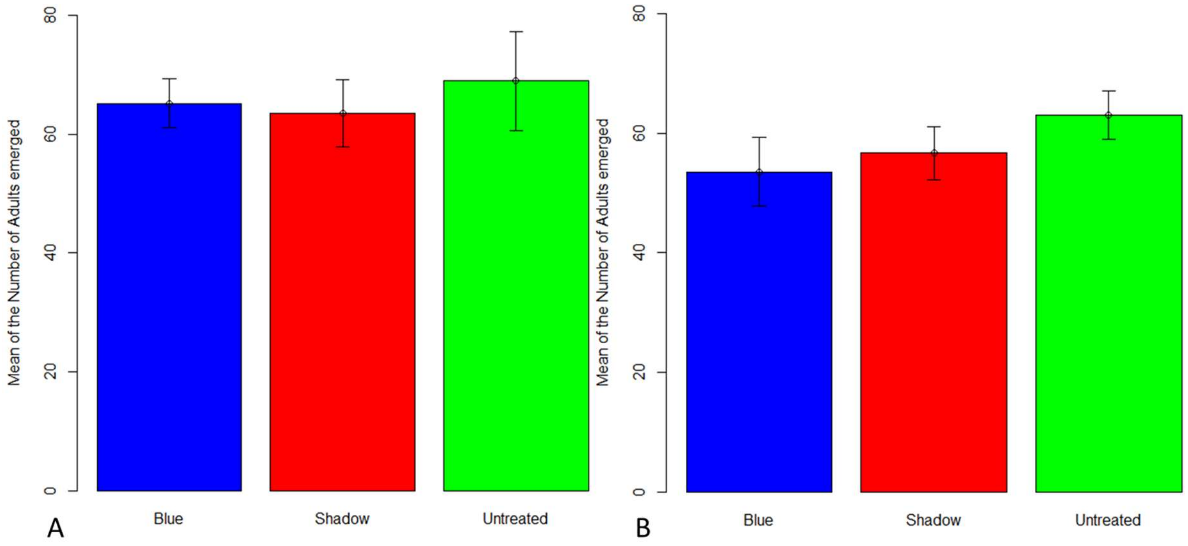

Figure 3. 5. Mean number (±SE) of adults emerged from the three treatments (tap, blue and shadow) in **A**. summer and **B**. autumn.

Table 3.1. Tukey post doc tests comparing adult emergence from the three treatments (tap, blue and shadow) in summer and autumn.

Interaction	**Summer**		**Autumn**	
	Z	***P***	**Z**	***P***
Blue-Shadow	0.738	0.741	-1.327	0.38
Blue-Tap	-1.262	0.417	-4.056	<0.001*
Shadow-Tap	-2.001	0.112	-2.738	0.017*

3.3.3 Natural colonisation of containers

The only species recorded breeding in the small experimental bins was *Culex pipiens (sensu lato)*. Larval and pupal numbers were analysed by season; summer (August-Middle of September) and autumn (Middle to September to October). No significant differences were observed in overall larval and pupal densities between treatments (larval $F_{1, 32} = 0.318$, $P = 0.576$; pupal $F_{1, 33} = 2.611$ $P = 0.116$) (Figure 3.6). However, untreated bins showed overall higher pupal densities in summer season compared with treated bins ($F_{1, 16} = 6.317$, $P = 0.023$) (Figure 3.7A). This was because pupal density varied significantly in weeks 5, 6 and 7 between treated and untreated bins ($F_{1,16} = 5.254$, $P = 0.036$). The total number of larvae varied significantly between seasons, with higher numbers in the summer ($F_{1, 32} = 14.528$, $P < 0.001$) (Figure 3.7B). However, pupal abundance did not vary between seasons ($F_{1 ,32} = 1.861$, $P = 0.172$).

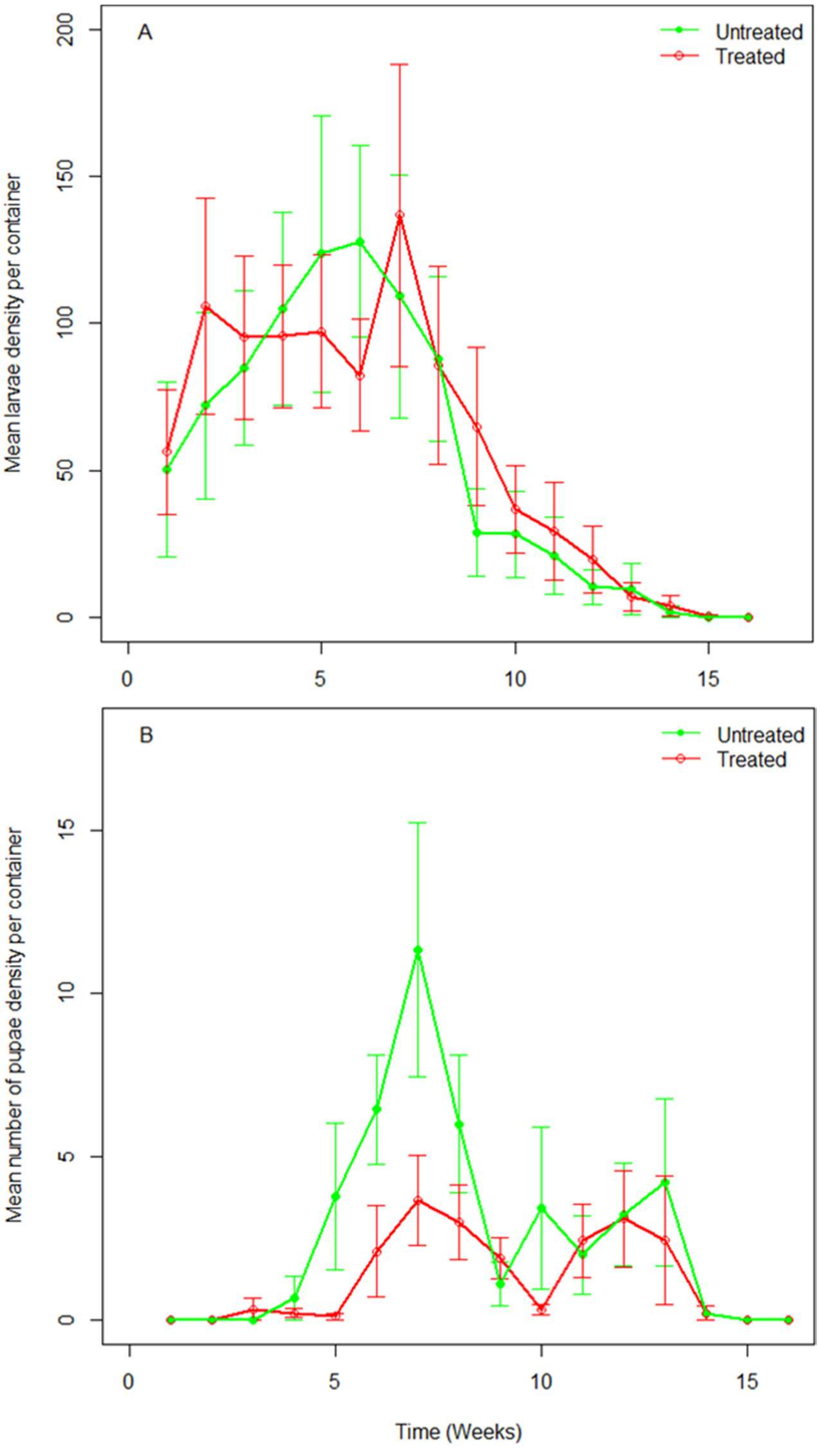

Figure 3. 6. Mean (±SE) *Culex pipiens* abundance in untreated and shadow dye-treated small bins across the sampling period. **A**. larvae. **B.** pupae.

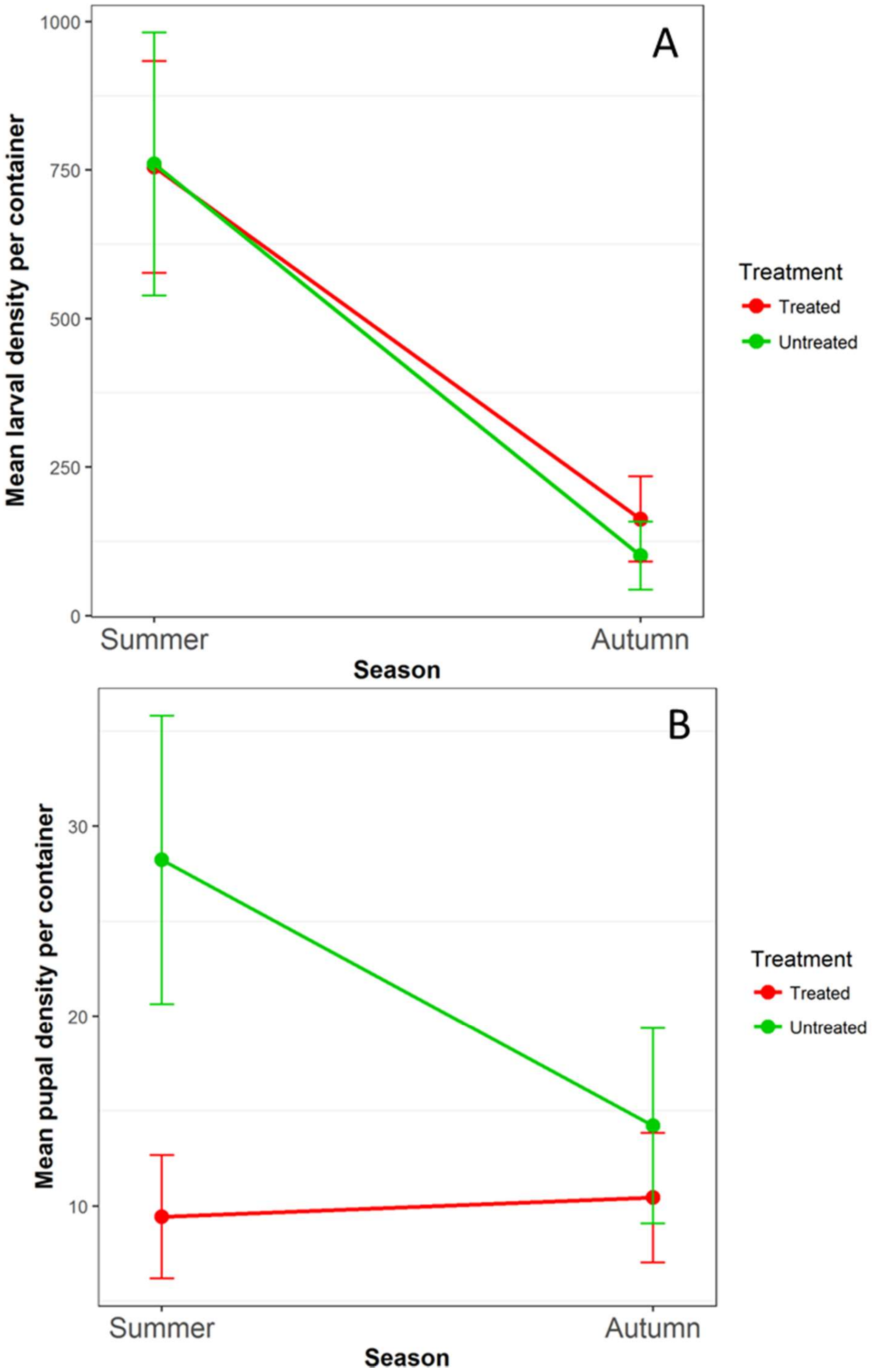

Figure 3. 7. Mean (±SE) abundance between untreated and treated small bins. **A**. larvae and **B**. pupae mosquito density.

3.4 Discussion

We previously demonstrated that gravid female *Culex* mosquitoes preferred to lay eggs in black dyed water (Ortiz and Callaghan, 2017). However, we show here that blue and shadow (colourless) pond dyes had no effect at all on oviposition in either laboratory tests or in the semi-field study in the tent. Given these results, it would seem that the black dye colour does have more attractive or stimulant properties than either the blue or shadow dyes. Possible explanations for these results are that mosquitoes choose to oviposit in black water because i) it indicates depth and therefore a lower threat of desiccation before juveniles develop, ii) it might indicate a higher concentration of organic matter providing nutrition (Hoel *et al.*, 2011; Williams, 1962), iii) it mimics shading of the water body (Vezzani *et al.*, 2005) and iv) black dyed water holds heat longer than undyed and mosquitoes may be able to visually sense near-infrared radiation (700 to >900 nm) (Hoel *et al.*, 2011).

Although there was no sustained effect on egg laying, blue and shadow dyes had an impact on *Culex* sp mosquito survival. For both dye treatments, significantly fewer adults emerged from containers that had been placed outside and covered to prevent colonisation by other mosquitoes or macroinvertebrates. These results are similar to those previously reported by Ortiz and Callaghan (2017) (using an identical experimental design) where the number of adults emerging from a black dye treatment were significantly lower than the control. This was not easily explained since a toxicological assay found no significant larval mortality following exposure to any of the dyes, at various concentrations over a 48 hour period. If the experiment had been in a treated natural pond full of algae, and if that algal population was affected by the treatment, then an explanation of the results might be a reduction in the availability of food, since algae form a significant proportion of the larval diet. However, mosquito larvae are not discriminatory and their diet will consist of detritus and microorganisms as well as algae (Clements, 1992). In this particular experiment, tap water was used with guinea pig food (and, potentially, resulting microorganisms) for larvae to eat. Therefore algae should not have been a limiting factor.

My previous work on the effect of black pond dye failed to detect any measurable impact on mosquito abundance when the experiment was conducted in a naturally colonised container rather than under controlled conditions (Ortiz and Callaghan, 2017), despite a very strongly significant impact on survival and oviposition under controlled conditions. However, in contrast, the shadow dye treatment had a significantly negative impact on pupal abundance in a naturally colonised container in late summer. The containers all had high abundances of *Culex pipiens* mosquitoes which generates competition and can have a significant impact on the development rate (Reiskind and Zarrabi, 2012; Costa *et al*., 2010; Couret *et al*., 2014) and survival of conspecific mosquitoes (Marinho *et al*., 2016; Harrington *et al*., 2008; Torrisi and Hoback, 2013). It is likely that, since these dyes are not oviposition attractants, a lack of difference between treatments (apart from the impact on pupae during one season) is more related to overcrowding and competition having a greater impact than the dye. The relationship between mean larval numbers converted into mean pupal numbers shows a very low survival rate (between 1% minimum and 7% maximum) compared to the emergence rates in the controlled experiment (around 60%).

Vision is a long-range cue used for oviposition site location by many mosquito species (Kennedy, 1942). A number of studies have looked at the behavioural ecology of oviposition choice, including colour, for a variety of mosquitoes of the genera *Aedes*, *Culex*, *Anopheles*, and *Toxorhynchites* (Dhileepan, 1997; Panigrahi *et al*., 2014; Hoel *et al*., 2011; Collins and Blackwell, 2000; Huang *et al*., 2007; Li *et al*., 2009; Oliva *et al*., 2014). Black, blue and red colours all seem to be attractive to species including *Aedes albopictus*, *Culex annulirostris, Aedes albopictus* and *Aedes aegypti* (Panigrahi *et al*., 2014; Hoel *et al*., 2011). However, many of these studies result from laboratory studies, which instructive, may not accurately reflect the cues that are used in the field. Whilst laboratory studies that measure the total number of mosquito eggs (or egg batches) laid in test versus control conditions can provide useful information on oviposition stimulants and repellents, these studies can say little about the impact of the chemicals on oviposition in nature. This has certainly been the case in our experimental research.

It has been suggested that the terminology used in laboratory and field studies should be clarified, so that oviposition attractants or repellents are terms used when mosquitoes are using long-to middle-range cues resulting in a reorientation of flight direction (Day, 2016). In the case of short range or contact cues, such as those used in laboratory studies, Day (2016) suggests that the term stimulant or deterrent would be more accurate.

Conclusion

These results show that pond dyes have an impact on mosquito behaviour and survival. Although the blue and shadow dyes had no impact on oviposition (unlike black dye (Ortiz and Callaghan, 2017)), the emergence of adults in dyed water was significantly impacted. These results do imply that the dye is in some way toxic to the mosquitoes over a long period of time, although it is not clear what is happening.

Populations of mosquitoes are likely to change as landscape and climate changes, and it has been suggested that towns and cities represent some of the highest risk areas for potential transmission of bird-related mosquito-borne diseases (Snow and Medlock, 2006a). The ornithophagic habit of *Cx. pipiens* limits its potential as a bridge vector but seasonal abundance and other eco-behavioural characteristics predispose this species to serve as a potential enzootic vector of WNV, capable of maintaining cycles among bird populations, in the UK (Medlock *et al.*, 2005b). It is important to understand environmental factors that might impact on mosquito population success in urban habitats, particularly if these factors are anthropological in nature. The results presented here and in our previous work show that dyes are not totally neutral and can reduce fecundity as well as act as attractants (Ortiz and Callaghan, 2017). Mosquito larvae are normally one member of a freshwater ecosystem that includes other macroinvertebrates. We know that these interact with each other and so the next stage will be to look at mosquito populations in dyed ponds containing whole communities.

CHAPTER 4. The effect of pond dyes on macroinvertebrate communities in freshwater ponds.

Declaration

The data in this chapter of macroinvertebrates recorded in freshwater ponds once a week were collected by student, Sophie Connor. All other work in this chapter is the work of Natali Ortiz.

CHAPTER 4. The effect of pond dyes on macroinvertebrate communities in freshwater ponds.

4.1 Introduction

Freshwater ecosystems are one of the most endangered ecosystems in the world (Dudgeon *et al.*, 2006; Sala *et al.*, 2000), supporting around 6% of all described species (Hawksworth and Kalin-Arroyo, 1995). Ponds are widely recognised as supporting greater regional invertebrate diversity than most other freshwater ecosystems in the UK and across Europe (Davies *et al.*, 2009; Williams *et al.*, 2003). They represent a hotspot for biodiversity and possess a disproportionately high level of biological richness and endemism (Combes, 2003; Dudgeon *et al.*, 2006; Gledhill *et al.*, 2008; Ormerod *et al.*, 2010; Strayer and Dudgeon, 2010; Sayer *et al.*, 2012; Collen *et al.*, 2014; Penaluna *et al.*, 2016; Schmidt-Kloiber *et al.*, 2016). Ponds also provide freshwater species with a network of distributed, discrete habitat patches (“pondscapes”) that allow them to disperse across landscapes (Pereira *et al.*, 2011; Hassall, 2014). This network also enables species to migrate in response to climate change e.g. to shift a distribution northward (Céréghino *et al.*, 2014), offering a refugia for highly mobile adults such as Odonata.

Urban ponds tend to be anthropogenic in nature and traditionally have been considered as providing insignificant biodiversity to the regional species pool compared to larger habitats such as lakes and rivers (Céréghino *et al.*, 2008). Whilst it is true that the average garden pond may only contain relatively few species, as a pondscape they can provide a variety of abiotic and biotic conditions. This in turn creates a network of diversity in ecological communities and contributes to landscape-level biodiversity, often exceeding the biodiversity of larger more homogenous wetlands (Hassall, 2014). Some studies suggest that urban ponds differ markedly from those of non-urban ponds, supporting high taxonomic richness and contributing to local faunal diversity (Wood *et al*., 2003; Hill and Wood, 2014). A recent UK wide study of urban vs non-urban ponds found similar alpha diversity (i.e. diversity of each pond) of aquatic macro-invertebrates at a family and species level and similar gamma diversity (the diversity of the landscape) at family level, although non-urban ponds recorded higher estimated gamma diversity on at species scale (Hill *et al*., 2017). However biological communities of urban ponds exceeded those from non-urban ponds (Hill *et al*., 2017).

It is estimated that across the UK the number of ponds in domestic gardens is between 2.5-3.5 million (Davies *et al*., 2009). Management of these ponds is therefore vital if urban ponds are to play a role in conserving the diversity of macroinvertebrates. Unfortunately, domestic gardens are often managed for aesthetic purposes and difficulties arise in maintaining normal ecosystem function whilst retaining desirable aesthetic qualities (Hunter and Hunter, 2008).

Algae are a natural component of ponds, providing food to zooplankton, the primary consumers in the community (Lukešová and Frouz, 2007). However, overgrowth of algae, which in garden ponds is sometimes caused by too much nutrient in the water (often from tap water), leads to algal blooms with gardeners acting to remove or otherwise control the primary producers. Algal blooms have previously been removed through a variety of methods depending on the size of the pond, including dredging, ultrasound, chemical agents such as copper algaecides and biological control (Wang *et al*., 2012). Dyes have also been used previously to limit algal growth by inhibiting light penetrating the water surface. An early example was the use of aniline dye to test the concept of reducing light entering a pond (it was never intended to be applied outside the experimental plot) (Buglewicz and Hergenrader, 1977). While effective at reducing blue-green algal growth and totally eliminating aquatic macrophytes, it was a particularly hazardous chemical which has since been banned.

Manufacturers of modern pond dyes claim that they are environmentally friendly because they are non-toxic, meeting European Food Additive regulations (DyoFix, 2015c). They are available for use in residential ponds but also commercially on large bodies of water such as lakes and have been used by the National Trust and advocated on gardening TV programmes. These dyes act as red light filters to restrict photosynthesis, blocking peak absorption by chlorophyll at 650nm (Simis *et al*., 2012). However, there are no investigations into any secondary effects dyes may have on organisms and no long-term studies investigating the use of the products in freshwater ecosystems. Using freshwater experimental ponds, this study examines the effects of the DyoFix black pond dye on individual taxa and overall species community in newly established ponds. As products enter the market claiming to be environmentally friendly whilst improving pond aesthetics, it is important to evaluate these claims to prevent future mismanagement of such important ecosystems.

4.2 Materials and Methods

4.2.1 Study site and ponds

Thirty two ponds were dug in experimental grounds at the University of Reading, Berkshire, England (51.4419°N, 0.9456°W) in 2012 (Figure 4.1). Eight of these ponds were randomly selected for use in this 2015 study (Figure 4.2). Each pond consisted of a sunken bucket lined with pond liner, (diameter 48 cm depth 30 cm). The ponds had been colonised by macroinvertebrates in the three previous years. These were all removed by passing the pond water through a sieve (dimensions 6 x 12 cm; 250 µm pore size) and placing contents onto a white plastic sampling tray (25 x 35 x 5 cm) with some water. Based on high abundance, five species were reintroduced in the same proportion into each pond which contained approximately 20 L of water: 20 lesser water boatman (*Corixa punctata*), 15 non-biting midge larvae (*Chironomus plumosus*), 10 mosquito larvae (*Culex pipiens*), 3 dragonfly larvae (Anisoptera sp.) and 500 *Daphnia pulex*.

Figure 4. 1. Location of the 32 ponds dug in experimental grounds at the University of Reading. The map was taken from Digimap: http://digimap.edina.ac.uk.

Figure 4. 2. Ponds in experimental grounds at the University of Reading.

4.2.2 Pond treatment.

Two treatments were applied to the ponds: 4 ponds were treated with black pond dye and 4 were used as a control (Figure 4.3). For each treatment, on 29 th of June 2015, 100 g dye per 2000ml of water was applied according to the manufacturer's instruction (DyoFix, 2015c). Sampling began on 6th of July 2015 and was performed weekly until 9th of November 2015. On 11th of September 2015, halfway through the sampling period, the black colour dye was reapplied on the recommendation of the company; 15% of the original amount was re-added as per the manufacturer's instruction.

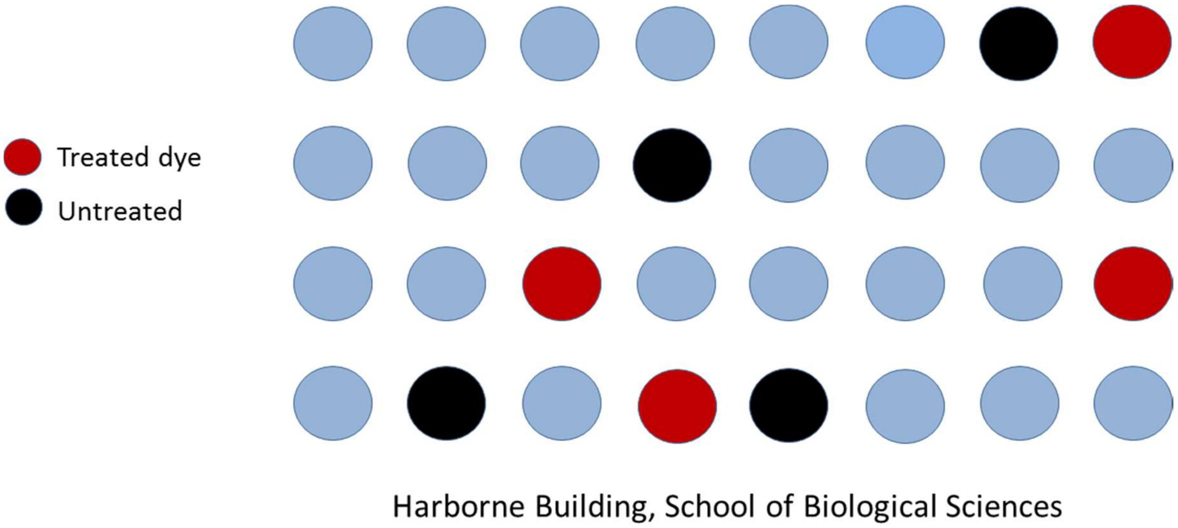

Figure 4. 3. Diagram of the control ponds and with dye treatment. Ponds coloured with red denote those with dye colour and the black colour are the control ponds.

4.2.3 Sampling and identification

Each pond was sampled using a 6 x 12 cm aquarium fish net. Sampling involved moving the net in an S shape through the water column. Organisms collected were placed into white sampling trays (25 x 35 x 5 cm) with 500 ml of water from each pond and were taken to the laboratory to be identified and counted. Specimens were counted under a stereomicroscope (x10 magnification) and identified using the key of Croft (1986), Cranston *et al*.,(1987), Snow (1990), and Greenhalgh and Ovenden (2007). The majority of specimens were identified to species or genus level with the exception of beetle and meniscus larvae which were identified to family level. All individuals were returned to their pond to prevent skewing of the data from unrealistic species loss.

4.2.4 Statistical analysis

The effects of pond dye application on pond macroinvertebrate communities were explored using Redundancy Analysis (RDA) in Canoco 5 (Šmilauer and Lepš, 2014). A pooled record of all the macroinvertebrates recorded throughout the whole sampling period was used to test the effects of pond dye treatment whilst also taking into account the effect of season (summer/autumn). Seasonality was taken account, from the first week of sampling until the second dye application (11th September) was taken as summer season and from 18th September until November was autumn.

Weekly analyses were also performed to identify how the community composition within treated (Collen *et al*., 2014) and control (no dye) ponds changed over time. Species data (response variables) were log(x+1) transformed and the significance of the explanatory variables was determined by $p < 0.05$ based on 999 Monte Carlo permutations in all cases. Log (x+1) was used as zero values were present in the data collection during the weeks.

Results were presented as bi-plots with the explanatory variables (pond dye treatment and season) represented as symbols and the response of macroinvertebrates represented by arrows. The length of an arrow is a measure of fit for that species correlation with the ordination axes (i.e., arrows that point directly towards a treatment symbol correlated most strongly with that treatment).

All statistical analyses were performed using R version 3.4.0 (R Development Core, 2016). The Shannon-Wiener Diversity Index infer all species are represented in a sample which they are randomly sampled between different habitats (Ortiz-Burgos, 2016) and Evenness how individuals are distributed over different species in a community (Heip *et al*., 1998; Molinari, 1989). Shannon-Wiener Diversity Index and evenness value and individual abundance in ponds were modelled through a generalised linear model with a negative binomial distribution using package MASS (Ripley 2015).

4.3 Results

4.3.1 Effects on biodiversity

4.3.1.1 *Biodiversity and evenness index*

The Shannon-Wiener diversity index was significantly lower in untreated than in treated ponds (Figure 4.4A). From July to September there was no significant variation between treatments, but there was a significant between treated and untreated ponds in autumn season (Table 4.1). The evenness value produced from the Shannon-Weiner biodiversity index was higher in treated ponds compared with untreated treatments but this was not significant (Table 4.1) (Figure 4.4B).

Table 4. 1 Summary of the analysis of Shannon-Wiener diversity index and evenness index collected in the ponds. *All sampling period (5 months).

	Season	**Estimated**	**Std. Error**	**z Value**	***P***
Shannon-Wiener diversity index	*	0.4040	0.1812	2.230	***P*= 0.026**
	Summer	0.1468	0.2502	0.587	*P*= 0.557
	Autumn	0.6821	0.2779	2.455	***P* = 0.014**
Evenness index	*	0.2081	0.6852	0.304	*P*= 0.761
	Summer	-0.1123	1.0126	-0.111	*P*= 0.912
	Autumn	-0.244	0.3896	-0.626	*P*= 0.531

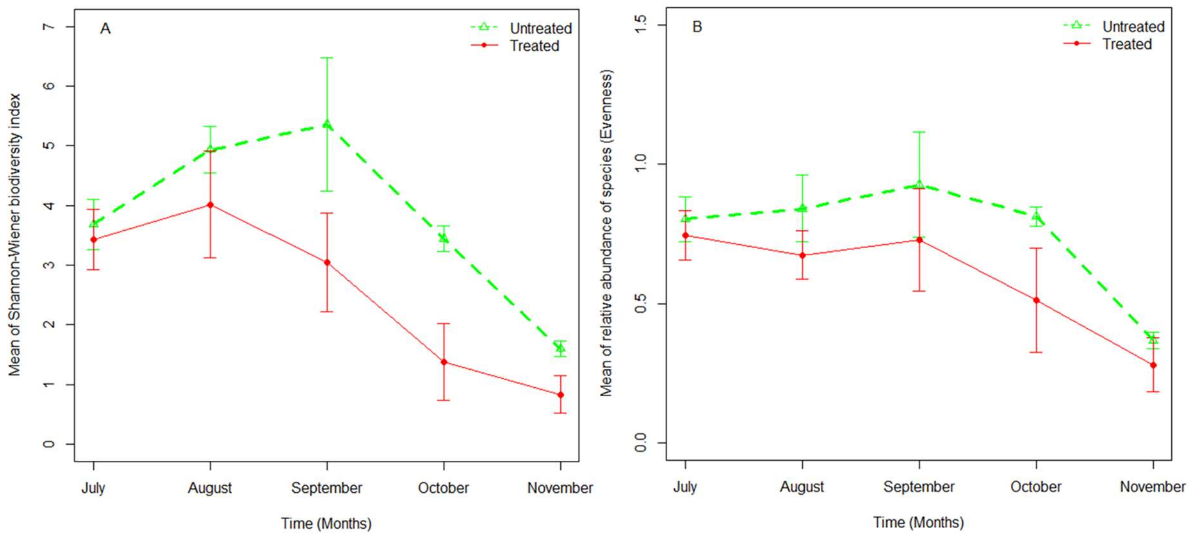

Figure 4. 4. A. Mean number of Shannon-Wiener index (±SE) and **B** average of species evenness (±SE) for no dye and dye ponds.

4.3.1.2 *Effects on individual species*

A total of 13 species were observed across all ponds for the whole collection period (Table 4.2). The most abundant species were not necessarily those added at the start of the experiment, demonstrating that the ponds were being colonised very rapidly.

Table 4. 2. Macroinvertebrate species found in the ponds with overall abundance (mean and standard deviation). ǂ indicates species added at the beginning of the experiment.

Common name	Species	Abundance Control	Abundance Dye
Predators			
Diving beetle	Dytiscidae	19.37±12.47	12.05±6.61
Dragonfly	Anisoptera ǂ	0.10±0.46	0.31±1.38
Water mite	*Hydrachna* sp	0.21±0.42	0
Herbivores/Detrivores			
Lesser waterboatman	*Corixa punctata* ǂ	21.00±14.99	4.68±3.67
Pond snail	*Lymnaea* sp.	50.42±67.22	12.84±12.76
Pond olive	*Cloeon dipterum*	148.89±93.26	204.05±86.50
Cyclops	*Cyclops* sp.	213.16±363.18	23.68±51.01
Mosquito	*Culex pipiens* ǂ	11.31±17.11	20.21±23.29
Non-biting midge	*Chironomus plumosus* ǂ	4.53±3.69	2.58±2.39
Meniscus midge	Dixidae	3.05±3.66	2.47±2.91
Water flea	*Daphnia pulex* ǂ	9642.16±3583.87	2957.90±1466.98
Omnivore			
Seed Shrimp	*Ostracoda* sp.	2.42±3.32	1.74±2.38

The abundance of *Daphnia pulex* and lesser water boatman (*Corixa punctata*) were significantly lower in treated ponds compared to the untreated ponds (Figure 4.5A and 4.5B) Differences were observed between treatments before (summer) and after (autumn) the reapplication of the dye in both species (Table 4.3). However, trends in *C. punctata* abundance presented a decrease three weeks after initial dye application and further reductions in abundance after the reapplication of dye (week 10), especially in the number of individuals in untreated ponds.

The negative binomial test found no statistically significant differences in pond olive (*Cloeon dipterum*) population between ponds treated with dye and control (Figure 4.5C) or in mayfly larvae abundance during the summer and autumn (Table 4.3). Although no general trend was found in abundance of mayfly during the sampling period, control ponds were observed to have large fluctuations in abundances whereas treated ponds remained largely stable. A significant effect was observed on coleopteran larvae population between untreated and treated ponds (Table 4.3). This was because after reapplication of the Dyofix dye resulted in a sharp decrease in abundance whereas there was no significant effect before the reapplication of the dye (Figure 4.5D).

Table 4. 3. Summary of the analysis of *Daphnia pulex*, *Corixa punctata*, *Cloeon dipterum* and coleopteran larvae collected in the ponds. *All sampling period (5 months).

Specie\Common name	**Season**	**Estimated**	**Std. Error**	**z Value**	***P***
Daphnia pulex	*	1.1817	0.2127	5.555	***P* < 0.001**
	Summer	-0.9782	0.2524	-3.875	***P* < 0.001**
	Autumn	-1.3858	0.2904	-4.773	***P* < 0.001**
Corixa punctata	*	-1.5003	0.3537	-4.241	***P* < 0.001**
	Summer	-1.2208	0.4368	-2.795	***P* < 0.005**
	Autumn	-1.8921	0.5022	-3.768	***P* < 0.001**
Cloeon dipterum	*	0.3151	0.2976	1.059	*P* = 0.29
	Summer	0.3371	0.5286	0.638	*P* =0.524
	Autumn	0.3051	0.3375	0.904	*P* =0.366
Coleopteran larvae	*	-0.4744	0.219	-0.2166	***P* = 0.030**
	Summer	-0.6647	0.3159	-2.104	***P* = 0.035**
	Autumn	-0.2712	0.2683	-1.011	*P*= 0.312

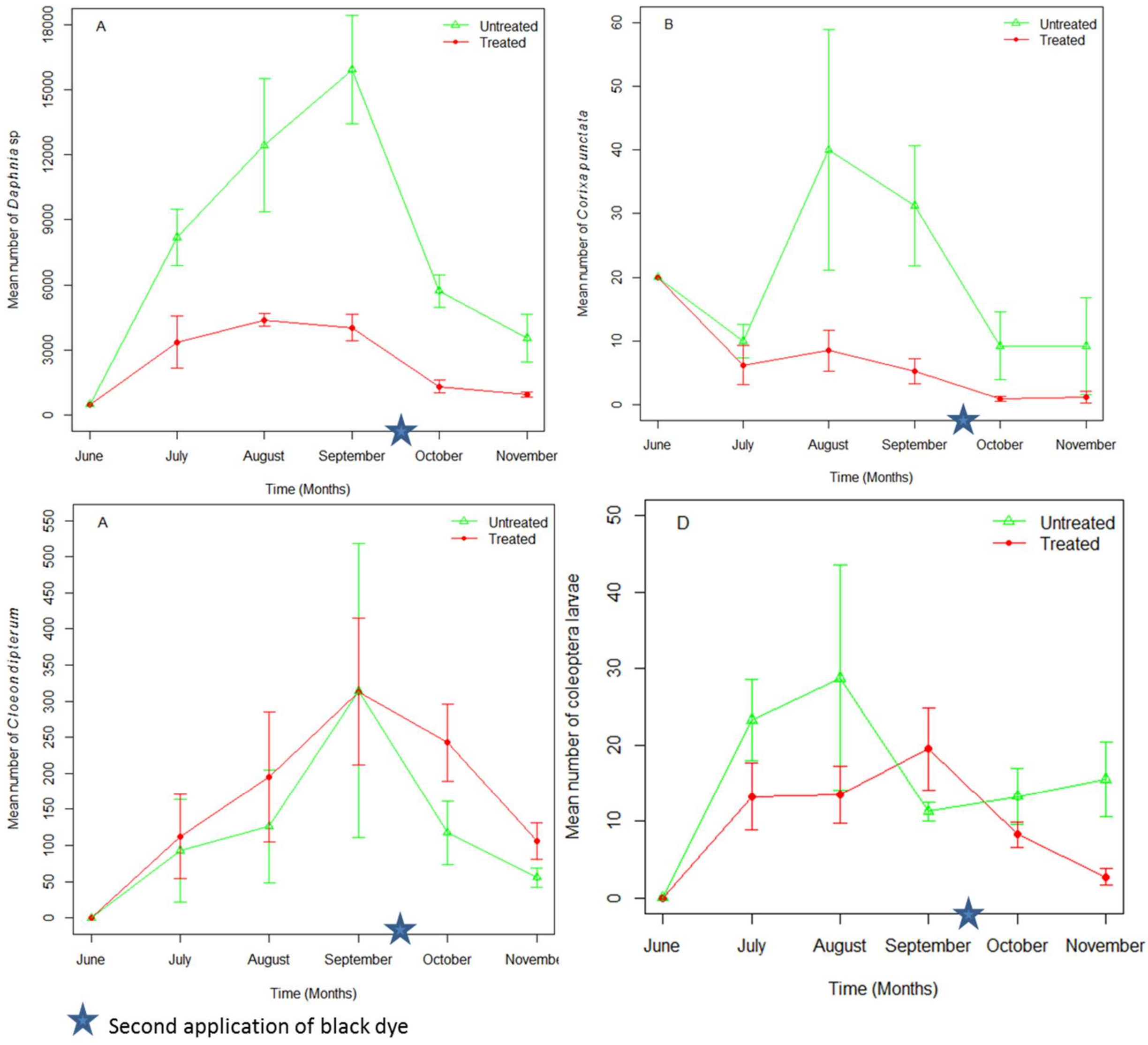

Figure 4. 5. Comparison of the mean populations ± SE for each treatment across the sampling period. **A**. *Daphnia pulex*. **B**. Lesser water boatman *(Corixa punctata)*. **C**. Pond olive *(Cloeon dipterum)*. **D**. Coleoptera larvae

The model showed that there was no statistically significant differences in mosquito larvae (*Culex pipiens*) abundance between ponds treated with black colour dye and control (Table 4.4). Both treatments appear to show similar trends in abundance over the sampling period, but it is evident that there is a higher, if not significant, abundance of mosquito larvae in treated ponds than control ponds (Figure 4.6A). Mosquito larval abundance started to decline after week 2 in treated ponds. No mosquito larvae were found after week 14 in either untreated and treated ponds (Figure 4.6A).

Pond snails were discovered in some of the ponds during week five of sampling. Pond snails were found in all four control ponds and persisted in two of these. They were also found in two treated ponds. The median abundance for treated ponds and control ponds were not significantly different (Table 4.4). Control ponds show low abundance from August when abundances begin to rise, with a peak in October (Figure 4.6B). Pond snail abundance was significantly reduced by dye treatment before (summer) and after (autumn) the reapplication of the dye (Table 4.4).

Table 4. 4. Summary of the analysis of *Culex pipiens* and pond snail collected in the ponds. *All sampling period (5 months).

Specie\Common name	Season	Estimated	Std. Error	z Value	*P*
Culex pipiens	*	0.58	0.6546	0.886	*P* = 0.376
	Summer	0.5356	0.5322	1.007	*P* = 0.314
	Autumn	1.0116	1.3047	0.775	*P* = 0.438
Lymnaea sp.	*	-1.3677	0.8155	-1.677	*P* = 0.093
	Summer	1.2287	0.2321	5.2941	***P* < 0.001**
	Autumn	-1.7519	0.776	-2.257	***P* = 0.024**

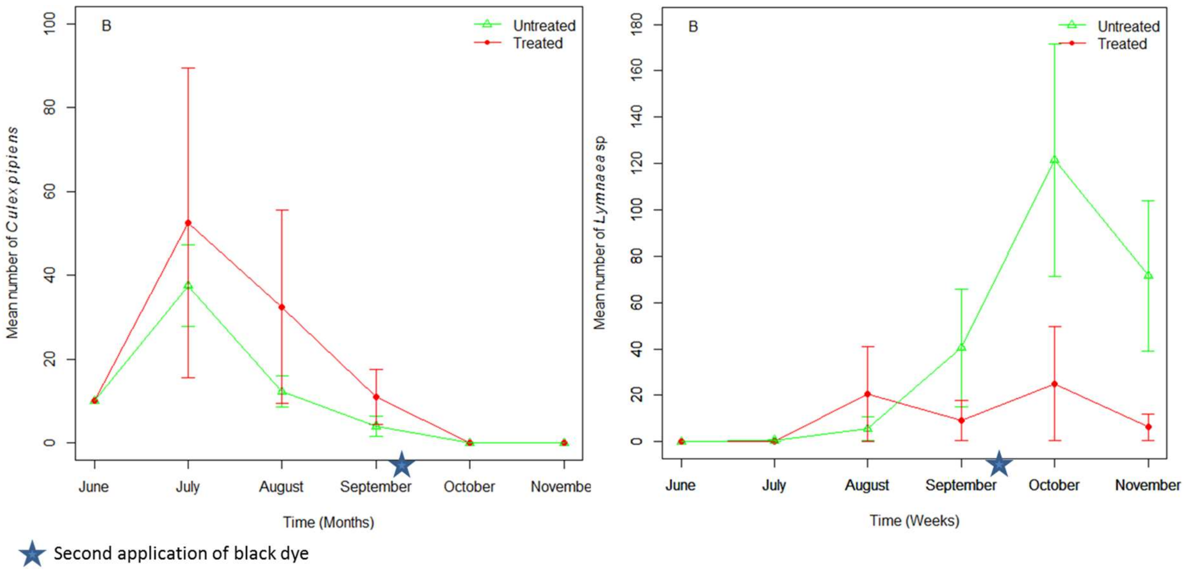

Figure 4. 6. Mean (x ±SE) of *Culex pipiens* larvae and pond snail (*Lymnaea* sp.) for each treatment between June and November. **A.** *Culex pipiens*. **B**. Pond snail (*Lymnaea* sp.)

Chironomus plumosus larvae were observed intermittently throughout the entire sampling period in both treatments (Figure 4.7A). Investigating differences between treatments revealed the mean abundance for treated ponds and control ponds were not significantly different. No differences were observed before (summer) or after (autumn) the second application of the dye colour pond (Table 4.5).

The abundance of *Cyclops* was higher in untreated ponds (Figure 4.7B). *Cyclops* abundance was significantly lower in treated ponds before September and following reapplication (Table 4.5)

Table 4. 5. Summary of the analysis of *Chironomus plumosus* and *Cyclops* sp.collected in the ponds. *All sampling period (5 months).

Specie\Common name	**Season**	**Estimated**	**Std. Error**	**z Value**	***P***
Chironomus plumosus	*	-0.9578	0.5076	-1.887	*P* = 0.059
	Summer	-0.07696	0.84825	-0.091	*P* = 0.928
	Autumn	-1.9981	0.5965	-3.35	***P* < 0.001**
Cyclops sp.	*	-2.19722	0.04969	-44.22	***P* < 0.001**
	Summer	-1.7346	0.06262	-27.7	***P* < 0.001**
	Autumn	-2.7515	1.1246	-2.447	***P* = 0.014**

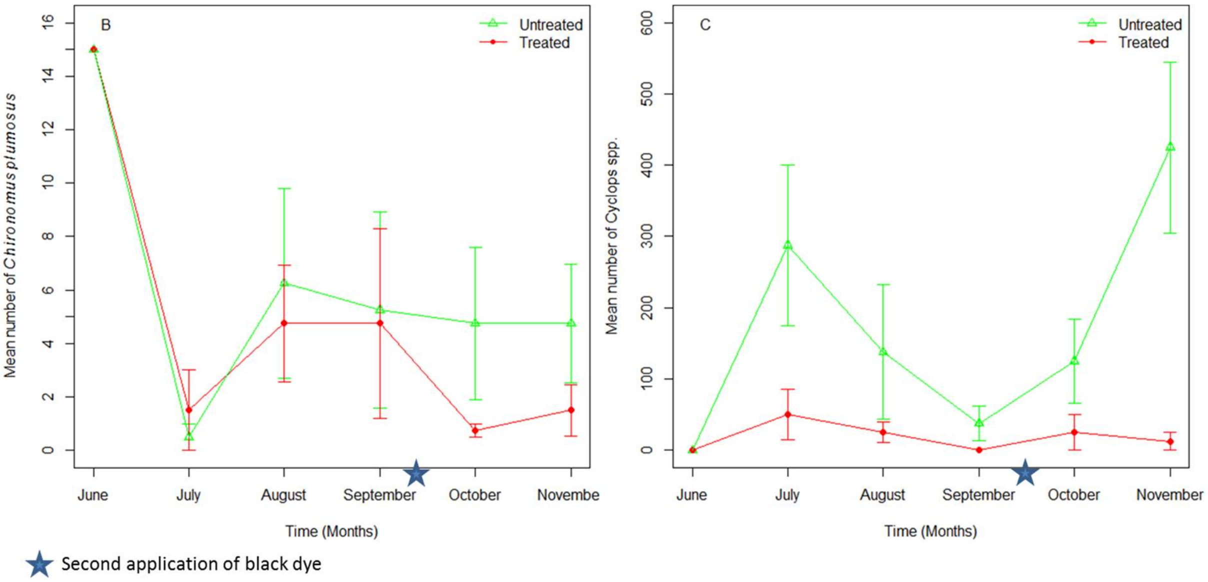

Figure 4.7. Mean of non-biting midge larvae and *Cyclops* (x ±SE) between June and November in treated and untreated ponds. **A**. *Chironomus plumosus*. **B.** *Cyclops* sp.

Ostracoda sp. were observed intermittently throughout the sampling period, with no observations being recorded for many of the weeks and many of the ponds. A similar trend was observed in both treatments, an increase in the abundance was observed with a peak in August (Figure 4.8A), but both untreated and treated ponds started to decrease after the peak until the end of the sampling period. No statistically differences in *Ostracoda* sp. population between treated and control were observed. No differences in abundance were observed between untreated and treated ponds before or after reapplication (Table 4.6).

No statistically significant differences were observed in meniscus midge (*Dixidae*) abundance between untreated and treated ponds during the sampling period (Figure 4.8B). Similar results were observed before and after the second application of the dye (Table 4.6).

Table 4. 6. Summary of the analysis of *Ostracoda sp.* and Meniscus midge *(Dixidae)* collected in the ponds. *All sampling period (5 months).

Specie\Common name	**Season**	**Estimated**	**Std. Error**	**z Value**	***P***
Ostracoda sp.	*	-0.3321	0.7583	-0.438	*P*= 0.661
	Summer	-0.2097	0.8674	-0.242	*P*= 0.809
	Autumn	-1.0986	1.0923	-1.006	*P*= 0.315
Meniscus midge *(Dixidae)*	*	-0.2103	0.5151	-0.408	*P*= 0.683
	Summer	0.4925	0.4369	1.127	*P*= 0.26
	Autumn	-1.1856	1.004	-1.181	*P*= 0.238

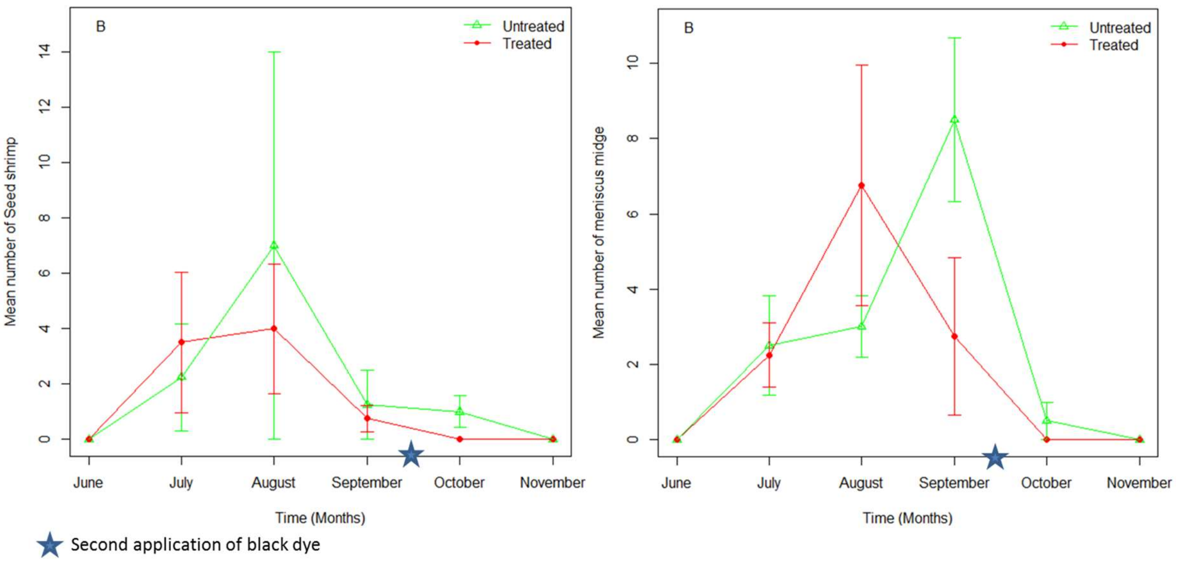

Figure 4. 8. Mean of *Ostracoda sp.* and meniscus midge (*Dixidae*) (x ±SE) between June and November in treated and untreated ponds count for each treatment across the sampling period. A. Seed shrimp *Ostracoda sp.*population. B. Meniscus midge (*Dixidae*).

4.3.2 Redundancy analysis

The summary of the effects of treatment on macroinvertebrate communities over the 6 months can be seen in Figure 4.9. This figure suggests that factors other than dye treatment are important and most likely reflect the seasonal differences in abundance. The RDA analysis showed significant differences in community composition between treated and untreated ponds (*P* = 0.044) and months (*P* = 0.002). *Corixa punctata, Daphnia pulex, Cyclops* sp. and coleoptera larvae were more abundant in ponds without dye (Figure 4.9). *Culex pipiens* larvae, *Ostracoda* sp., dipteran larvae and *Anisoptera* sp were affected more by season (summer) than the dye application. Pond olive nymph (Clo.dip), *Dixidae* pupae and

Chironomus plumosus did not respond either to dye or season. This was observed in the previous results, no significant differences were observed in season and between treated and untreated ponds. It is likely that another factor is affecting abundance. No species abundance was positively correlated to the dye.

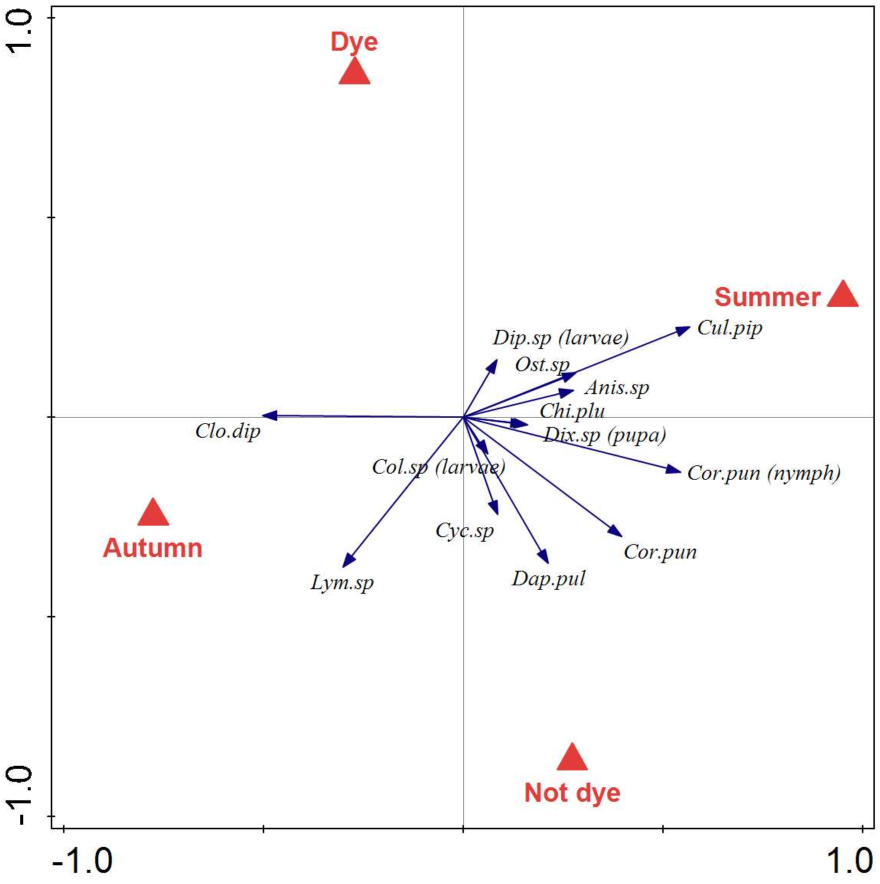

Figure 4. 9. RDA results of the abundance of species in both treatments (Dye and Not dye) and season (Summer and Autumn) across the sampling period (July-November). Species Key: *Chironomus plumosus* (Chi.plu), Coleoptera larvae (Col.sp), *Culex pipiens* larvae (Cul.pip), dragonfly-*Anisoptera* (Ani.sp (nymph)), Lesser waterboatman-*Corixa punctata* (Cor.pun), Meniscus midge-*Dixidae* (Dix.sp), Ostracoda (Ost.sp), pond snail-*Lymnea* sp. (Lym.sp), pond olive-*Cloeon dipterum* (Clo.dip) and water mite-*Hydrachnidiae* (Hyd.sp).

From the five species reintroduced in the ponds only *Chironomus plumosus, Daphnia pulex*, and *Corixa punctata* were recorded during the nineteen weeks of sampling. *Anisoptera* disappeared after the first week and *Culex pipiens* disappeared at week 13. Eight species colonised ponds after the reintroduction, Coleoptera larvae, pond snail (*Lymnea* sp), *Cyclops* sp., Ostracoda, meniscus (*Dixidae*) and water mite. Pond olive *Cyclops* sp., meniscus larvae and Coleoptera larvae colonized from the first week and were collected until November but *Cyclops* sp. was not sampled frequently and meniscus larvae were not recorded from week 16. Ostracoda and pond snail colonized in week two and four respectively.

The abundance of species was significantly different between untreated and treated ponds after the eleventh week where the second application was added to the ponds (Figure 4.10 and 4.11). Pond olive-*Cloeon dipterum* (Clo.dip) exhibited a stronger response to ponds treated with dye (Figure 4.9 and 4.10) but no significant differences were observed (week 7 t= 0.125, df = 6, *P* = 0.905; week 8 t = -0.446, df = 6, *P* = 0.671). Lesser waterboatman (larvae and nymphs), *Dixidae* pupae, *Daphnia pulex* and pond snail were more prevalent in control ponds. One possible reason is that abundance could be affected more by season in contrast with dye colour.

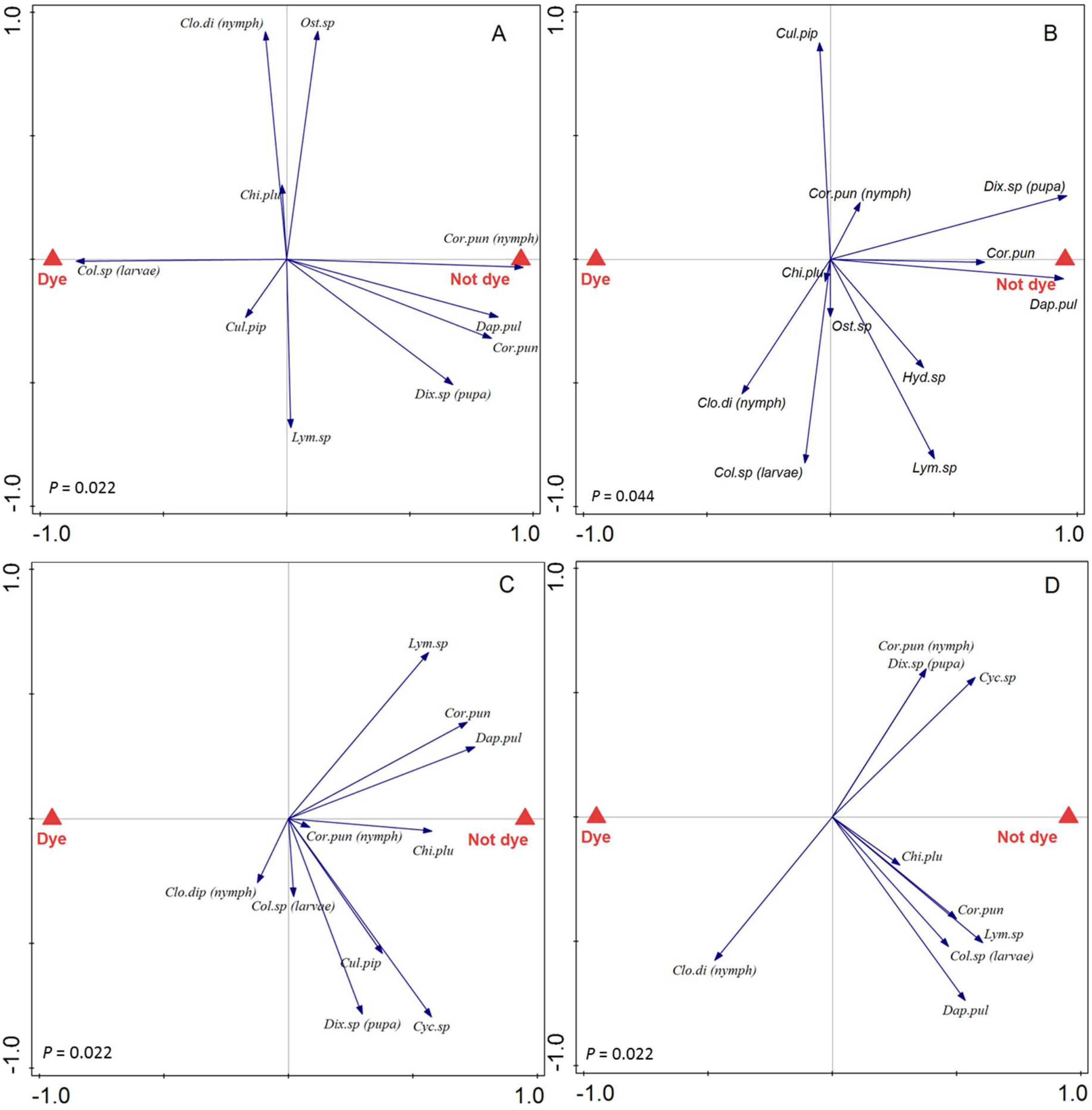

Figure 4. 10. Constrained ordination analysis for weeks and *P* values where the variation in species composition was significant between treatments. **A.** Week 10. **B.** Week 11. **C.** Week 13. **D.** Week 14.

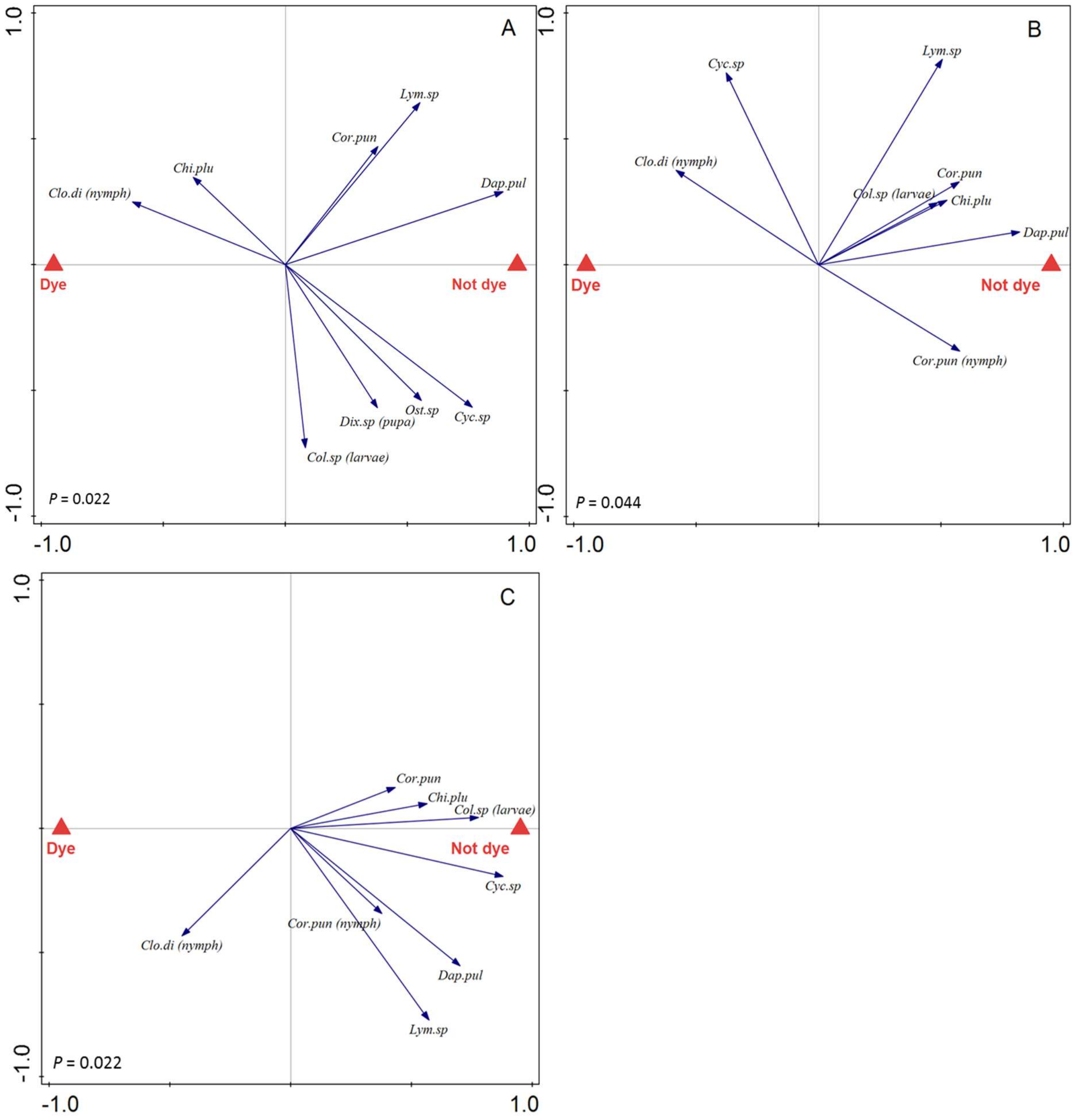

Figure 4. 11. Constrained ordination analysis for weeks and *P* values where the variation in species composition was significant between treatments. **A.** Week 15. **B.** Week 17. **C.** Week 18.

4.4 Discussion

The creation of urban ponds can be a means of enhancing the biodiversity value of gardens (Gaston *et al*., 2005). The ponds used here were relatively small, although together they represented a reasonably sized “pondscape”. Ponds were initially established with five species per replicate but all were rapidly colonised with other species of invertebrate, regardless of the treatment type, with a total of at least 12 species in the ponds. These results are in line with previous work showing community saturation in zooplankton can be achieved very rapidly with timescales of between 6 months and two (Jenkins and Buikema, 1998; Cáceres and Soluk, 2002; Sartori *et al*., 2014). This is also evidence to suggest that the dye is not repellent to colonising animals. In fact for some species, there is evidence that it is an attractant (Ortiz and Callaghan, 2017). Black dye was shown to be an attractant for *Culex pipiens* oviposition in laboratory studies, although it had a significant negative impact on the survival of mosquitoes through to adults (Ortiz and Callaghan, 2017). However in a more natural system with other organisms sharing resources, the impact of the dye was not evident (Ortiz and Callaghan, 2017).

In our ponds, addition of the dye significantly increased diversity and evenness as measured using Shannon-Weiner. However, dye was associated with a significant reduction in abundance of *Daphnia*, lesser water boatmen, pond snails and *Cyclops*, particularly after the second application of the dye at week 11. A constrained redundancy analysis and individual species data found mixed impacts of the dye on abundance but generally reflected the individual data analysis.

These data demonstrate that pond dyes, although acutely non-toxic, are selectively altering the pond community. It is possible that the increase in biodiversity results from a selective effect on key species. A trade off in species' success, particularly where normally there is competitive asymmetry, can be important for stabilizing coexistence in communities and thus contribute to biodiversity (Juliano, 2009). Another explanation for this is that in a manipulated ecosystem a single stress might produce a number of stresses due to the complex nature interactions in the aquatic habitats (Frost *et al.*, 2001; Frost *et al.*, 1999). Little Rock lake in Northern Wisconsin was acidified to investigate the ecosystem response to the lower pH (Frost *et al.*, 1999). The authors found that multiple stresses could be derived from a single stress which is accompanied by a number of changes in the lake processes and biodiversity and abundance of many species.

Daphnia and *Culex* are competitors for resources (Duquesne *et al.*, 2011). *Daphnia* sp. are frequently found in freshwater environments, earmarking it as an important animal in algal bloom control, food-web dynamics and as the key of pond system stability (Steiner, 2004; Steiner, 2002; Miner *et al.*, 2012). Egg production and rate of growth depend on algae as a food source and therefore anything that affects the growth of algae, such as the dye, would be reducing the availability of food (Choi *et al.*, 2014). The extremely high number of *Daphnia* in the ponds will potentially be driving many interactions and particularly competition for resources. Water boatmen, mosquitoes, pond snails and *Cyclops* all feed mostly on algae and dead plant material (detritus) (Popham, 1959; Blaustein and Chase, 2007; Brönmark *et al.*, 1991).

Changes in *Daphnia* behaviour could reduce food resources available, but also may result in a change in community structure due to shifts in predator behaviour (Wissel *et al.*, 2003). *Daphnia* sp. has a phototactic behaviour where they move downward during daytime to avoid predators (Ebert, 2005). Since less light will penetrate the water after the application of lakes and pond colours, *Daphnia* may adapt their behaviour and not migrate to a low enough depth to avoid predation. Previous studies have shown that local adaptation in *Daphnia* significantly influenced zooplankton community composition (Pantel *et al*., 2015). They observed that local adaptation in *Daphnia* suppressed some of the taxa such as Chydoridae, although Cyclopoida and *Simocephalus vetulus* were positively benefited. *Daphnia* is a strong competitor and considerable work has shown that this cladoceran species may have a negative impact on the development of *Cx. pipiens* larvae (development more slowly) and pupae (reduction in pupal size) (Stav *et al*., 2005). *Daphnia* and *Cx. pipiens* both feed on phytoplankton in the water column creating a possible negative correlation between them in untreated ponds (Stav *et al*., 2005; Kroeger *et al*., 2013a). In laboratory and field studies, mosquito larval population are negatively affected by the predatory and competitive behaviour of copepods, snail and Crustacea: Ostracoda (Kroeger *et al*., 2013a; Kroeger *et al*., 2013b; Lardeux *et al*., 1992; Rey *et al*., 2004; Rossi *et al*., 2011; Knight *et al*., 2004; Duquesne *et al*., 2011; Rowbottom *et al*., 2015)

Cx. pipiens was abundant in ponds treated with dye although a significant difference between treatments could not be found. This reflects previous experiments on natural populations of mosquitoes colonising water butts containing dye where known negative effects of dye in laboratory studies are not apparent (Ortiz and Callaghan, 2017). Habitat type was far more important in determining the number of larvae and pupae, with a darker woodland habitat producing significantly more pupae compared to those in an unshaded greenhouse area (Ortiz and Callaghan, 2017). We have here a potential explanation for these results, in that the negative effect of dye on competitors such as *Daphnia* have resulted in a balanced compensation, i.e. although the dye influences negatively on *Culex* survival, in comparison to ponds without dye, there is more food available which improves *Culex* survival. However, the RDA analysis in the experiments recorded here suggested that for *Ostracod*a and *Culex*, the season was a more powerful factor in explaining abundance than the presence of dye.

No pond snails had been originally found when clearing the ponds before sampling and they were not deliberately introduced. I suggest that they colonized the ponds as a result of the close interaction between other ponds in the area. Pond snails are controphic herbivores which grow on the substrate and consume on periphytic algae (Brönmark *et al.*, 1991; Blaustein and Chase, 2007; Brönmark, 1989). As such, snails can affect mosquito survival since they are competitors for food (Knight *et al.*, 2004). Knight *et al.* (2004) observed that mosquito larvae and pupae density and adults emergence were reduced in mesocosms where snails were present. However, in the study, there was little overlap in populations of mosquitos and snails to suggest that the negative effect of the dye on snails was having a corresponding positive impact on *Culex*.

Pond dye restricts algal growth and it could be possible that primary food resources would be limited leading to interspecific competition. It is speculation to suggest that a change of the structure of the aquatic community has occurred as a result of the presence of the dye but there is strong evidence to show that individual taxa are impacted. Reduced nutrient levels could cause a shift in predator behaviour and an increase of intraspecific competition among the spatial niches.

There is little information regarding the effect pond dye has on aquatic biodiversity. The application of Dyofix pond black serves to limit algal growth (DyoFix, 2015c); however, algae is a major food source for invertebrate larvae and zooplankton (Campeau *et al.*, 1994; Dobson and Frid, 2008) which is an important resource of many macroinvertebrates sampled.

The dose of dye during the experiment was the recommended by DyoFix. DyoFix pond and lakes can be used up to 10 times the minimum dose without affecting fish, wildlife and domestic animals (DyoFix, 2015a). I suggest that even at recommended doses the application of lakes and pond colours might change community structure and reduce abundance and biodiversity (Connor, 2016). The findings highlight the importance of the implications of the pond dyes to aquatic macroinvertebrates and the aquatic community.

CHAPTER 5. Can ponds dyes have an effect on communities in freshwater habitats?

Declaration

The data collected in this chapter was in collaboration with students Alice Hurley and Tania Smith.

CHAPTER 5. Can ponds dyes have an impact on communities in freshwater habitats?

5.1 Introduction

Freshwater ecosystems are a key conservation priority and a considerable amount of research has been undertaken in recent years focussing on pond flora and fauna (Dudgeon *et al.*, 2006; Céréghino *et al.*, 2014). These habitats have the capacity to support high proportions of rare and endemic species, a factor important in anthropogenically dominated urban landscapes (Williams *et al.*, 2003; Davies *et al.*, 2008; Biggs *et al.*, 2005; Sayer *et al.*, 2012; Céréghino *et al.*, 2014). Ponds are often abundant in urban landscapes and are created for a variety of reasons, including ornamental ponds in gardens and parks (Goertzen and Suhling, 2015).

Many macroinvertebrate species are active colonisers, migrating between ponds, so that a well-connected urban network of ponds can support very high regional macroinvertebrate biodiversity (Gledhill *et al.*, 2008; Williams *et al.*, 2008). Given issues with the loss of ponds in the wider rural, agriculturally dominated landscape, urban ponds provide replacement biodiversity hotspots both in terms of species composition and biological traits, and in so doing have been shown to be a habitat equivalent of natural ponds. Ponds also affect positively on urban ecosystems more broadly, providing food and shelter for amphibians, bats, birds and invertebrates. Evidence is also accumulating that ponds provide a network of habitats that allow species of amphibians and invertebrates to respond to global climate change by migrating northward (Ott, 2001; Rosset and Oertli, 2011; Walther *et al.*, 2002).

Given, therefore, the potentially high ecological value of ponds, it is surprising that relatively little attention is given to the management of garden ponds.

In the UK at least two-thirds of native animals and plants have been described in freshwater ecosystems (Williams, 1999). It is estimated that across the UK the number of ponds in domestic gardens is between 2.5-3.5 million (Davies *et al.*, 2009). Management of these ponds is therefore vital if urban ponds are to play a greater and more quantifiable role in conserving the diversity of macroinvertebrates. In consequence of this objective, in 2008 UK ponds were designated as habitats of conservation importance in Annex 1 of the Habitats Directive (EEC 1992), where they meet the required criteria. They continue, however, to be frequently created and managed for primarily aesthetic purposes, a situation fuelled by a plethora of media encouragement, with conservation objectives secondary or absent (Hunter and Hunter, 2008). The practice of maintaining ponds primarily or at least equally importantly for their ecosystem functions, if more widely and vigorously promoted in a way appealing to the gardening public, could lead to significant gains in respect of their wildlife value and conservation importance, at a time when many species and habitats are in decline. This cultural shift, however, to be widely adopted, requires active promotion of an understanding that it is not necessarily deleterious to their appeal, and in some ways might enhance it, providing additional amenity benefits, for example by encouraging highly visual and aesthetically attractive macro-invertebrates, such as dragonflies and damselflies, as well as birds and other animals.

This study arose from a trip to a National Trust garden, where a heavily dyed pond was in evidence. The use of dyes was found to be quite common on further observation, with garden designers at prestigious gardening events, such as the Chelsea Flower Show, and TV gardeners advocating their use for aesthetic reasons. Around Reading, leisure parks such as Maidenhead Sailing Club lake, Taplow Water-ski lake and the Cotswold and South Cerney Sailing club's lakes have all applied DyoFix dyes (DyoFix, 2015c). The dye is used to inhibit algal growth and to improve the overall appearance of the water body, particularly if a reflective surface is part of the desired appearance. The suppliers claim that they work by blocking red light from entering the water, interrupting the photosynthetic process and therefore inhibiting the growth of certain aquatic plants such as algae (DyoFix, 2015c). As such, they are promoted as non-toxic, environmentally friendly alternatives to using algaecides such as copper.

Algae are primary producers in aquatic ecosystems which form the base of many food chains, as well as contributing to the maintenance of oxygen and nutrient flows (Baltazar-Soares *et al.*, 2014; McCormick and Cairns, 1994). They are consumed by primary consumers including zooplankton and rotifers (Downing and Leibold, 2002). Algal blooms can occur when natural grazing is reduced, eutrophication of a water body occurs, or in response to other physical changes in their environment. Such blooms occur rapidly, reducing the amount of light available for photosynthesis and hence causing the death of some other aquatic plants. The dead vegetation which decomposes can reduce the amount of available oxygen in the water, which kills other aquatic life including fish (Chislock *et al.*, 2013). Copper based on algaecides have always been the mainstay of algae treatment (Mastin and Rodgers Jr, 2000). Their toxicity towards non-target organisms such as *Daphnia* has led to a move away from these algaecides, to alternative chemical and more naturally derived herbicides, such as pond dyes and barley straw. The manufacturers of pond dyes advertise that their dyes are non-toxic to fish and invertebrates. However, in the last few years, previous studies showed that products such as dye_Crystal Blue-Ocean (CBO) or Aquashade reduced the dissolved oxygen (DO) and affected the fish growth (Tew, 2003; Martin *et al*., 1987).

Previous studies showed that methylene blue and rose bengal reduced the dissolved oxygen up to 43 and 35% (Martin *et al*., 1987). Given that their function is to limit the growth of algae, we hypothesise that it is likely that they have a profound impact on the wider community of invertebrates that co-exist with the target algae.

So far, my work has shown that black pond dyes are attractants to at least one species of Dipteran (*Culex pipiens,* Common House Mosquito) while having a negative impact on their survival (Ortiz and Callaghan, 2017). However, a study of their impact on mosquito numbers in a naturally colonising habitat found no effect. In Chapter 4, identical ponds were stocked with an identical number and variety of fauna and treated with dye. After the initial set up they were left to naturally colonise. The dyes had a negative impact on abundance of an important keystone species (*Daphnia*) that may have driven an increase in biodiversity and evenness. Here a similar experiment was undertaken, but bearing in mind differences found between controlled and uncontrolled conditions with the mosquito work, unmanipulated, naturally colonising ponds were used.

5.2 Materials and Methods

5.2.1 Study site and pond

Mesocosm experimental ponds were created by sinking thirty-two plastic buckets outdoors in experimental grounds, at the University of Reading, Berkshire, England (51.4419°N, 0.9456°W) in 2012 (Figure 5.1). Each pond comprised a sunken bucket (diameter 48 cm, depth 30 cm) lined with pond liner, which was colonised by macroinvertebrates during 2012-2015, prior to the commencement of the current research in 2015. Twenty-four randomly selected ponds were used; eight had been used in a previous experiment in 2014.

Figure 5. 1. Location of the 32 ponds dug in experimental grounds at the University of Reading. The map was taken from Digimap: http://digimap.edina.ac.uk.

5.2.2 Pond treatments

Three different treatments were applied to the ponds (Figure 5.2):

- 8 were treated with black pond dye for the first time;
- 8 were re-treated with black dye, having been treated the previous year with similar black dye; and
- 8 were not treated with dye, and formed the controls.

An initial pre-sampling of invertebrates on each pond was performed on 15 June 2015 before the application of the dye on 16 June 2015. 100 g dye per 2000 ml of water was applied to the appropriate ponds according to the manufacturer's instruction (DyoFix, 2015c). Regular sampling as described below began on 24 June 2015 and was performed weekly until 7 December 2015.

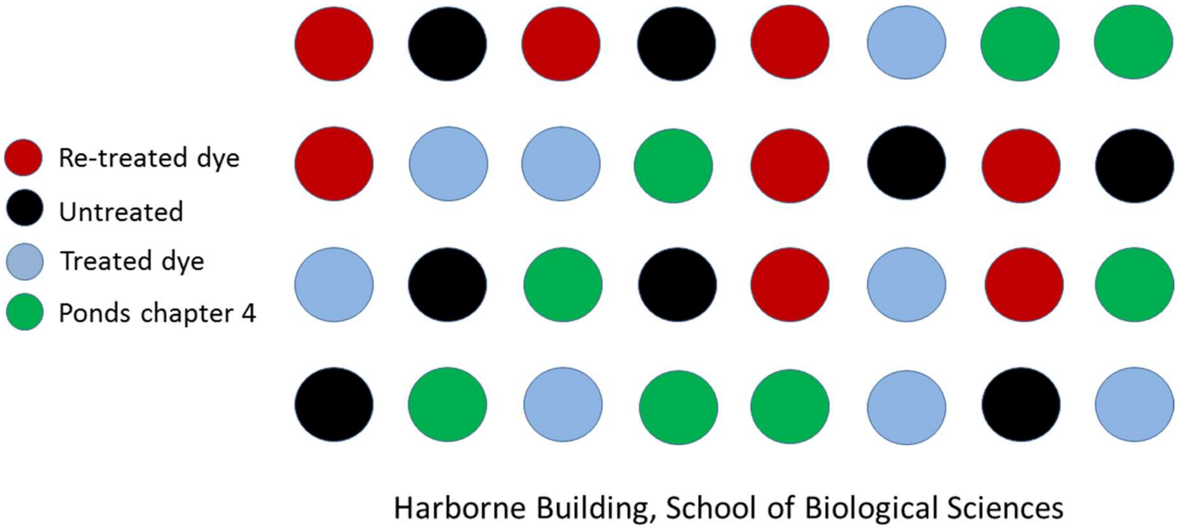

Figure 5. 2. Diagram of the control ponds and with dye treatment. Ponds coloured with red denote those with dye colour and the black colour are the control ponds.

5.2.3 Sampling and animal identification

Each pond was sampled for macroinvertebrates using a 6 × 12 cm aquarium fish net. The net was dropped into each pond with an "S" shaped movement, twice, from one side to the other and approximately 10 centimetres from the bottom. Netted macroinvertebrates were transferred into rectangular sampling trays (25 × 35 × 5cm) with 500 ml pond water and immediately transported to the nearby laboratory for identification and counting. Counting was undertaken with a stereomicroscope (10X magnification). Animals were identified using a number of freshwater keys Croft, (1986), Cranston *et al.*, (1987), Snow (1990) and Greenhalgh and Ovenden (2007). Most animals were identified to species or genus level with the exception of beetle and meniscus midge larvae, which were identified to family level, since the time taken to identify them might have been fatal to them. All sampled invertebrates were returned to their pond of origin to avoid skewed results that might arise from artefactual loss of individuals.

Daphniidae were observed to be present in very high numbers so were counted using a sub-sampling method. The content of *Daphnia* was poured into a 600 ml beaker and 500 ml of distilled water was added. The sample was swirled and 10 ml sample was collected with by a pipette in a circular motion. To calculate the abundance, the individuals collected were counted and then multiplied by 50 (n x 50 = 500 ml, n = sample number of *Daphnia*).

5.2.4 Statistical analysis

The effect of pond dyes on macroinvertebrate communities was explored using Redundancy Analysis (RDA) in Canoco (Šmilauer and Lepš, 2014). The effects of the pond dye treatment in each pond and seasonal (summer/autumn) effects were analysed from the time series sampling data collected for all macroinvertebrates throughout the sampling period. Summer and autumn were taken into account because climatic conditions associated with seasonality as factor that can affect the abundance of macroinvertebrates, and potentially, therefore, interacts with the effects of the dye. Summer was taken as commencing at the first week of sampling, 16 June 2015, and continuing until the middle of September, 18 September, and autumn from 19 September until the end of sampling on 7 December 2015.

To identify how macroinvertebrate communities within treated and control ponds changed over time, weekly analyses were performed. The response variable (species number) were Log (x+1) transformed. Log (x+1) was used as zero values were present in the data collection during in some weeks. The significance of the canonical axes derived from the explanatory variables was determined at 95% confidence level ($p < 0.05$), based on 999 Monte Carlo permutations in all cases.

Results with explanatory (pond dye and season) and response variables (macroinvertebrate populations) are presented as bi-plots; pond dye and season are represented symbolically and the macroinvertebrate responses as arrows. The direction of an arrow is a measure of fit for the correlation of the species with the ordination axes (i.e., arrows pointing directly towards a treatment symbol correlate most strongly with that treatment).

All statistical analyses were performed using R version 3.4.0 (R Development Core, 2016). The Shannon-Wiener Diversity Index infer all species are represented in a sample which they are randomly sampled between different habitats (Ortiz-Burgos, 2016) and Evenness how individuals are distributed over different species in a community (Heip *et al.*, 1998; Molinari, 1989). Shannon-Wiener Diversity Index and evenness value and individual abundance in ponds were modelled through a generalised linear model with a negative binomial distribution using package MASS (Ripley 2015).

5.3 Results

5.3.1 Effects on Biodiversity

5.3.1.1 *Biodiversity and evenness index*

Biodiversity index The Shannon-Wiener diversity index was not significant between treated and untreated ponds (Figure 5.3A). Similar results were obtained in summer and autumn season measures (Table 5.1). The evenness value produced by the Shannon-Weiner diversity index was not significantly different between untreated and treated ponds (Figure 1B). The evenness value in untreated ponds was bigger in summer compared with the autumn, but no differences were observed between seasons in the three treatments (Table 5.1).

Table 5. 1. Summary of the analysis of Shannon-Wiener diversity index and evenness index collected in the ponds. *All sampling period (6 months).

	Season	Treatment	Estimated	Std. Error	z Value	*P*
Shannon-Wiener diversity index	*	Retreated	0.05692	0.38099	0.149	*P*= 0.881
		Untreated	0.075	0.37933	0.198	*P*= 0.843
	Summer	Retreated	0.08009	0.50591	0.158	*P*= 0.874
		Untreated	0.19656	0.49238	0.399	*P*= 0.690
	Autumn	Retreated	0.006084	0.585788	0.01	*P*= 0.991
		Untreated	-0.12586	0.605386	-0.208	*P*= 0.835
Evenness index	*	Retreated	0.11566	0.41043	0.282	*P*= 0.778
		Untreated	0.09409	0.41253	0.228	*P*= 0.820
	Summer	Retreated	0.1779	0.5585	0.319	*P*= 0.750
		Untreated	0.2537	0.5491	0.462	*P*= 0.644
	Autumn	Retreated	0.0421	0.6059	0.069	*P*= 0.945
		Untreated	-0.1179	0.6312	-0.187	*P*= 0.851

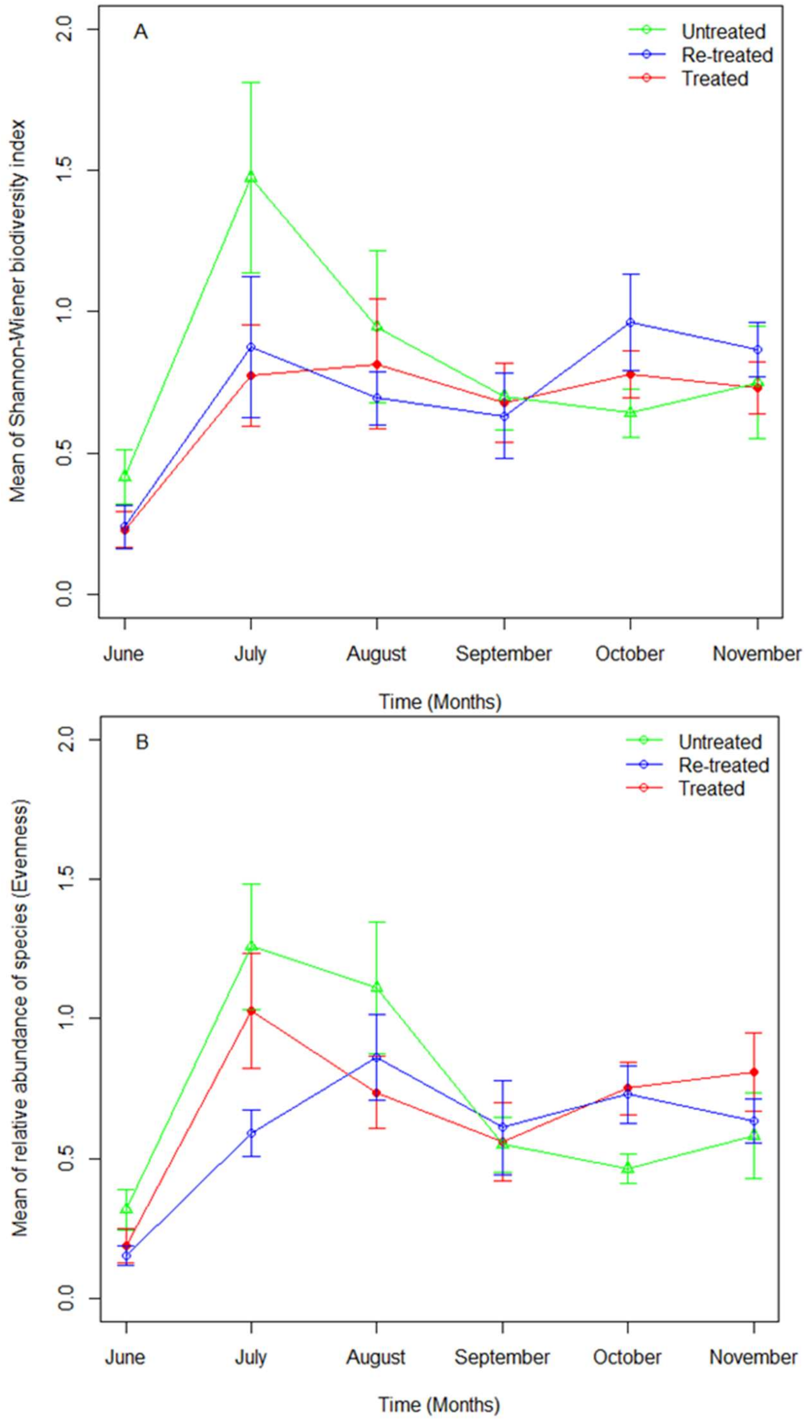

Figure 5. 3. Shannon-Weiner diversity index and evenness results. **A** – Mean of Shannon-Wiener index (±SE); and **B** mean of species evenness (±SE), for untreated (Control and Prevention), treated and re-treated treatments.

5.3.2 Effects of Treatment and Season on individual species

5.3.2.1 *Effects on individual species*

A total of 15 taxa were identified in samples across all ponds, although only twelve were present throughout the entire sampling period (Table 5.2); a single rat-tailed maggot and dragonfly larva were observed at the beginning of the experiment in a pond treated with the dye.

Daphnia pulex was by far the most abundant species recorded (Table 5.2). *Daphnia* abundance was not significantly different between the two dye treated and untreated ponds (Figure 5.4A). Similar results were obtained, *Daphnia* abundance was not significantly different between all three treatments in summer and autumn (Table 5.3).

Lesser water boatman numbers were lowest in re-treated ponds and highest in untreated ponds, these differences were significant in retreated ponds (Table 5.3) (Figure 5.4B). In seasonal observations, retreated ponds were significantly different in summer; but in autumn differences were seen in untreated ponds (Table 5.3).

Table 5. 2. List of macroinvertebrate species found in pond samples and their abundances in the three treatments. Macroinvertebrates found by sampling in each of the experimental conditions, with an indication of their overall abundance (abundance and standard deviation).

Common name / Group	**Species**	**Treatment** (abundance, SD)		
		Control	*Dye*	*Re-Dye (Second application)*
Predators				
Diving beetle	Dytiscidae	0.072 ±0.284	0.072 ±0.416	0.059 ±0.237
Water mite	*Hydrachna* spp.	0.046 ±0.370	0.099 ±0.770	0.855 ±0.459
Herbivores/Detrivores				
Lesser water boatman	*Corixa punctata*	2.52 ±4.639	1.855 ±6.420	0.803 ±1.527
Pond snail	*Lymnaea* sp.	1.960 ±4.520	1.375 ±3.047	0.612 ±1.823
Pond olive	*Cloeon dipterum*	4.039 ±7.873	6.678 ±14.756	6.572 ±13.635
Cyclops	*Cyclops* sp.	0.237 ±0.8514	1.006 ±6.506	0.441 ±3.285
Mosquito larvae	*Culex pipiens*	5.401 ±15.182	7.243 ±19.500	2.783 ±5.334
Mosquito egg batches	*Culex pipiens*	0.908 ±3.072	3.171 ±38.195	0.803 ±2.058
Mosquito pupae	*Culex pipiens*	0.158 ±1.245	0.066 ±0.376	0.230 ±1.258
Non-biting midge larvae	*Chironomus plumosus*	0.144 ±0.894	0.644 ±5.069	0.770 ±2.787
Meniscus midge	Dixidae	0.080 ±0.534	0.052 ±0.277	0.118 ±0.788
Water flea	*Daphnia pulex*	447.579 ±705.335	577.710 ±855.220	689.703 ±1049.494
Omnivores				
Seed Shrimp	*Ostracoda* sp.	1.697 ±10.109	4.993 ±41.501	3.875 ±29.364

Table 5. 3. Summary of the analysis of *Daphnia sp*. and *Corixa punctata* in the ponds. *All sampling period (6 months).

Specie\Common name	**Season**	**Treatment**	**Estimated**	**Std. Error**	**z Value**	***P***
Daphnia sp	*	Retreated	0.1881	0.2164	0.869	*P*= 0.385
		Untreated	-0.2489	0.2164	-1.15	*P*= 0.250
	Summer	Retreated	0.2184	0.2396	0.911	*P*= 0.362
		Untreated	-0.3786	0.2396	-1580	*P*= 0.114
	Autumn	Retreated	-0.02627	0.20422	-0.129	*P*= 0.898
		Untreated	0.31865	0.20416	1.561	*P*= 0.119
Corixa punctata	*	Retreated	-0.8337	0.3162	-2.637	***P* < 0.001**
		Untreated	0.345	0.3066	1.125	*P*= 0.260
	Summer	Retreated	-1.0198	0.37	-2.754	***P* < 0.005**
		Untreated	0.2289	0.3588	0.638	*P*= 0.523
	Autumn	Retreated	0.1092	0.4325	0.252	*P*= 0.801
		Untreated	1.04597	0.40753	2.567	***P*= 0.010**

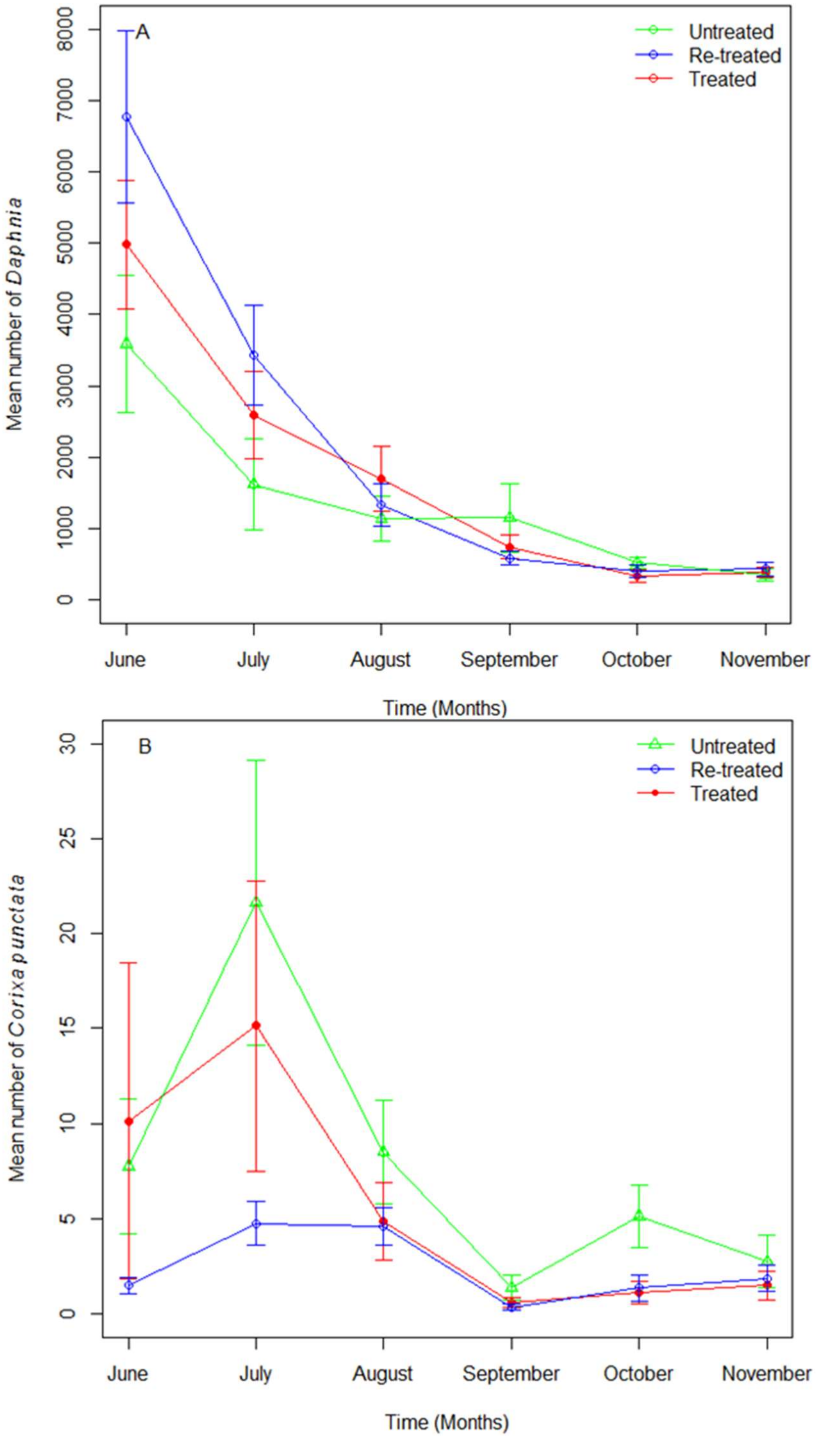

Figure 5. 4. Comparisons of the monthly variations in mean populations for three prominent taxa. Comparisons of populations (±SE) for each of the three treatments, untreated (Control and Prevention), treated and re-treated, through the sampling period. **A** - *Daphnia pulex*. **B** - Lesser water boatman (*Corixa punctata)*.

Similar results were obtained for pond snail, a higher abundance was observed in untreated ponds and the lowest number in re-treated ponds across the sampling period (Figure 5.5). The model found statistically significant differences in retreated ponds in contrast to the control (Table 5.4). In the summer season, retreated and untreated pond did not show significant differences; however, in autumn season retreated ponds are highly different (Table 5.4).

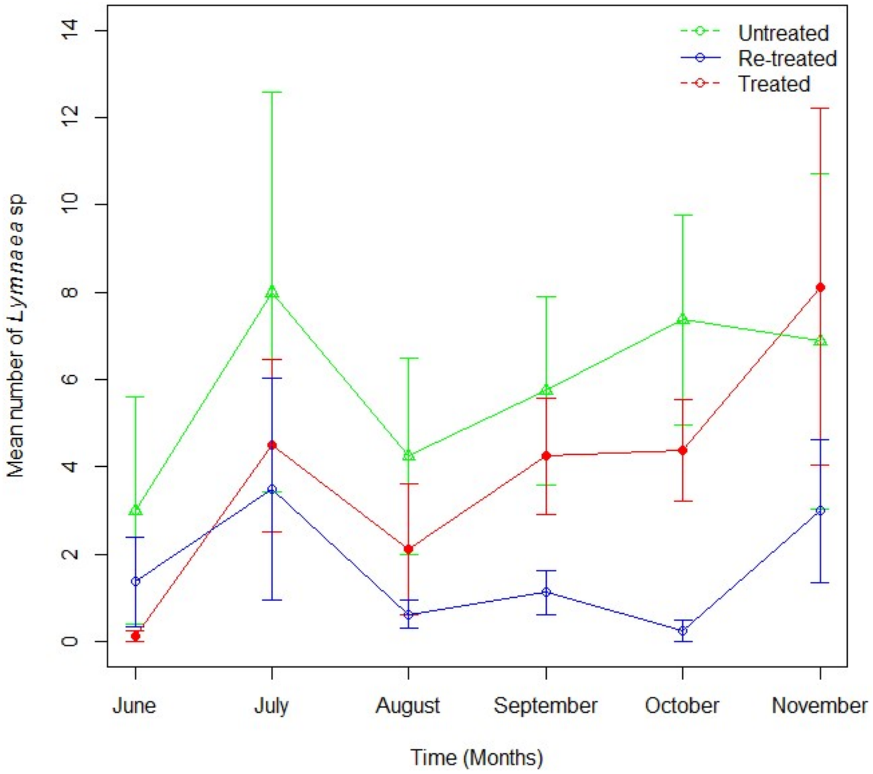

Figure 5. 5. Comparisons of the monthly variations in mean populations for Pond Snail (*Lymnaea* sp.). Comparisons of populations (±SE) for each of the three treatments, untreated, treated and re-treated, through the sampling period.

Table 5. 4. Summary of the analysis of *Lymnaeat* in the ponds. *All sampling period (6 months).

Specie\Common name	Season	Treatment	Estimated	Std. Error	z Value	*P*
Lymnaea sp.	*	Retreated	-1.251	0.3682	-3.397	***P* < 0.001**
		Untreated	-0.3321	0.3578	-0.928	*P*= 0.353
	Summer	Retreated	-1.0198	0.5985	-1.704	*P*= 0.088
		Untreated	-0.6451	0.5926	-1.089	*P*= 0.276
	Autumn	Retreated	-1.4816	0.4468	-3.316	***P* < 0.001**
		Untreated	-0.1391	0.4225	-0.329	*P*= 0.742

No significant differences were observed in larval and pupal densities among all treatments sampled in artificial containers (refer to results) (Ortiz and Callaghan, 2017). The abundance of *Culex pipiens* egg batches peaked in control and re-dyed ponds in July and in dyed ponds in August (Figure 5.6A). Larval and pupal densities showed more complex monthly variation. Larvae number (Figure 5.6B) peaked in July in control and re-treated ponds. In treated treatments, their levels increased only slightly until August after which they declined. High numbers of pupae (Figure 5.6C) were found in June for both the treated and re-treated treatments. These declined in July but numbers were at their highest peaks in August. Their numbers in the untreated ponds; however, peaked twice, in July and September, with September the highest. No significant differences in the abundances of eggs batches or pupae densities were observed between all treatments (Table 5.5). Similar results were observed between seasons in retreated ponds in contrast to the controls, no significant differences were presented in the abundance of pupae or egg batches. Larvae are statistically significant in retreated ponds all sampling period and in summer season.

Table 5. 5. Summary of the analysis of larvae, pupae and mosquito eggs (*Culex pipiens*) in the ponds. *All sampling period (6 months).

Specie\Common name	Season	Treatment	Estimated	Std. Error	z Value	*P*
Larvae of *Culex pipiens*	*	Retreated	0.9521	0.3924	2.426	***P*= 0.015**
		Untreated	0.6558	0.3928	1.67	*P*= 0.095
	Summer	Retreated	0.9545	0.3709	2.5474	***P*= 0.010**
		Untreated	0.6523	0.3714	1.757	*P*= 0.079
	Autumn	Retreated	0.97538	0.71641	1.361	*P*= 0.173
		Untreated	0.75633	0.71921	1.052	*P*= 0.293
Puape *of Culex pipiens*	*	Retreated	1.2528	0.7738	1.619	*P* = 0.105
		Untreated	0.8755	0.7822	1.119	*P*= 0.263
	Summer	Retreated	1.3545	0.7959	1.702	*P* = 0.089
		Untreated	-0.2877	0.8763	-0.328	*P*= 0.743
	Autumn	Retreated	0.6931	1.5493	0.447	*P*= 0.655
		Untreated	2.1972	1.4853	1.479	*P*= 0.14
Egg batches *of Culex pipiens*	*	Retreated	0.4096	0.3808	1.076	*P* = 0282
		Untreated	0.5328	0.3795	1.404	*P*= 0.263
	Summer	Retreated	0.4823	0.3241	1.488	*P* = 0.137
		Untreated	0.5248	0.3235	1.622	*P*= 0.105
	Autumn	Retreated	-0.3567	0.9283	-0.384	*P*= 0.701
		Untreated	0.5878	0.88	0.668	*P*= 0.504

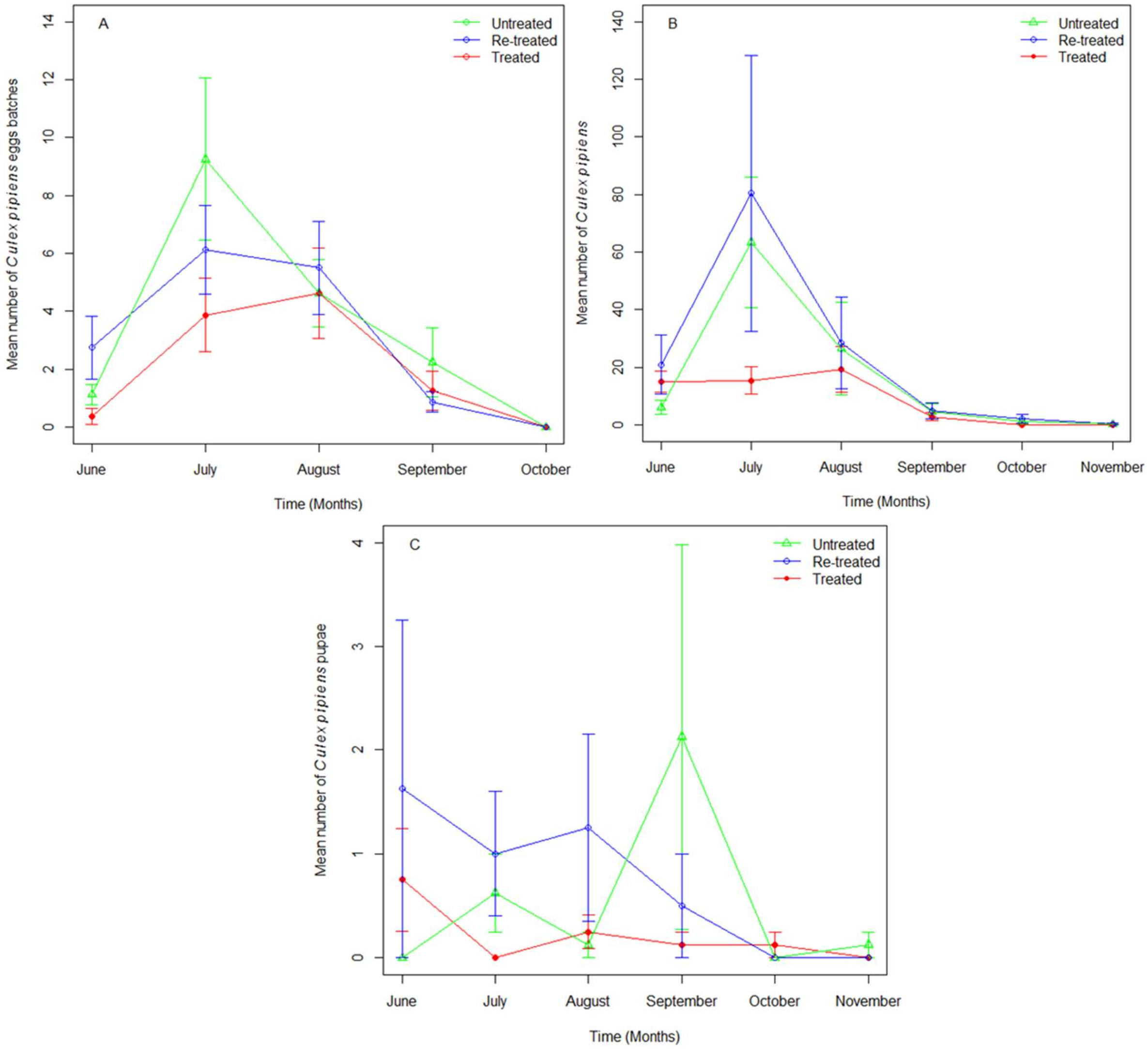

Figure 5. 6. Comparison of monthly variation in abundances of three wild mosquito (*Culex pipiens*) life stages. Egg batches, larvae and pupae abundances (±SE) for each treatment, untreated (Control and Prevention), treated and re-treated, from June to November. **A** - Mosquito egg batches. **B** - Mosquito larvae. **C** - Mosquito pupae.

Mayfly larvae (*Cloeon dipterum*, pond olive) numbers were generally low in samples from July to late August, but from September numbers increased in all treatments. However, no significant differences in pond olive populations between treated and untreated ponds were observed (Figure 5.7A). Analysis of the summer period showed no differences on the abundances of pond olive larvae in both treated and re-retreated and untreated treatments; however, the autumn season had a slight effect in the abundance of pond olive populations in the untreated treatment (Table 5.6).

Coleoptera larvae (*Dysticidae* sp.) were only found in samples during the summer months (June-August) (Figure 5.7B). No general trend was observed among treatments through the sampling period, and the total abundance of Coleoptera larvae was similar between all three treatments, untreated, treated and re-treated ponds (Table 5.6).

Table 5. 6. Summary of the analysis of mayfly larvae (*Cloeon dipterum*, pond olive) and coleopteran larvae in the ponds. *All sampling period (6 months).

Specie\Common name	Season	Treatment	Estimated	Std. Error	z Value	*P*
Cloeon dipterum	*	Retreated	0.04507	0.32868	0.137	*P*= 0.891
		Untreated	-0.46366	0.32974	-1.406	*P*= 0.160
	Summer	Retreated	0.225	0.599	0.376	*P*= 0.707
		Untreated	0.3108	0.5985	0.519	*P*= 0.603
	Autumn	Retreated	0.01848	0.29017	0.064	*P*= 0.949
		Untreated	-0.63282	0.29209	-2.166	***P*= 0.03**
Coleopteran larvae	*	Retreated	-0.1018	0.5355	-0.19	*P* = 0.849
		Untreated	-0.3429	0.5446	-0.63	*P*= 0.529

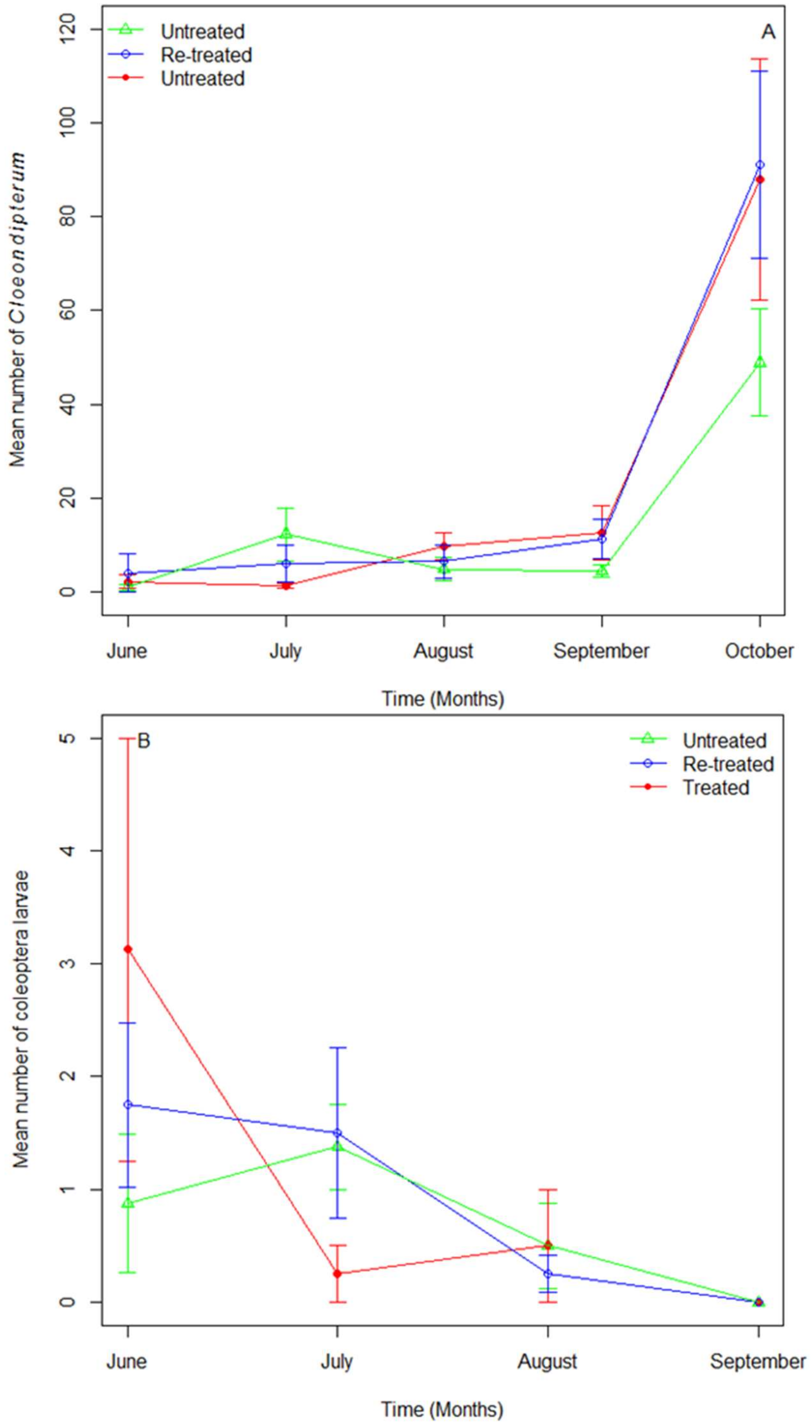

Figure 5. 7. Comparison of monthly variation in the mean populations of mayfly and diving beetle larvae. Population abundances (±SE), for untreated, treated and re-treated treatments for the sampling period. **A.** -Mayfly larvae (*Cloeon dipterum,* pond olive). **B** - Diving beetle larvae (coleoptera, *Dysticidae* sp.).

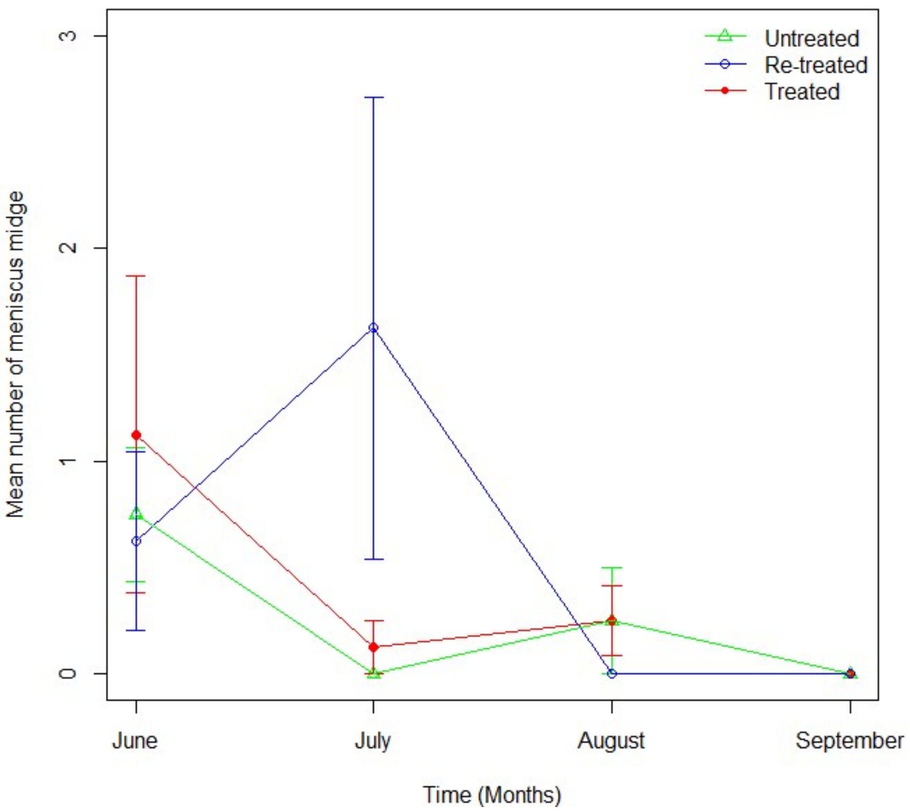

Figure 5. 8. Comparison of monthly variation in the mean populations of meniscus larvae. Population abundances (±SE), for untreated, treated and re-treated treatments for the sampling period.

Meniscus midge (Dixidae) abundance had the highest peak in July for the re-treated treatment (Figure 5.8). Only a few individuals were recorded in untreated and treated ponds during the study period. No significant differences in midge populations were found between treated and re-treated and untreated ponds (Table 5.7).

Table 5. 7. Summary of the analysis of Meniscus midge (Dixidae) in the ponds. *All sampling period (6 months).

Specie\Common name	Season	Treatment	Estimated	Std. Error	z Value	*P*
Meniscus midge (Dixidae)	*	Retreated	0.8109	0.7697	1.054	*P* = 0.292
		Untreated	0.4055	0.7876	0.515	*P*= 0.607

A decline in the number of seed shrimp (*Ostracoda* sp.) was seen after June in all treatments. *Ostracoda* populations were not found to be significantly different between untreated and both treated and re-treated ponds (Figure 5.9A). Despite the high June numbers, the total abundance of seed shrimp in summer did not vary significantly, but in the autumn season the number of seed shrimp is significantly different in untreated ponds (Table 5.8)

In *Cyclops* (*Cyclops* spp.), a high number of individuals were recorded in August in the re-treated treatment, but in the treated treatment the peak was in October (Figure 5.9B). As with the previous results, the abundance of *Cyclops* from was significantly different in control treatments (Table 5.8). In summer season no significantly different in the abundance of *Cyclops* was observed in contrast to the autumn season. However, in autumn season abundance of *Cyclops* is significantly different in both treatments retreated and control (Table 5.8)

Table 5. 8. Summary of the analysis of Ostracoda and *Cyclops* in the ponds. *All sampling period (6 months).

Specie\Common name	Season	Treatment	Estimated	Std. Error	z Value	*P*
Ostracoda	*	Retreated	-0.2706	0.6248	-0.433	*P*= 0.665
		Untreated	-1.0776	0.6265	-1.72	*P*= 0.085
	Summer	Retreated	-0.221	0.7786	-0.284	*P*= 0.777
		Untreated	-1.0174	0.78	-1.304	*P*= 0.192
	Autumn	Retreated	-0.976	0.7217	-1.352	*P*= 0.176
		Untreated	-2.0369	0.7704	-2.644	***P* < 0.005**
Cyclops	*	Retreated	-0.7087	0.6646	-1.066	*P* = 0.286
		Untreated	-1.6895	0.6839	-2.47	***P*= 0.013**
	Summer	Retreated	0.8056	0.8917	0.903	*P*= 0.366
		Untreated	0.3509	0.9678	0.363	*P*= 0.717
	Autumn	Retreated	-2.1518	0.7834	-2.747	***P* < 0.005**
		Untreated	-2.0266	0.7784	-2.604	***P* < 0.005**

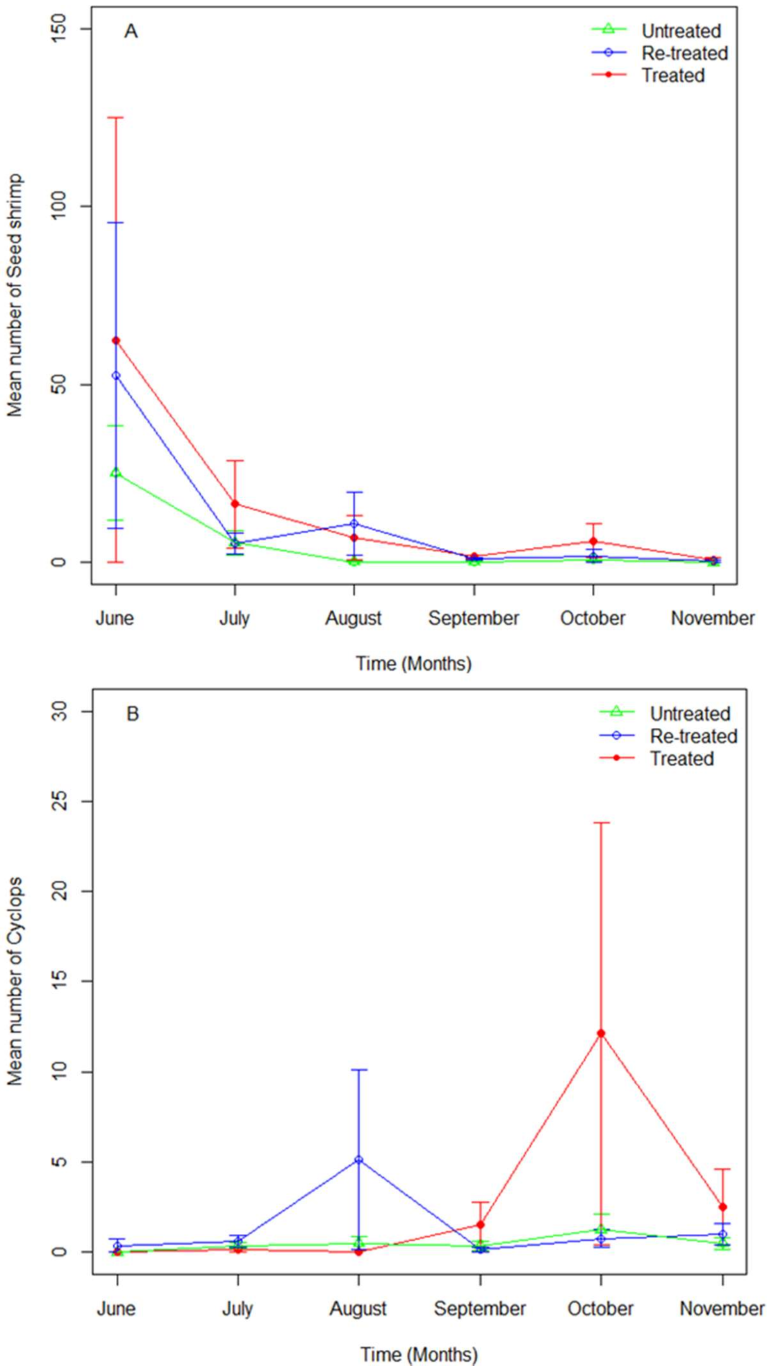

Figure 5. 9. Comparison of monthly variation in the mean populations of Ostracoda and *Cyclops*. Population abundances (±SE), for Untreated (Control and Prevention), treated and re-treated treatments for the sampling period. **A.** Ostracoda. **B** *Cyclops*.

No general trend was observed in Water mite (*Hydrachna* sp.) abundance from June to October (Figure 5.10A) in the untreated or either of the treated treatments. Water mite abundance was not impacted by the addition of dye during the entire sampling period or in summer and autumn (Table 5.9).

Non-biting midge larvae (*Chironomus plumosus,* Bloodworms) were present mostly in the summer season, July to August (Figure 5.10B). In July, re-treated and treated ponds were found to have the highest numbers of Bloodworm larvae. However, no significant differences were observed between all three treatments for this specie during the entire sampling period, but in the summer season, the abundance of bloodworms is significantly different in control ponds (Table 5.9).

Table 5. 9. Summary of the analysis of Water mite (*Hydrachna* sp.) and *Chironomus plumosus,* Bloodworms.* in the ponds. All sampling period (6 months).

Specie\Common name	**Season**	**Treatment**	**Estimated**	**Std. Error**	**z Value**	***P***
Water mite (*Hydrachna* sp.)	*	Retreated	-0.1431	0.9801	-0.146	*P*= 0.884
		Untreated	-0.7621	1.0132	-0.752	*P*= 0.452
	Summer	Retreated	-0.2231	1.0034	-0.222	*P*= 0.824
		Untreated	-0.7621	1.0326	-0.738	*P*= 0.460
Chironomus plumosus, Bloodworms	*	Retreated	0.1772	0.742	0.239	*P* = 0.811
		Untreated	-1.4939	0.7665	-1.949	*P*= 0.051
	Summer	Retreated	0.1772	0.7016	0.253	*P*= 0.80
		Untreated	-1.6946	0.7343	-2.308	***P*= 0.021**

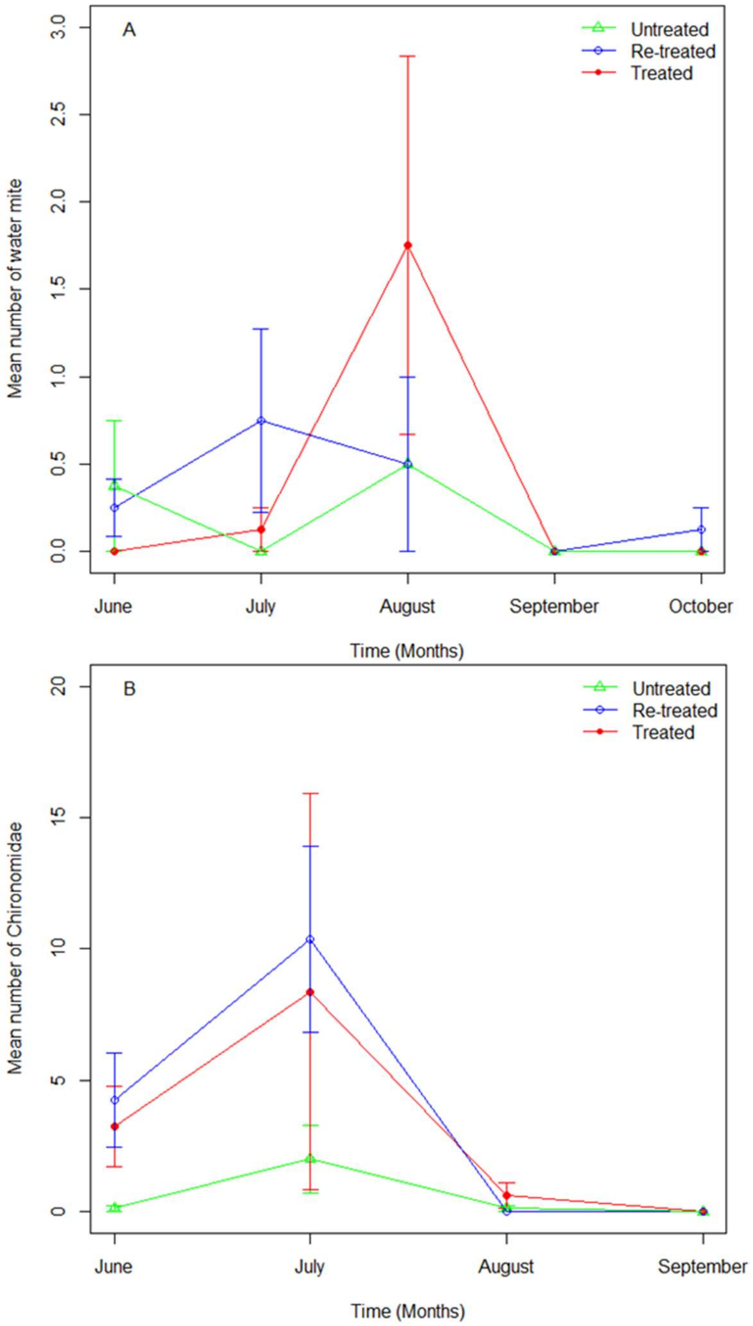

Figure 5. 10. Comparison of monthly variation in the mean abundances of water mites and non-biting midge larvae. Population abundances (±SEM), for untreated (Control and Prevention), treated and re-treated treatments for the sampling period. **A** - Water mite (*Hydrachna* sp.). **B** non-biting midge larvae (bloodworms, *Chironomus plumosus*).

5.3.3 Redundancy analysis (RDA)

A summary of the effects of the two dye treatments on macroinvertebrate individual communities over the 6 month study period can be seen in Figure 5.11. The RDA analysis showed that seasonality was correlated with a strong response in species abundance ($P < 0.001$). Most of the species were more abundant in the summer season (as defined) except for pond snail, diving beetle, *Cyclops* and pond olive which were more abundant in the autumn season.

Both dye and re-treated treatments and untreated control ponds showed significant differences in the abundance of species ($P = 0.028$). Lesser water boatman and dragonfly larvae (*Anisoptera* sp.) numbers responded negatively to treated ponds in comparison to untreated ponds. The constrained ordination results for five individual weeks selected at regular intervals throughout the study season (weeks 1, 5, 10, 15 and 19) (5-weekly samples) are shown in Figure 5.11. Lesser water boatman (Cor.pun, as depicted in Figure 5.11) were present over most of the sampling period and in all 5-weekly samples, their highest abundance was in week 5 in untreated ponds (Figure 5.12B). However, at the end of the sampling period, they were most abundant in dye treated ponds (Figure 5.12E). Dragonfly larvae (Ani.sp) strongly favoured untreated ponds in the first 5-weekly sample (Figure 5.12A), but were not recorded thereafter. Mosquito larvae (Cul.pip) were most abundant in re-treated ponds in the week 1, 15 and 19 samples; but in the week 5 and 10 samples were most abundant in untreated ponds. Mosquito pupae were not often recorded in samples, and at the end of the sampling period (October and November) were not found in most of the samples, this may be due to seasonal effects.

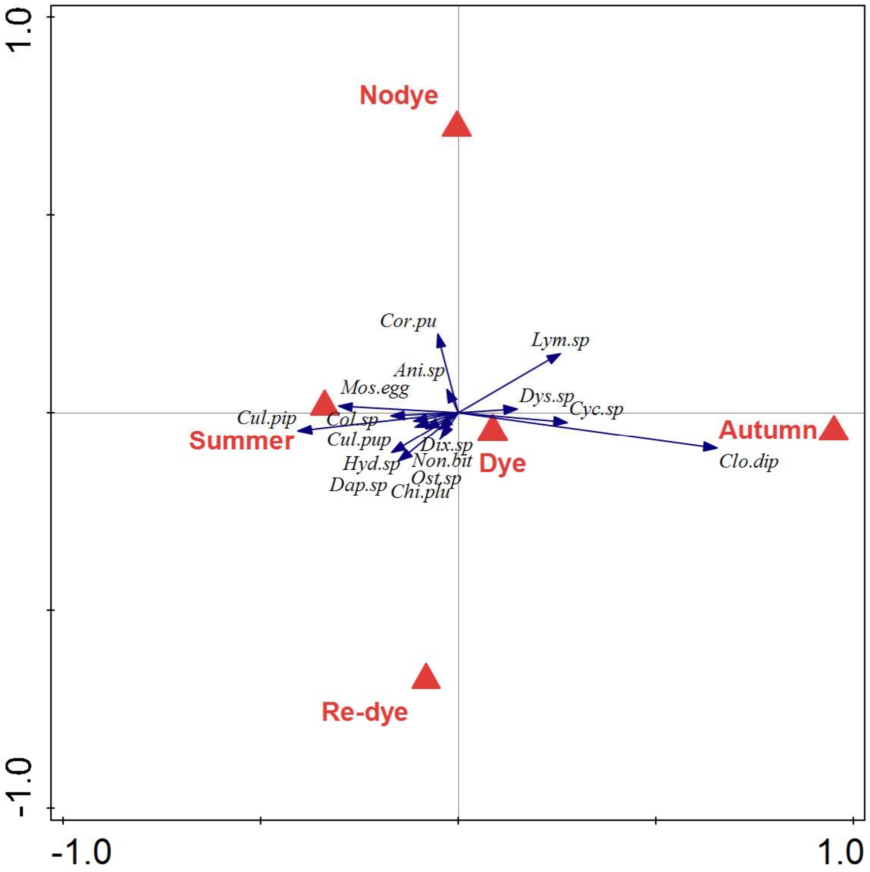

Figure 5.11. RDA Results. Variation partitioning bi-plot, illustrating the abundance of species by season (X-axis) and treatment, no-dye (Untreated) (Control and Prevention), dye (Treated) and re-dye (re-treated) (Y-axis). Taxa larvae (chart abbreviation): Bloodworm (*Chironomus plumosus)* (Chi.plu), diving beetle (Coleoptera, Dysticidae) (Col.sp), mosquito (*Culex pipiens)* and Mosquito pupae (Cul.pip), mosquito egg batches (Mos.egg), Dragonfly (*Anisoptera* sp.) (Ani.sp), lesser water boatman (*Corixa punctata)* (Cor.pu), meniscus midge (*Dixidae)* (Dix.sp), seed shrimp (*Ostracoda* sp.) (Ost.sp), Pond snail (*Lymnaea* sp). (Lym.sp), pond olive (*Cloeon dipterum)* (Clo.dip), water mite (*Hydrachna sp.)* (Hyd.sp), *Cyclops* (*Cyclops* sp.) (Cyc.sp), *Daphnia* (*Daphnia pulex*) (Dap.sp).

Daphnia pulex, Culex pipiens, Corixa punctata, Lymnaea sp. and *Cloeon dipterum* were in all the 5-weekly samples, *Ostracoda* sp., however, was absent during the Weeks 3, 5 and 11. The abundance of all species collected in each 5-weekly sample is not highly significant between all three treatments, except in week 5, the second 5-weekly sample ($P = 0.032$) (Figure 5.12B).

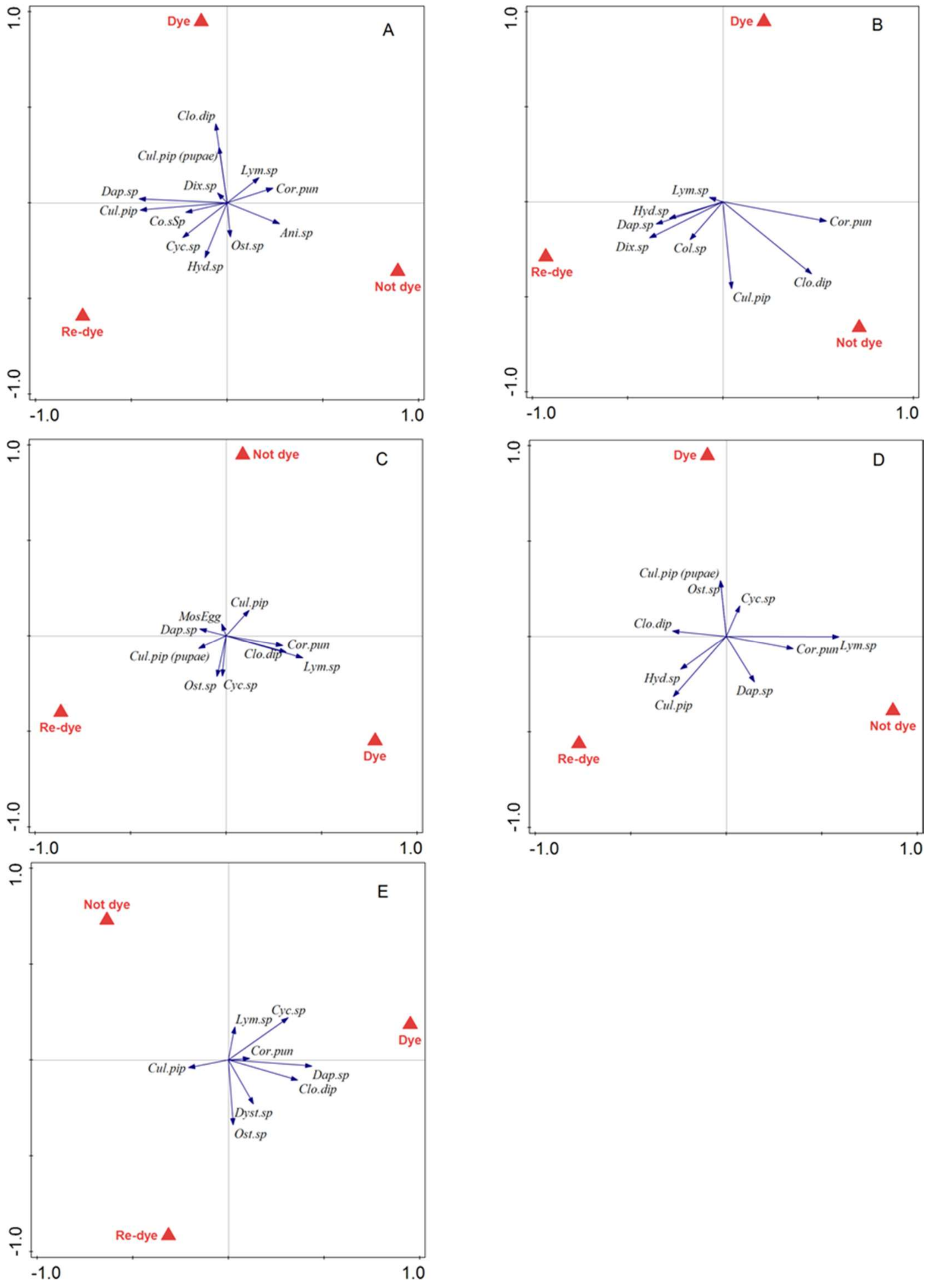

Figure 5.12. RDA Constrained ordination analysis from samples one week in five of the sampling period. All three treatments are ordinated against a sub-set of the study taxa. A - Week 1. B - Week 5. C - Week 10. D - Week 15. E - Week 19.

5.4 Discussion

Algae are at the base of the food chain in aquatic ecosystems and are an important food source for aquatic organisms living in ponds, lakes and streams, they also generate oxygen which plants, fish and other aquatic organisms depend upon (Addy and Green, 1996). Algal densities play a role in the aquatic ecosystem, influencing the distribution and abundance of macroinvertebrates (Fuller *et al.*, 1986; Addy and Green, 1996). Lesser water boatmen, mayfly larvae, *Daphnia*, mosquito larvae, Bloodworms, *Cyclops* and pond snail are herbivorous and most of their larval stages feed on organic debris, detritus and algae (Kriska, 2013; Becker *et al.*, 2010; Forró *et al.*, 2008; Fryer, 1987; Sanseverino and Nessimian, 2008; László *et al.*, 1987; Brönmark *et al.*, 1991). Therefore the expectation was that addition of a dye that blocked algal growth to the ponds would have a significant impact on the pond community. A constrained RDA found mixed impacts from dye treatments and seasonality in macroinvertebrates communities.

Black DyoFix dye did not affect macroinvertebrate species diversity and evenness. These results are in contrast to those presented in Chapter 4 where evenness and diversity were significantly higher in treated than in untreated ponds. A possible explanation might be that black DyoFix did not affect all algae species present in the ponds during the experiment. Spencer (1984) observed *Anabaena flos-aquae and Scenedesmus quadricauda* presented a small reduction at 1 ppm but at 3 ppm the rate growth of the algae were reduced at least 50%.

Daphnia was the most abundant animal in the ponds and their reduction in number in the previous experiment was given as an explanation for the differences observed. They have been shown to cause order-of-magnitude biomass reductions in phytoplankton and are often the most significant herbivore in the water (Lampert *et al.*, 1986). They are generally relatively unselective feeders and can affect both protist and bacterial assemblages which secondarily effect other zooplankton (Zöllner *et al.*, 2003). *Daphnia* can also determine water quality as a direct consequence of their consumption of algae. Anthropogenic induced changes in growth, behaviour and reproduction can all reduce their competitive ability relative to other zooplankton species, altering population dynamics (Hanazato and Dodson, 1995). Therefore if dyes are deleterious to *Daphnia*, changes induced by their presence are likely to drive changes in community composition. In chapter 4 a reduction in the number of *Daphnia* was associated with a change in biodiversity and community composition, whereas here, no differences in *Daphnia* numbers or biodiversity were found. The results in this chapter showed no significant impact of the dye on *Daphnia* abundance or on most of the taxa present. This suggests that dyes are not having the deleterious effects mentioned above.

Pond snail numbers appeared to be influenced by dye in both this and the work in chapter 4. Pond snails are generally herbivores and graze on periphytic algae (Brönmark *et al.*, 1991; Osenberg, 1989), but so do Mayfly larvae which did not have a negative effect by the dyes (McCafferty and Provonsha, 1986; Campbell, 2012). If dyes were successful in reducing algal abundance, they would likely increase competition for food. Previous studies have reported that chlorophyll “a” and “b” decrease with increasing concentrations of commercial blue and brown aniline dye added to pond water (Buglewicz and Hergenrader, 1977). Primary productivity is decreased in these conditions, affecting algae and aquatic macrophytes, due to a reduction in light intensity in the water (Buglewicz and Hergenrader, 1977). Dyofix dyes claim to control the growth of unicellular green algae by reducing the pigment complex-chlorophyll and by reducing the penetration of light (DyoFix, 2015c). However, we have no evidence that algal abundance was reduced and some studies have suggested that wavelength-blocking dyes are not very effective in controlling algae or in reducing chlorophyll a (Tew, 2003; Spencer, 1984).

In both chapters, lesser water boatman numbers fell in dye-treated waters. These diurnal bugs must re-surface regularly in order to replenish the air stored under their abdomen, a behaviour that exposes them to predators (Frolov, 2015). They have excellent vision and relatively large eyes (Frolov, 2015), not for predation, since they are detrivores, but to escape predation. The lower abundance of these animals in treated ponds is probably a behavioural escape response (they can fly) to find clearer and safer water. Further studies are, therefore, needed to understand the wider consequences on species, individuals and communities in control or field conditions of the addition of dye treatment chemicals.

In Chapter 2, I demonstrated that black dye (DyoFix) is an oviposition attractant for *Culex spp.* gravid females and that it has a significant negative impact on mosquito survival under controlled conditions. However, in a “natural” water butt freely colonised by other macroinvertebrates, no differences were found between treatments. No evidence of severe toxicity to *Culex* larvae over 48 h dyes was found in laboratory tests (data not shown). The results in this chapter confirm that dye has no measurable impact on mosquito numbers when in a natural environment. There have been many instances where laboratory experiments yield very different results to field experiments (Campbell *et al*., 2009). In this instance, it could be because dyes are affecting some other part of the ecosystem that was not measured, causing a secondary effect. The controlled experiments on dye were to allow the manipulation of variables in order to measure the effect of the dye. Experiments were designed to exclude heterospecific interactions in order to concentrate on the effect on *Culex*.

The results of this study, that black dye does not have an effect on biodiversity and abundance in most of the aquatic invertebrates studied when applied to an established pond community, might be explained by adaptation of the macroinvertebrates to the presence of the pond dye. Organisms could respond to the dye by changing behaviour (predation, competition or foraging strategies) (Wong and Candolin, 2015). *Daphnia* in particular are known to display phenotypic plasticity in response to chemicals such as toxins released by cyanobacteria (Fradkin and Gilbert, 1996) and fish kairomones (Stibor and Navarra, 2000). Lampert (1994) describes behavioural changes in *Daphnia* under extreme conditions, such as low algae concentrations, *Daphnia* will grow slowly and produce fewer neonates. Korinek *et al.* (1986) support these findings, reporting a morphological change in the third and fourth pairs of legs, used as large filter combs, at low concentrations of food in daphnids.

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Chapter 6: Predation of *Culex quinquefasciatus* and wild mosquito larvae and functional response of *Chaoborus flavicans* and *Gammarus pulex* in presence of black dyes.

CHAPTER 6. Predation of *Culex quinquefasciatus* and wild mosquito larvae and functional response of *Chaoborus flavicans* and *Gammarus pulex* in presence of black dyes.

6.1. Introduction

Pond dyes are cosmetic products commercially available for use in domestic ponds and in large water bodies. They are used to improve appearance by creating a black, reflective surface: they also inhibit algal growth through the disruption of photosynthesis, blocking light in the red portion of the spectrum. Dye products are marketed as environmentally friendly and manufacturers claim they are harmless to wildlife, specifically fish, plants and insects (DyoFix, 2015b). However, the results in chapter 4 revealed that pond dyes are associated with changes in the diversity and abundance of macroinvertebrate species and that competition and predation may be affected by their application. This result was not confirmed when the work was repeated using a different experimental design (Chapter 5). When a more natural community was treated with the pond dye, there were no significant differences in the biodiversity, evenness and abundance of individual species as a result of the application of dye colours. Most differences observed were related to the season rather than the dye.

Predation in freshwater habitats is considered one of the most important biotic interactions. Predation determines and regulates the species composition of communities (De Bernardi, 1981; Fischer *et al.*, 2013) and a large number of natural mosquito enemies have been described in different habitats supporting their eggs, larvae and pupae. Copepods (Schaper, 1999; Dida *et al.*, 2015), Hemiptera (Dida *et al.*, 2015), Coleoptera larvae (great diving beetle) (Onyeka, 1983), Odonata (dragonfly and damselfly) nymphs (Onyeka, 1983), fish

(*Gambusia* spp.) (Blaustein and Karban, 1989), amphibian tadpoles (Ramsdale and Snow, 1995) and *Gammarus duebeni* (amphipod crustaceans) (Roberts, 1995) have all been reported as predators of mosquito larvae and pupae. *Gammarus* is widely distributed in freshwater habitats of northern Eurasia and America, including still, running and brackish waters (MacNeil *et al.*, 1997; Sutcliffe, 2010). *Gammarus pulex, G. lacustris* and *G. duebeni* are common species in England (Dick, 2008; Hynes, 1954; Hawksworth, 2003). A few studies have shown that *G. duebeni* (Roberts, 1995) and *G. pulex* (Service, 1977) can be predators of mosquito larvae.

Chaoborus (phantom midge, glassworm) is a common dipteran in semi-permanent and permanent water bodies (Becker *et al.*, 2010). *Chaoborus* has predatory aquatic larval stages (Borkent, 1980) which are important primary predators in ponds and lakes (Schröder, 2013; Cressa and Lewis, 1986). *Chaoborus* spp. larvae feed on a wide range of prey (Repka *et al.*, 1995; Boucher, 1988; Moore, 1988) including *Daphnia* spp. (Repka *et al.*, 1995), *Bosmina longirostris* and *Ceriodaphnia reticulata* (Boucher, 1988) and rotifer species including *Synchaeta pectinata, Keratella testudo, and K. cochlearis* f. *typica* (Moore, 1988). A few studies have reported that *Chaoborus* larvae are mosquito predators (Sailer and Lienk, 1954; James and Smith, 1958; Helgen, 1989). *Chaoborus* midge larvae were found in high numbers in the water butts in Chapter 2 and it was hypothesised that their presence may have reduced the number of larvae found (data not shown). Therefore this final experimental chapter of the thesis aimed to look at the relationship between mosquito larvae predation and the presence of dye.

6.1.1. Functional Responses

In the late 1950's, Crawford Stanley Holling proposed a theory of how prey density is related to a predator's rate of prey capture (Denny, 2014). The theory, named "Functional Response", describes the relationship between the average number of prey eaten by a predator (individual rate of consumption) and prey density (Weterings *et al.*, 2015; Holling, 1966; Holling, 1959).

The functional response is classified into three types, according to the saturation point, where no more prey are consumed by an individual predator. The functional response regulates the population dynamics of predator-prey relationships in an ecosystem (Ghosh and Chandra, 2011; Schenk and Bacher, 2002). Type I functional response is a linear regression between prey density and the number of prey consumed (Figure 6.1), this means that there is not as the prey density increase, the predator rate on prey does not show a gradual saturation (Seo and DeAngelis, 2011; Dawes and Souza, 2013). A Type II functional response shows that the number of prey eaten by the predator at the start rises faster as the density of prey increases but then is a saturation point where the curve decreases with increasing the prey density. The saturation point is the maximum number of prey that a predator can eat. This type of functional response is considered to have the potential to destabilise prey populations since high levels of predation at low prey densities can be detrimental (Dawes and Souza, 2013). Type III functional response is similar to the type II, however, initially a low prey consumption is observed, followed by rapid predation, may result in stability of prey numbers, by providing refugia at low densities (Holling, 1959). It might assume that depending on how dense the prey populations are, the predators may make choices (Ganter and Peterson, 2006; Ganter and Peterson, 2005).

In biocontrol programs, the functional response can determine the efficiency of a predator in controlling prey populations, describing the rate at which a predator kills the prey in different prey densities (Murdoch and Oaten, 1975). O'Neil (1990) explained that the search, capture and handling time might be an important and informative resources in biological controls. One of the reasons is that as the density of a prey increases, less time will be invested on searching but the time on handling time increases (Ganter and Peterson, 2006). The handling time is the time that predators spend on manipulating, killing and eating the prey (Ganter and Peterson, 2006).

This chapter investigates whether predation is affected by dye application in aquatic habitats by considering the effect of black pond dye on the Functional Response in *Chaoborus flavicans* and *Gammarus pulex* as predators on immature stages of the prey species wild *Culex* spp. and *Culex quinquefasciatus*.

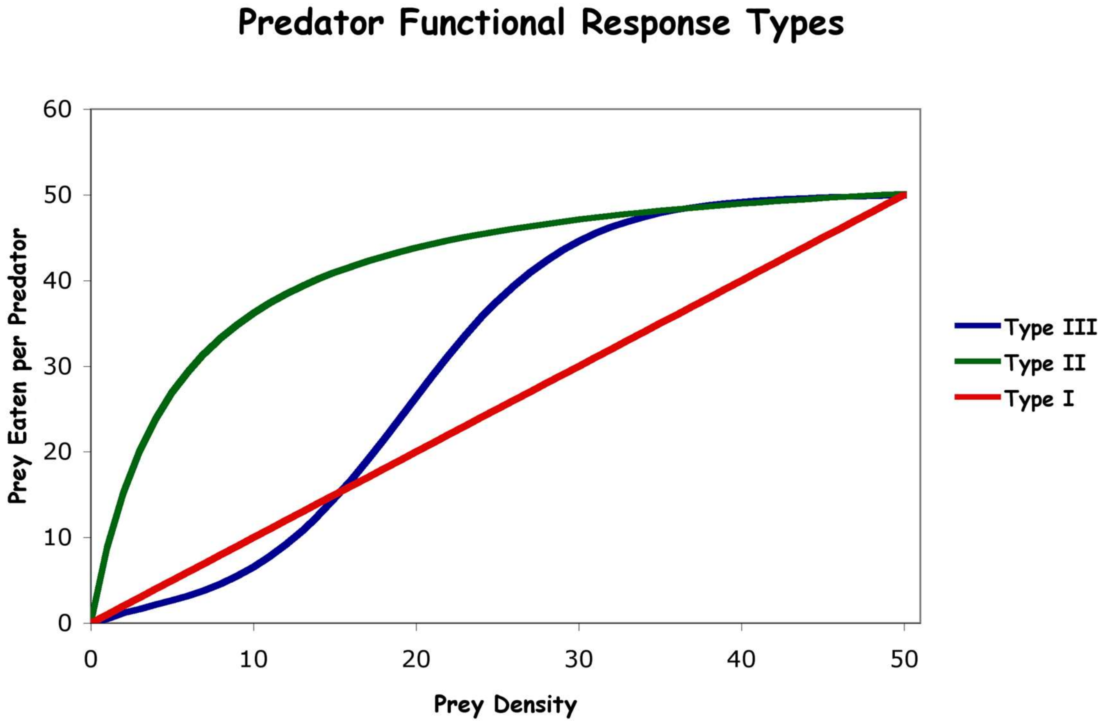

Figure 6. 1. Predator functional response types (Ganter and Peterson, 2006).

6.2 Methods

6.2.1 Study site, animal collection and maintenance

6.2.1.1 *Gammarids*

G. pulex were sampled from streams located in Dinton Pastures Country Park, Wokingham, Berkshire, England (51.4399° N, 0.8726° W) (Figure 6.2). Gammarids were collected using 3-minute kick-sampling with a pond net (Figure 6.3); then they were transferred to 1 L plastic bottles filled with stream water and some aquatic plants for transportation (Sago Pondweed). *G. pulex* was maintained in laboratory conditions (19 ± 2°C), in normal light/dark photoperiod (16:8 h) at the University of Reading. Gammarids were maintained in groups of maximum 40 individuals to avoid overcrowding and cannibalism in 5 L tanks with food (aquatic weed and stones) and deionized tap water (RO water). The RO water was continuously aerated using an air pump before and after adding the *G. pulex* to the tank.

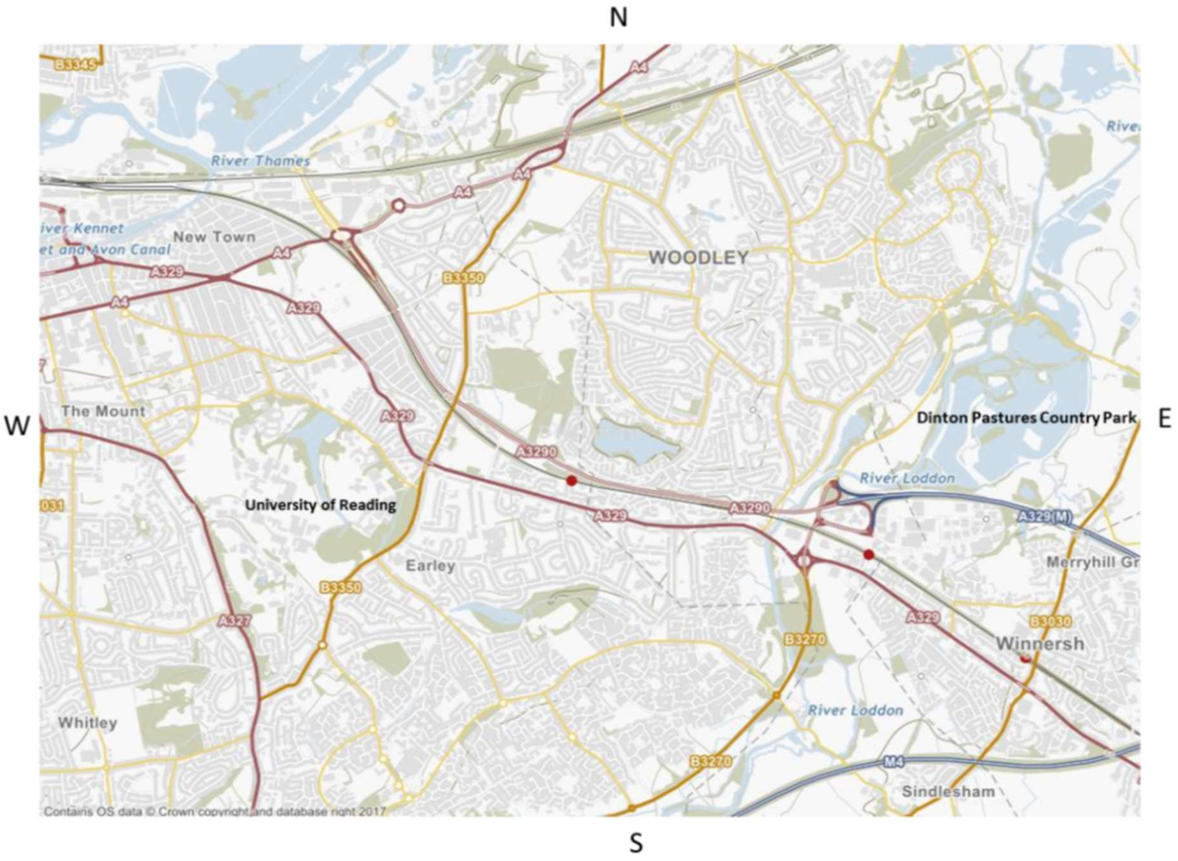

Figure 6. 2. Dinton Pastures Country Park, Wokingham, Berkshire, England (51.4399° N, 0.8726° W). Map from Digimap: http://digimap.edina.ac.uk.

Figure 6. 3. *Gammarus pulex* collection. The author collecting *G. pulex* using a pond net and kick-sampling.

6.2.1.2 *Chaoborus flavicans*

Approximately 200 individuals of *C. flavicans* larvae (Figure 6.4) were purchased mail-order from a commercial supplier (Northampton Reptile Centre). The larvae were reared in laboratory conditions (19 ± 2°C), in normal light/dark photoperiod (16:8 h) in 5 L tanks containing deionized tap water (RO water) and fed, as recommended by the supplier, on "jelly pot" comprising honey, lactic acid and fruits including mango, melon, banana and strawberry. Populations were maintained at 200 individuals maximum.

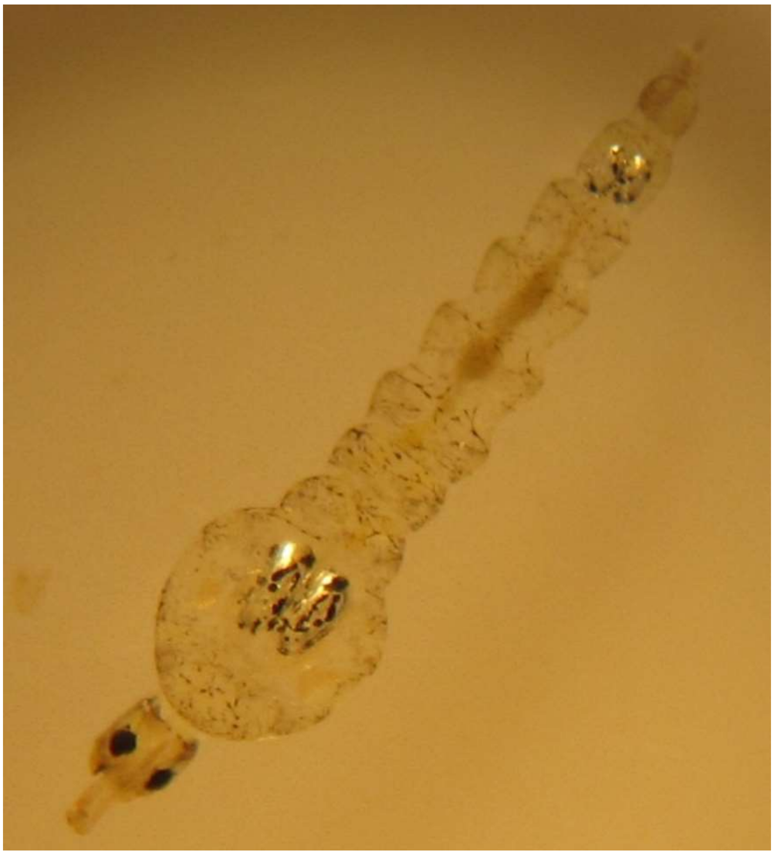

Figure 6. 4. *Chaoborus flavicans* larva**.**

6.2.1.3 Wild *Culex* egg batches collection

Wild *Culex pipiens (S.L.)* egg batches (Figure 6.5 A) were collected from water butts in the glasshouse area of the Whiteknights campus of the University of Reading, Berkshire, England (51.4419° N, 0.9456° W) using a modified larvae net using a plastic spoon adapted with a fine mesh from net used to cover mosquito cages (Figure 6.5 B). Egg batches were transferred to 200 ml plastic tubs (Figure 6.5 B) containing a small amount of water. They were then transferred to laboratory conditions (19 ± 2°C) and normal light/dark photoperiod (16:8 h). The egg batches were moved into a 2 L plastic containers (10 x 10 x 22 cm) containing 1.5 L of tap water and allowed to hatch. Larvae were fed 1.2 g of guinea pig food per 2 L plastic container every other day. Five egg batches maximum per container were maintained, to avoid overcrowding of larvae.

Figure 6. 5. Egg raft and holding tub and spoon. **A**. Eggs batches from wild mosquitoes collected from bins on the University of Reading Campus, **B**. The adapted spoon and tub, with some egg batches already in place.

6.2.1.4 *Culex quinquefasciatus* colonies

Culex quinquefasciatus were originally collected in Cyprus in 2005 by Dr A. Callaghan and have been reared in laboratory conditions since then. The mosquito samples were reared in net cages (30 x 30 x 30 cm) kept in a constant temperature-humidity room CTH, (Temp 25 ± 0.2 °C, RH 70 ± 5%) under a 16:8 light:dark photoperiod regimen. Adult mosquitoes were maintained at densities of 100-200 adults per cage and fed on a 10% sucrose solution from Fisher-Scientific. Blood feeding was facilitated through an artificial membrane feeding system (Hemotek) (Figure 6.6 D) using a parafilm membrane and a power unit connected to five heated cylinders (blood feeders) supplied by Sigma-Aldrich (Figure 6.6 A-B). Cylinders had a blood reservoir at the top protected by a parafilm membrane sealed with an O ring (Figure 6.6 B-C). Adult female mosquitoes were fed with defibrinated horse or chicken blood cells in Alsevers solution (TCS Bioscience, Botolph, Claydon, Buckingham, MK18 2LR, UK) two to three times per week for general colony maintenance (Figure 6.6).

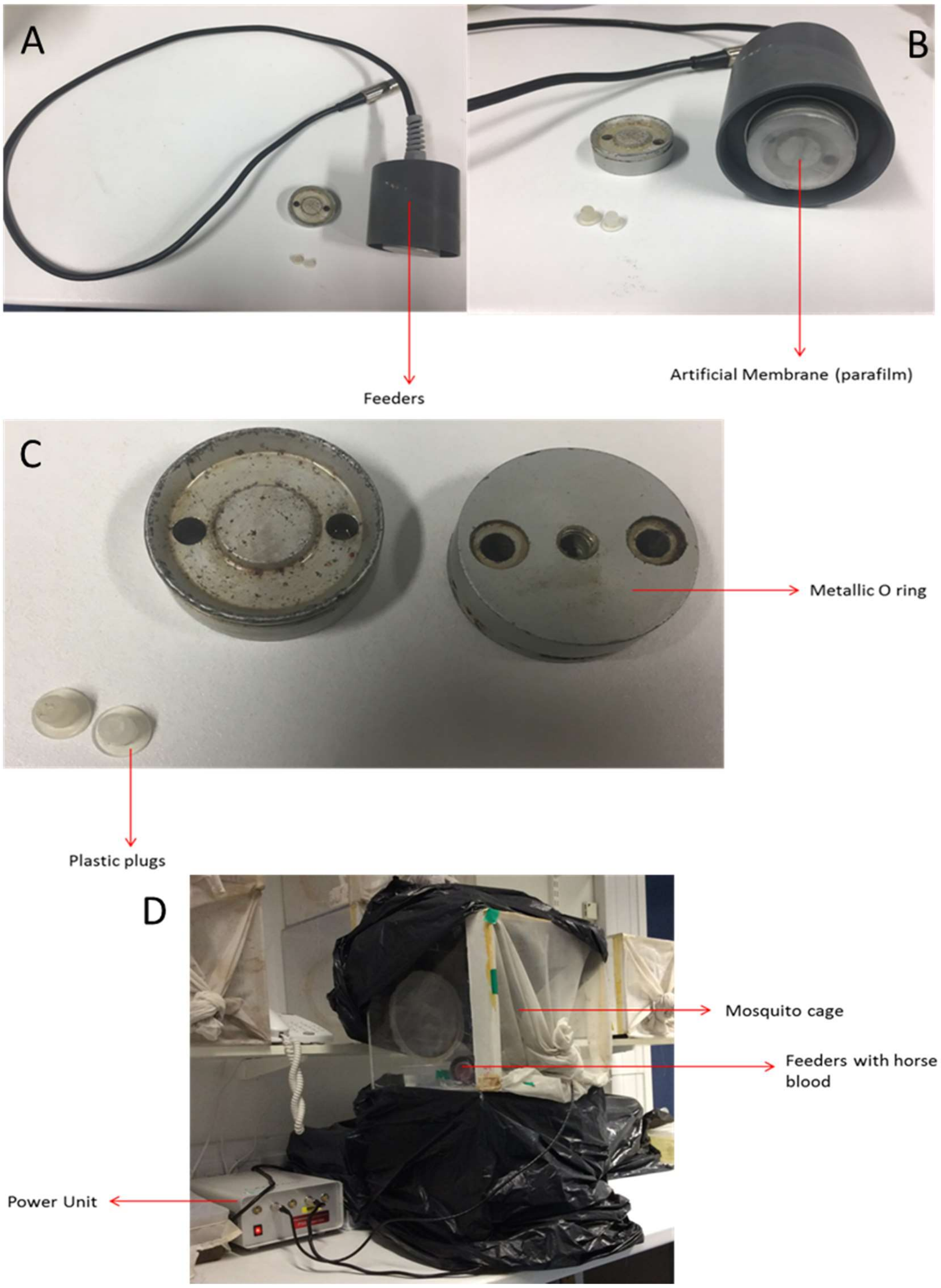

Figure 6. 6. Blood feeder. **A** (top left) whole feeder, metallic O ring detached. **B** (top right) with a metallic O ring in place and covered with the artificial membrane (parafilm). **C** (middle) the plastic plugs used to seal the metallic O ring. **D** the artificial membrane feeding system (Hemotek) in situ on a mosquito cage.

Immediately after blood feeding, a small cup (12 x 5 cm) containing tap water was placed in each cage, to encourage female mosquitoes to lay eggs. Five freshly laid egg rafts were placed in 2 L (14 x 21 x 110 cm) plastic containers with 1.5 L of tap water for hatching. Mosquito larvae were fed on guinea pig food pellets (Burgess, Yorkshire, England), approximately 1 pellet (1.2 g) per container every other day. The plastic containers were cleaned when their water became cloudy, the content was sieved through a plastic spoon with a fine net (Figure 6.5. B) and larvae transferred to a clean container with fresh water and food. Pupae were removed with a 3 ml plastic pipette and placed in cups with 200 ml of tap water in adult cages for emergence.

6.2.2 Experimental design

For both experiments a black dye solution was prepared according to the manufacturer's recommendation for domestic use, 0.3 g/L of black dye (DyoFix, 2015b) was added to RO water.

6.2.2.1 Predation of *Gammarus pulex* on *Culex* wild mosquito larvae.

To determine the Functional Response and assess the number of mosquito larvae that *G. pulex* consumes in different dye treatment conditions, two experiments were performed: trials were run with and without dye and in the presence and absence of the predator. The second experiment was a repetition of the first experiment using the same conditions and densities of mosquito larvae.

Gammarids size matched to 1.5-2 cm body length were selected for the experiments and a sample of 30-35 individuals was moved to a 5 L container (30 x 20 cm) containing aerated RO water but no food and starved for 24 h before the trials.

The experiments were run at 19 ± 2 °C with 2-4th instar mosquito larvae at six different densities: 1, 3, 6, 20, 30 and 40 (n = 3 replicates per experimental treatment). Experiments were undertaken in 50 ml glass beakers containing 40 ml of aerated RO water with and without the dye. *Cx. pipiens* larvae (1st-3rd instar) were transferred to separate beakers, followed one hour later by *G. pulex*. Individuals and beakers were allocated randomly. After 24 h, predators were removed and the number of mosquito larvae in each beaker recorded, with absent larvae counted as prey.

6.2.2.2 Predation of *Chaoborus flavicans* on wild *Culex pipiens* and *Cx. quinquefasciatus* larvae.

To determine the Functional Response and assess the number of mosquito larvae that *C. flavicans* consumes in different dye treatment conditions, two experiments were performed: trials were run with and without dye and in the presence and absence of the predator.

C. flavicans larvae selected for the experiment were those beneath the water surface, to avoid individuals in bad condition. Individual larvae were transferred to 20 ml glass beakers with RO water but no food and starved for 24h before the trials. *Cx. pipiens* and *Cx. quinquefasciatus* larval mortality was measured with and without dye and with and without *C. flavicans*.

Experiment 1, to determine the functional response of *C. flavicans* without dye, was undertaken in 50 ml glass beakers containing 40 ml of RO water. The experiment was run at 19 ± 2 °C with 2-4th instar of *Cx. quinquefasciatus* larvae at "high" (2, 4, 10, 20), "medium" (2, 4, 6, 10) and "low" (1-2-4-6) (n = 3 replicates per experimental treatment), mosquito densities. Individuals and beakers were allocated randomly. After 24 h, predators were removed and the number of mosquito larvae in each beaker recorded, absent larvae were counted as prey.

Experiment 2, both wild *Cx. pipiens* and *Cx. quinquefasciatus* larvae were used to test the functional response in both dyed and undyed treatments with *C. flavicans*. Larval densities were 6, 15, 20, 30 and 40 (n = 4 replicates per experimental treatment). Individuals of the first and second stage mosquito larvae were deposited randomly into 50 ml glass beakers containing 40 ml RO water or dye treatment and *C. flavicans* added later as previously.

6.2.3 Statistical Analyses

All statistical analyses were performed using R version 3.4.2 (R Development Core, 2015). To derive functional responses, we used a logistic regression of prey killed as a function of prey density. A Type II Functional Response is evident when results show a significant negative first order term, while a Type III response is evident from a significantly positive first order linear coefficient, followed by a significantly negative second order coefficient (Trexler *et al.*, 1988; Juliano, 2001). To account for conditions in non-prey replacement we used the FRAIR package in R. Attack rate ‘a’ and handling time ‘h’ values were estimated using Roger’s equation for prey depletion (Juliano, 2001).

$$N_e = N_0 (1 - \exp (a (N_e h - T)))$$

Where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack constant, h is the handling time and T is the total experimental period, for these experiments T is 24h. Because of the implicit nature of the random predator equation (Rogers, 1972), which does not have a single, readily determined solution, the Lambert W function (Bolker, 2008) was used in R, to estimate attack rate, handling time and maximum feeding rate for 95% confidence intervals around FR curves, data was bootstrapped ($n = 2000$). Finally, overall consumption with ‘predator’, ‘dye’, and ‘supply’ as factors was analysed using a generalised linear model (GLM) and ANOVA to account for overdispersion in the model and assuming a Poisson distribution.

6.3 Results

6.3.1 Functional Response- predation by *Gammarus pulex* on *Cx. pipiens* wild mosquito larvae.

Predation of mosquito larvae by *G. pulex* was noted in casual observations during the trials, however, no functional responses were detected and there was no statistically significant relationship between predation and prey densities of 1, 3, 6, 20, 30 or 40 in *Cx. pipiens*. In the first experiment, more wild larvae mosquito were consumed by the predator in the undyed treatment than the dyed, but the difference was not significant ($F_{1,5} = 1.119$, $P = 0.294$) (Figure 6.7 A). In contrast, in the second experiment, the number of larvae consumed by *G. pulex* was higher and significant in the dyed treatment ($F_{1,5} = 12.900$, $P < 0.001$) (Figure 6.7 B). A significant effect of predation was found when the prey supply was high in the wild mosquito larvae in both experiments (Table 6.1).

Table 6. 1. Results of the logistic regressions for the effect of predation by *G. pulex* on wild mosquito populations in high prey supply conditions; both experiments.

	Prey supply	Estimate	Sd. Error	t value	*P*
First experiment	6	1.658	1.357	1.222	$P = 0.226$
	10	1.91	1.333	1.433	$P = 0.157$
	20	2.546	1.291	1.971	$P = 0.053$
	30	2.918	1.277	2.285	$P = 0.025$
	40	3.418	1.264	2.704	$P = 0.009$
Second experiment	6	0.55	0.3566	1.543	$P = 0.129$
	10	1.1421	0.3261	3.502	$P < 0.001$
	20	1.8028	0.3064	5.883	$P < 0.001$
	30	1.9071	0.3043	6.267	$P < 0.001$
	40	2.4159	0.2963	8.153	$P < 0.001$

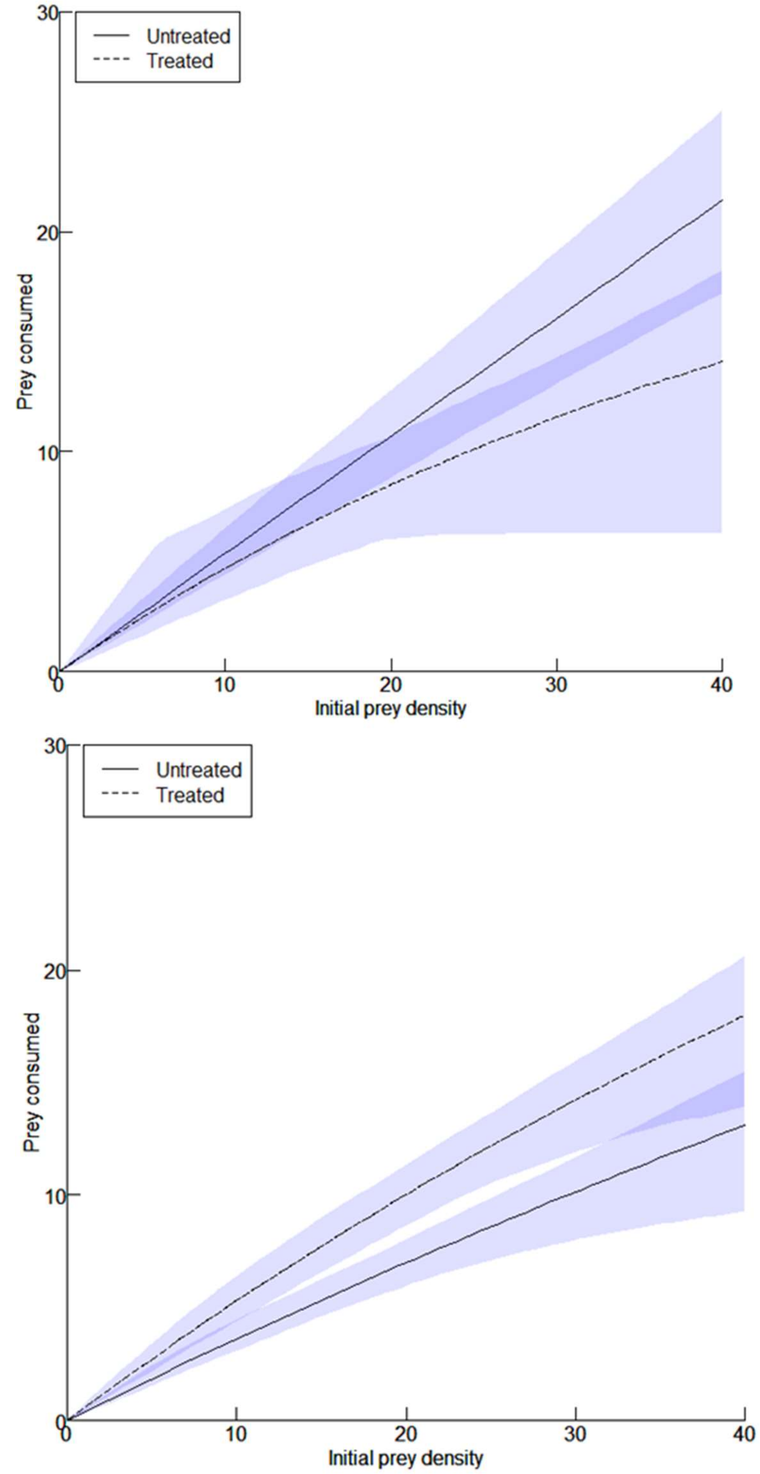

Figure 6. 7. Functional response of *G. pulex* in presence of *Cx. pipiens* wild mosquito larvae at four different prey densities, in two different experiments. **A** - First experiment. **B** - Second experiment.

6.3.2 *C. flavicans* predation of *Culex quinquefasciatus* larvae

C. flavicans was observed killing and eating mosquito larvae during the experiment (Figure 6.8). Survival of prey in the control was > 95% in all experiments. Experimental deaths were attributed to predation by *C. flavicans* because some *Cx. quinquefasciatus* larvae were counted as missed and partial consumption was observed after the 24 h.

A Type II functional response was observed at the highest density of mosquito larvae (Figure 6.9 C), GLM results of consumption of mosquito larvae by the predator in the high mosquito density were statistically highly significant ($z = -3.567$, $P < 0.001$). However, at medium and low larvae densities, the GLM found no statistically significant differences in the number of larvae consumed by *Chaoborus* ($z = -1.655$, $P = 0.098$, $z = -0.383$, $P = 0.702$, respectively), indicating that no functional response was detected (Figure 6.9 A-B).

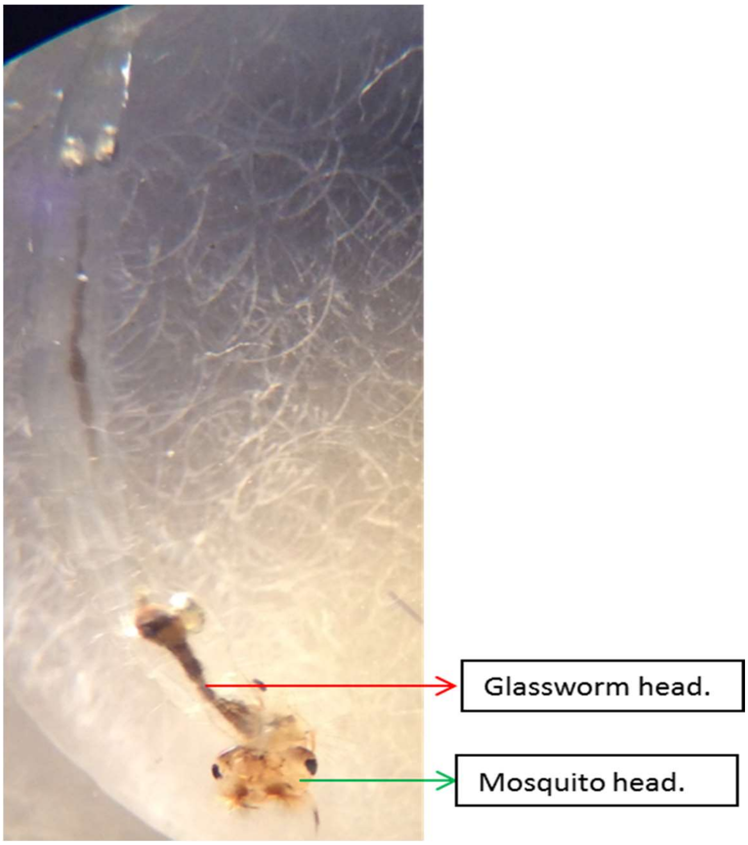

Figure 6. 8. Prey consumption (mosquito larvae) by *Chaoborus flavicans*. Observational evidence indicating that *Chaoborus* preys upon mosquito larvae under experimental conditions.

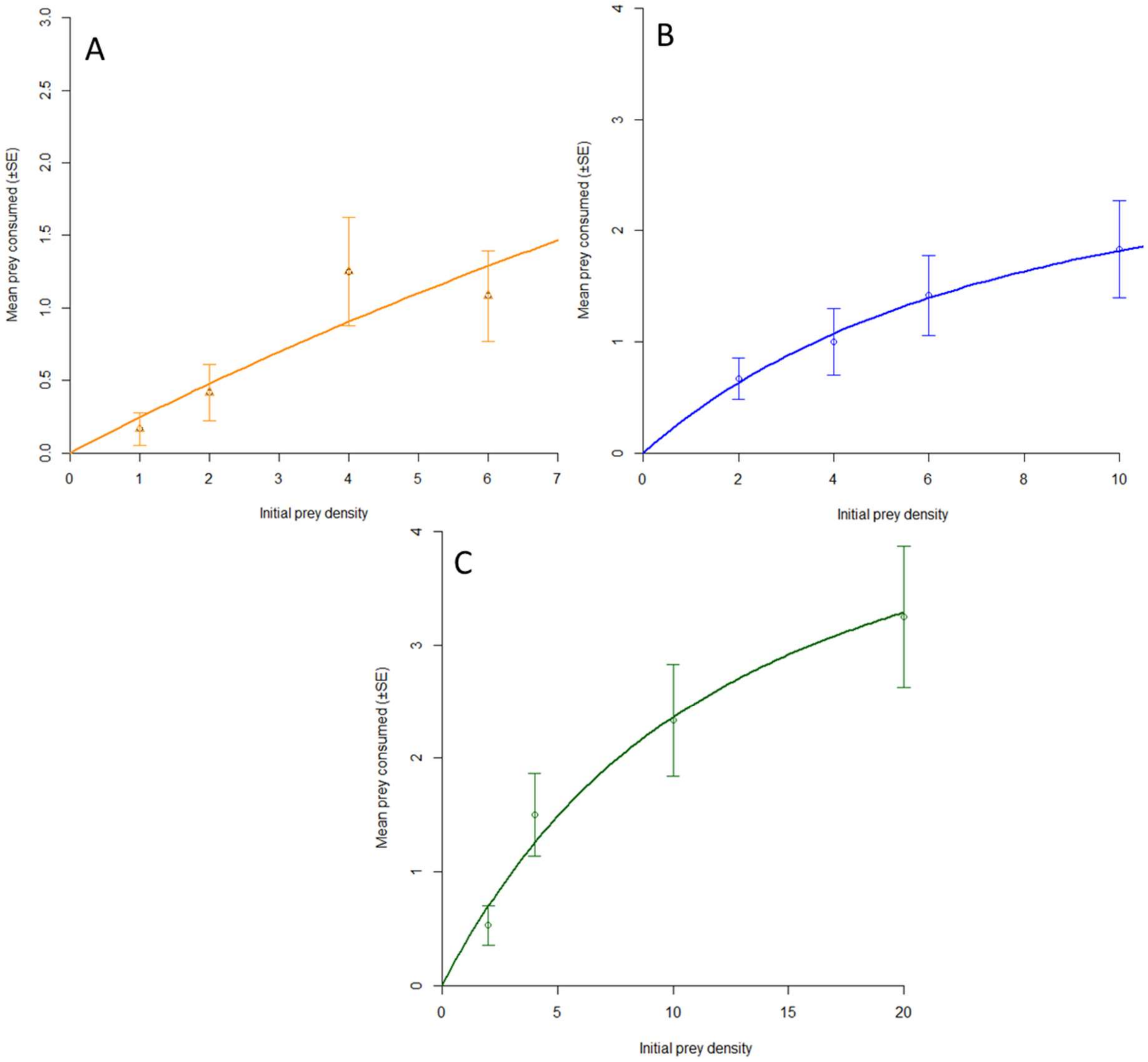

Figure 6. 9. Functional response of *Chaoborus flavicans* and *Cx. quinquefasciatus* larvae. Functional Response of *Chaoborus flavicans* and mosquito larvae at **A** - low, **B** - medium and **C** - high mosquito densities. Bars represent the error bars

6.3.3 *C. flavicans* predation on *Cx. quinquefasciatus* and *Cx. pipiens* wild mosquito larvae with and without dye.

Survival of prey in the control was > 86 % in all treatments. Experimental deaths were attributed to predation by *C. flavicans.*

The number of prey consumed was higher in wild mosquitos compared with *Cx. quinquefascitus* (Figure 6.9), however, no differences were observed between the numbers of wild mosquito and *Cx. quinquefasciatus* larvae consumed by *C. flavicans* in dyed and undyed treatments (t = -0.509, *P* = 0.612).

Significantly different numbers of *Cx. pipiens* and *Cx. quinquefascitus* larvae were predated by *C. flavicans* at 10, 20, 30 and 40 prey densities in dye treated conditions (10 larvae - t = 2.288, *P* = 0.028; 20 larvae - t = 2.235, *P* = 0.031; 30 larvae - t = 2.118, *P*= 0.040; 40 larvae - t = 2.235, *P* = 0.031). In undyed treatments of 20, 30 and 40 initial prey densities, the numbers of mosquito larvae consumed by *C. flavicans* were also significantly different between *Cx. quinquefascitus* and *Cx. pipiens* (20 larvae - t = 2.328, *P* = 0.056; 30 larvae - t = 2.169, *P* = 0.037; 40 larvae - t = 2.501, *P* = 0.017).

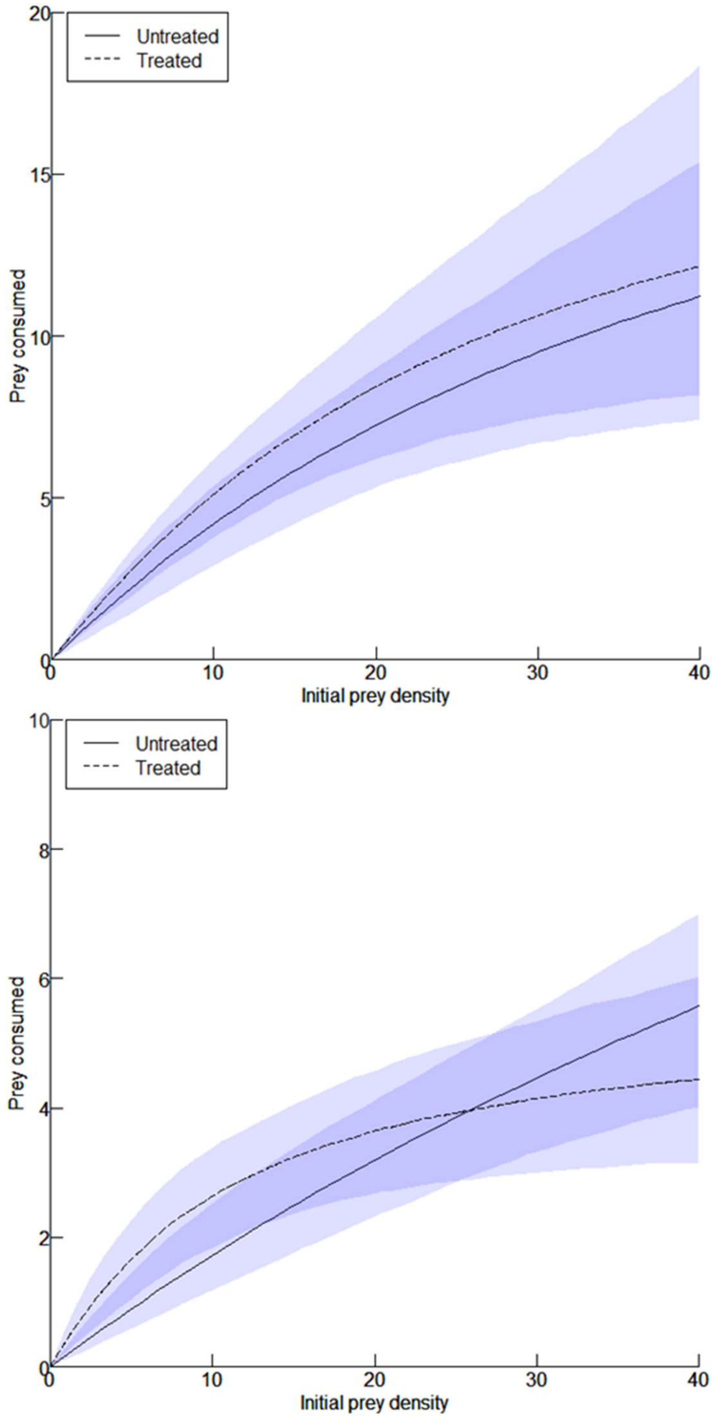

Figure 6. 10. Functional response of *Chaoborus flavicans* in the presence of wild *Cx. pipiens* and *Cx. quinquefasciatus* larvae at four different prey densities. **A**. wild *Cx. pipiens*. **B**. *Cx. Quinquefasciatus*

A Type II functional response was observed in both dyed and undyed treatments of wild mosquito larvae. No functional response was detected for predation of *Cx. quinquefasciatus*. Time spent pursuing, subduing and consuming each prey (handling time) and attack rate were shown, following calculation of the Lambert W function, to be significantly different between mosquito densities in both dyed and undyed treatments in wild *Cx. pipiens* mosquito larvae, but in *Cx. quinquefasciatus,* although attack rate remained significant, handling time was not significantly different in dyed and undyed treatments (Table 6.2).

Overall, prey consumption was not significantly different between undyed and dye treatments in *Cx. quinquefasciatus* or *Cx. pipiens* larvae (*Cx. quinquefasciatus* - $F_{1,6} = 0.199$, $P = 0.658$; *Cx. pipiens* - $F_{1,5} = 0.199$, $P = 0.468$), however, consumption increased significantly under increasing supply in both prey species (*Cx. quinquefasciatus* - $F_{1,6} = 9.913$, $P < 0.001$; *Cx. pipiens* - $F_{1,5} = 20.2754$, $P < 0.001$). Prey density had a significant impact on predation of mosquito larvae by *C. flavicans* (Table 6.3).

Table 6. 2. Results of the logistic regression of prey killed by *C. flavicans* as a function of prey density with attack rate and handling time. The P value for *Cx. quinquefasciatus* handling time in the undyed treatment indicates the relationship is not significant. *No Functional Response in *Cx. quinquefasciatus* in undyed treatment.

Species	**Treatment**	**First order term (P)**	**Attack rate (a)**	**Handling time (h)**
Cx. pipiens	Dyed	z = -4.223, *P* < 0.001	z = 4.689, *P* < 0.001	z = 4.583, *P* < 0.001
	Undyed	z = -2.090, *P* = 0.037	z = 3.831, *P* < 0.001	z = 2.556, *P* < 0.001
Cx. quinquefasciatus	Dyed	z = -4.400, *P* < 0.001	z = 3.095, *P* < 0.001	z = 4.471, *P* < 0.001
	Undyed	No FR*	z = 3.265, *P* < 0.001	z = 0.852, *P* = 0.394

Table 6. 3. Results of the logistic regression of the effect of initial prey supply of wild *Cx. pipiens* and *Cx. quinquefasciatus* on predation by *C. flavicans*.

Species	**Prey supply levels**	**P value**
Cx. quinquefasciatus	6	z = 2.078, *P* = 0.038
	10	z = 3.313, *P* < 0.005
	20	z = 3.894, *P* < 0.001
	30	z = 3.658, *P* < 0.001
	40	z = 4.059, *P* < 0.001
***Cx. pipiens* Wild mosquito**	6	z = 2.413, *P* = 0.203
	20	z = 3.063, *P* < 0.005
	30	z = 3.150, *P* < 0.005
	40	z = 3.185, *P* < 0.005

6.4 Discussion

C. flavicans and *G. pulex* are efficient predators of *Cx. quinquefasciatus* and wild *Culex pipiens* in both dyed and undyed treatments. Both predators exhibited an increase in consumption under increasing prey supply, with a Type II functional response evident in *C. flavicans* in both treatments, dyed and undyed. However, no functional response was observed in any of the experiments involving *G. pulex*. No differences in the number of larvae consumed by either predator, *C. flavicans* or *G. pulex,* were detected between the dyed and undyed treatments.

In the last few decades, *G. pulex* and *G. duebeni* have been reported as mosquito predators in the UK (Roberts, 1995; Service, 1977; Medlock and Snow, 2008). Service (1977) found evidence of *G. pulex* as a predator of *Aedes cantas* (Synonym *Ochlerotatus cantans*) and Lockwood (1986) and Roberts (1995) investigated the rate of feeding in mosquito larvae by *G. duebeni* in Hampshire, England, both studies found this brackish-water amphipod to be an efficient predator of *Aedes* larvae.

G. pulex is an invasive species in Northen Ireland and has been shown to negatively affect native species including *G. duebeni*, mayfly nymphs (*Baetis rhodani*) and blackfly larvae (Simuliidae) (Laverty *et al*., 2015; Dick, 2008). The normal habitat for this species is lakes and rivers, but it has been introduced in new habitats like ponds, where mosquito larvae are present (N. Ortiz pers. obs. October 2017). To date, there has been very little research of gammarids as predators of mosquito larvae. In Great Britain, in the last decade, no studies have been conducted which investigate the predatory potential of *G. pulex* on mosquito larvae. It is well documented that *G. pulex* is an aggressive species which can replace native species (*G. duebeni*) and drift native gammarids populations to extinction (Dick *et al*., 1995).

The results showed that, in laboratory conditions, gammarids can have a negative effect mosquito populations as a result of the aggressive behaviour, but more studies are needed to be undertaken to better understand how gammarids can impact mosquito populations.

In the trials, *G. pulex* showed a larvae consumption for 2nd and 3rd instars of *Cx. pipiens* wild mosquito and *C. flavicans* showed a preference for *Cx. pipiens* and *Cx. quinquefasciatus* 1st, 2nd and 3rd instars in both undyed and dyed treatments (N. Ortiz pers. obs. 2017). Similar predation behaviour was observed by Helgen (1989) who found that *Chaoborus americanus* preferred to consume *Aedes vexans* 1st, 2nd and 3rd instar with most rapid ingestion rates on *Aedes* 1st and 2nd instars (0.8-6 s). The differences in prey size preference by predators might be correlated to the differences in predator body size, since *G. pulex* is bigger (1.5-2 cm body length) than *C. flavicans* (1-1.5 cm body length).

Both predators were capable of consuming between 3 and 20 larvae in 24 h; these results confirm those of previous studies including Roberts (1995), and Sailer and Lienk (1954). Roberts (1995) observed that gammarids ate between 4 and 8 larvae in 24 h and Sailer and Lienk (1954) reported that fourth stage *C. flavicans* consumed 10 first stage *Aedes* larvae in laboratory conditions. They also described that, prior to pupating into adults, *C. flavicans* larvae killed some mosquito larvae without consuming them, this behaviour may be responsible for the otherwise unexplained deaths of some mosquito larvae, found after 24h, in these trials. Similar predatory behaviour was observed in *G. pulex* when *Cx. quinquefasciatus* was introduced into the trial containers (N. Ortiz pers. obs. 2017), some *Cx. quinquefasciatus* larvae were found dead after 24h, suggesting they may have been killed without consumption by *G. pulex*.

Sailer and Lienk (1954) also observed that *Chaoborus* larvae ate *Aedes* larvae tail-first, immediately after they were placed in the same container. In this experiment, it was observed that *Cx. pipiens* and *Cx. quinquefasciatus* were also attacked tail-first and then consumed by *C. flavicans* in all treatments (Figure 6.7). *G. pulex* attacked *Cx. pipiens* in the dorsal part of the thorax (segment I after the head).

A predatory behaviour observed in some *G. pulex* functional response studies is that a proportion of prey are only partially consumed. In this study, partial consumption was observed in gammarids, with a few tails and heads found at the end of the experiment. Paterson *et al.* (2015) considered that partial consumption was associated with high prey density. Similar results were described with the palaemonids (*Palaemonetes varians*) where mosquito larvae were killed and half-eaten (27-33 *Aedes* larvae were killed in 2h by palaemonids in laboratory conditions) (Roberts, 1995). Paterson *et al.* (2015) explained the partial consumption as a consequence of prey density since at high prey density (higher functional response) the selection by predators of the preferred prey body-part increases.

The primary objective was to determine if pond dyes can decrease mosquito predation by reducing the visibility of prey in the water. However, there appeared to be no impact of dye on either *Chaoborus* or *Gammarus* predation of *Culex* mosquito larvae. This may be because *Chaoborus* and *Gammarus* are non-visually hunting predators, instead, they may respond to vibrations in the water. Swift (1981) found that *Chaoborus* larvae possess mechanoreceptors that help to detect prey in the water, which supports this hypothesis.

Previous studies have shown that gravid *Culex* wild mosquitoes preferred to lay eggs in dye treated water in laboratory and semi-field conditions (Ortiz and Callaghan, 2017). Although the dyed water is a mosquito attractant in respect of oviposition, Ortiz and Callaghan (2017) observed that survivorship in wild *Culex* mosquitos was significantly reduced in black dyed water. Elsewhere our results show that, after the re-introduction of some aquatic species, the biodiversity and abundance of *Daphnia pulex*, lesser water boatman (*Corixa punctata*) and *Coleopteran* larvae were significantly reduced by the application of pond dye (Chapter 4). It is possible that, as a result of the reduction of algae, pond dye products might alter the dynamic of ponds and the interaction between individuals. However, black dyes do not directly affect the number of prey consumed by *Gammarus* or *Chaoborus* in experimental conditions.

Few studies in the functional response in small predator insects have been performed in field and laboratory conditions (Schenk and Bacher, 2002), most such experiments have been assessed in invasive predators like fishes or gammarids. Further work is needed to better understand the predatory activities of both predators (*C. flavicans and G. pulex*) because most of the gammarids studies have been examined the effect of the salt-marsh crustacean in the salt-marsh mosquito *A. detritus* larvae. *G. pulex* and *C. flavicans* are often described as animals that can be a potential mosquito predator and as natural enemies of mosquito larvae and pupae. As a result, these organisms may potentially contribute significantly to any strategy to control mosquito populations.

Chapter 7: General discussion

CHAPTER 7. General discussion

This study was conceived during a trip to Kingston Lacy (National Trust) on observing that the pond in their Japanese Garden was artificially dyed black to cosmetically enhance its beauty (Figure 7.1). It is widely acknowledged that dark or coloured waters are attractive to a variety of mosquito species for oviposition (Beckel, 1955; Harrington *et al*., 2008; Derraik, 2005; Torrisi and Hoback, 2013; Hilburn *et al*., 1983; Panigrahi *et al*., 2014). Therefore the first thought of a mosquito biologist on seeing the dyed pond was that it must surely be full of mosquitoes and was likely to provide an irresistible lure to gravid females.

Figure 7. 1. Japanese garden pond treated with black dye at Kingston Lacy, National Trust.

A small amount of research revealed that pond and lake dyes are widely used in small domestic ponds, at commercial lakes, at high profile garden shows like Hampton Court and have been featured on BBC Gardener's World in 2017. The dyes are marketed as non-toxic and environmentally friendly and the manufacturer states that they are free of herbicides, algaecides and pesticides (DyoFix, 2015b). However, toxicity is only one measure of effect on aquatic animals and plants. This thesis represents the first and only known study of the impact of this type of cosmetic pond dye on freshwater animals. The data in this thesis demonstrate that the although dyes appear to have no acute toxicity, they are not totally without effect.

7.1. Pond dyes and British Mosquitoes

A consequence of black pond dye use is potentially that of an increase in mosquito use of garden ponds for breeding, bringing mosquitoes in closer contact with humans. Mosquitoes are often presented with a choice between numerous water bodies in which to oviposit, particularly in urban and peri-urban environments where container-style habitats create dense arrays of potential patches. An important fact observed in urban areas is that the climatological change is linked to the growth in the urbanization. These climatic effects produce by urbanization have been documented in the past few years. Rural areas present a lower air and surface temperature in contrast with cities (Oke, 1973; Townroe and Callaghan, 2014). Townroe and Callaghan (2014) observed a significant Urban Heat Island (UHI) in Reading, England in 2011 and 2012 with water and air temperature higher at urban sites than in rural (water 1.3°C, air 0.9°C).

These environmental stress and human-induced habitat degradation have an effect on native species and provide a favourable habitat and environmental conditions for the establishment of the non-native species. Mosquitoes have been benefitted by the effect of man's modification of the atmospheric environment. In the past few decades, the geographic distribution of *Aedes* species have changed which *Aedes* sp has been reported in part of Europe (Benedict *et al.*, 2007; Lambrechts *et al.*, 2010; WHO, 2017). In our study, no invasive mosquitoes were observed at the end of the experimental collection. However, *Culex pipiens* is the most common mosquito in urban and rural areas in the British Isles (Snow and Medlock, 2008) and the most reported in this work.

Temporarily and permanent habitats contribute to mosquito populations but artificial breeding sites in urban areas are the most attractive aquatic habitat for mosquitoes due to the limited range of predators able to colonise them (Medlock and Vaux, 2011). Mosquitoes have become well adapted to the urban environment and they can be a nuisance problem in Great Britain. Artificial containers and urban trash as discharged tires, cans and bottles have risen due to urbanization (Vezzani, 2007b; McKinney, 2006). Artificial containers are suitable for many of species of mosquitoes in urban areas (Derraik, 2005). In our study, chapter 2, *Cx. pipiens*, *Cx. torrentiun*, *Culiseta annulata* and *Anopheles plumbeus* were reported in artificial containers in the woodland and the glasshouse habitat.

An. plumbeus is known as a dendrolimnic species because it is described as a tree hole mosquito (Snow, 1998; Schaffner et al., 2012a; Bradshaw and Holzapfel, 1991; Bueno-Marí and Jiménez-Peydró, 2011; Snow and Medlock, 2006b). As humans have provided niches opportunities and physical conditions for mosquitoes (McKinney, 2006), it is possible to suggest that *An. plumbeus* is shifting habitats and recently is exploring new larval artificial breeding sites in domestic and peridomestic areas such as plastic containers, septic tanks, tires, catch basins (Bueno-Marí and Jiménez-Peydró, 2011; Schaffner et al., 2012a). Previous studies had reported larval and pupal stages of *An. plumbeus* in residential gardens (domestic and peridomestic areas) (Schaffner et al., 2012a; Bueno-Marí and Jiménez-Peydró, 2011; Snow and Medlock, 2008). In the dry season, females can deposit eggs in domestic and peridomestic artificial containers (Bueno-Marí and Jiménez-Peydró, 2011).

In the dye treated and untreated experiment with artificial containers, larval and pupal stages of *An. plumbeus* were recorded in both treated and untreated artificial containers. However, *An. plumbeus* was only recorded in 2014. In 2015, old established containers supported only immature stages of *Cx. pipiens* and *Cs. annulata*. Our results of *An. plumbeus* agree with those presented by Townroe and Callaghan (2014). Townroe and Callaghan (2014) collected *A. plumbeus* in artificial containers in both urban and rural areas. This shifting behaviour was observed in *Aedes albopictus* where larvae breed originated in natural habitats (e.g. tree holes, bamboo stumps and bromeliads) (Li *et al*., 2014; Higa, 2011; WHO, 2017). As a consequence of uncontrolled urbanization, *Ae. albopictus* has adapted to urban areas and exploiting artificial containers as habitats for larvae and pupae stages (WHO, 2017; Leisnham and Juliano, 2012).

Recently in Europe, mosquito distribution, abundance, species composition, mosquito-host interaction, and the potential of arthropod-borne infection have changed (Medlock *et al*., 2012b; Prasad, 2010). Invasive mosquitoes have been introduced in Europe and established with the transmission and outbreaks of dengue and chikungunya virus (Medlock and Leach, 2015). In late September 2016, 37 eggs of *Aedes albopictus* were collected in one of the ovitraps in a lorry park, Kent (Medlock *et al*., 2017). In a future, in the British Isles, climatic conditions will be suitable for the establishment of *Aedes albopictus* (Medlock and Leach, 2015). Although the risk of a subsequent transmission of a vector-borne disease in the British Isles is low, an important consideration must be given to the changes in ecology and distribution of arthropod vectors.

The higher abundance of *Cx. pipiens* in artificial breeding containers suggests the flexibility to colonise urban containers. It was not unexpected to find *Cx. pipiens* in water butts as this species has been described as an urban mosquito. The breeding season of *Cx. pipiens* in the black containers in 2014 and 2015 was extended until December. *Culex torrentium* were present in lower numbers in the small buckets used for the shadow survival experiment (Chapter 3). *Cx.torrentium* presents similar ecological characteristics as *Cx. pipiens,* but it can be found more in artificial breeding sites and less in natural mesocosms (Townroe and Callaghan, 2014; Snow and Medlock, 2008).

Cx pipiens wild mosquitoes were in higher densities in the black and small containers compared to the collection of larvae and pupae of *An. plumbeus* and *Cs annulata*. In Great Britain, *Cx pipiens* feed on birds but the ornithophagic behaviour of *Cx pipiens* makes it a potential vector of West Nile Virus. To date, no local human cases of WNV have been reported in the UK and the risk of transmission with mosquitoes infected with this virus in urban and peri-urban areas is low (Buckley *et al*., 2003). However, there is some evidence that in Kent, the risk of transmission of WNV may be higher as the increasing of abundance of enzootic and bridge (non-primary) vectors (Golding *et al*., 2012).

The key to prevent a possible outbreak of a vector-borne disease is to understand the possible factors of mosquito ecological plasticity. The wide range of breeding sites in urban areas and the changes in climate allow some of the species of mosquito to spread worldwide. The diversity of oviposition sites is linked to the dispersal of eggs by gravid females. Gravid females can colonise between four-six different breeding sites (Abreu *et al.*, 2015). Therefore, the number of positive containers with a higher presence of mosquitoes could be higher in urban areas in contrast to suburban and rural habitats (Zahouli *et al.*, 2017). Selection of a breeding site by mosquito females is an important component of the life history of the mosquito and it has been observed that gravid mosquitoes do not deposit eggs randomly (Panigrahi et al., 2014).

Experiments with wild-caught *Cx. pipiens* and laboratory *Cx. molestus* and *Cx. quinquefasciatus* (data not shown in the thesis) revealed a strong preference for oviposition in black dyed water (Chapter 2). This aligns with prior studies demonstrating that black containers were chosen preferentially over white containers by gravid female mosquitoes when given a choice (Hoel *et al.*, 2011; Vezzani *et al.*, 2005; Williams, 1962). Indeed, the rearing of British *Cx. pipiens* in a laboratory is more successful if dark oviposition tubs are used over clear. The selectivity employed by mosquitoes between oviposition habitat choices is important to biological control, particularly when a species invests all of its eggs in one batch at a time (Wachira *et al.*, 2010). Natural selection will favour individuals that are able to select habitats where a high nutritional load will ensure survival and success for offspring (Thompson, 1988). The presence of dye may offer a false apparently bountiful nutritional load (Ortiz and Callaghan, 2017) allowing the manipulation of mosquito populations for control purposes.

In contrast, two other dyes, blue and shadow, were not attractive to females (Chapter 3). There was no significant difference in the number of eggs laid in water treated with the blue or shadow dye in either choice experiments: oviposition selection in a tent and in laboratory conditions. The preference for oviposition in the choice experiment in a tent and in laboratory conditions by gravid females in normal light/night or in darker condition was similar in both treated and untreated cups. This in itself is a surprising result since to the human eye, the dye blue and black waters do not look that dissimilar. It was not within the scope of this study to investigate the reason behind this difference, but the result is of interest to anyone who would like to dye their pond without attracting mosquitoes to breed in their garden.

All dyes had a significant negative effect on the survival of juveniles when mosquitoes were reared in containers without other macroinvertebrates (Chapters 2, 3). Adult mosquitoes still emerged from all three colour dyes but the black dye presented the lowest survivorship compared to the blue and shadow dye. The black and shadow dyes were used to determine whether a similar result was observed in containers that were allowed to be colonised naturally by other macroinvertebrates. Containers with shadow dye had fewer *Culex* pupae compared to the control. Since we know that there is no difference in oviposition between treatments, it suggests that the dye had a negative impact on development and/or survival (Chapter 3).

In contrast, there was no significant decrease on the abundance of mosquito larvae and pupae in containers treated with black dye that were allowed to colonise naturally. The water butts used for this experiment were placed in two different habitats (glass house and woodland) where light levels varied. The greenhouse habitat was exposed to bright sunlight in contrast with the woodland habitat where vegetation covered the water butts. The number of larvae and pupae recorded was higher in woodlands than in water butts exposed to sunlight. Results showed that habitat type was a more important factor than dye to explain differences in the number of larvae and pupae. These experimental results are difficult to interpret. Whereas only black dye is an oviposition attractant, all dyes have a negative impact on survival when mosquitoes have no competition or predators, despite a lack of evidence of acute toxicity. When in a more natural environment, the black dye has no effect but the shadow dye seems to reduce the abundance of mosquitoes. One explanation is that the negative impact on abundance with black dyes is balanced by an increase in oviposition, whereas the shadow dye, which is not an oviposition attractant, has no increased input of mosquitoes. Another possibility is that the dye is impacting other macroinvertebrates which may be competitors or predators of mosquitoes. Although the macroinvertebrate community in the water butts was not specifically measured, it was observed that a number of copepods and glassworms were present, some of which are known predators of mosquitoes (Baldacchino et al., 2017; Cuthbert et al., 2018). This prompted a study to see if the black dye had any impact on predation since it was presumed that sight would be an important component.

Predation is an essential biotic interaction in an ecosystem and important to control mosquito populations (Batzer and Murray, 2017). Functional responses detect the *per capita* intake rate of a resource (here mosquito larvae) as a function of its availability, or abundance (Holling, 1959; 1966). This approach can help inform the impact predators are likely to have over prey population stability; for example a Type II functional response could destabilise a population from high levels of predation at low prey densities, whereas in contrast a Type III functional response may impact prey population stability by providing refugia at low densities (Holling, 1959). In chapter 6, we observed that *C. flavicans* presented Type II functional response in both treatments, treated and untreated with black dye; however, no functional response was observed in *G. pulex* experiments. Even though, no functional response was detected in *G. pulex*, *G. pulex* might be a good mosquito predator.

A few studies have reported the efficiency of both predators in controlling larval stages (Helgen, 1989; Service, 1977; Sailer and Lienk, 1954). However, more studies are needed to understand how *Chaoborus* larvae and *Gammarus* sp could be efficient mosquito predators as part of a control strategy. The results in chapter 5 showed that *G. pulex* and *C. flavicans* are excellent mosquito predators but there was no evidence of any impact of the dye. In the case of gammarids, this is possible because they are themselves prey for many other invertebrate and fish species. To avoid predation they are often actively feeding in low light and low visibility which may have a strong limitation on the use of visual cues for locating prey (Lange *et al.*, 2005).

Chaoborus larvae have an important role in structuring pond zooplankton communities through predation and are second only to fish in importance (Dodson, 1972; Fedorenko, 1975; Yan *et al.*, 1991; Wetzel, 2001). Late instar *Chaoborus* larvae are well known predators of other dipteran larvae such as mosquitoes (Deonier, 1943) and elicit a migration response in zooplankton such as *Daphnia* (Kleiven *et al.*, 1996; Nesbitt et al., 1996). Their feeding strategy is primarily tactile rather than visual (Pastorok, 1980) and some species of *Chaoborus* actively avoid damaging UV light and remain in the middle of the water column on sunny days, whereas their distribution involved the full water column on cloudy days (Rautio *et al.*, 2003). Therefore pond dyes are unlikely to have any negative impact on their ability to detect prey as was shown in Chapter 5.

Up until this point in the study, all impacts of pond dyes were studied in relation to mosquitoes. However, the difference in results between the controlled vs natural emergence studies in chapters 2 and 3 led me to think about the impact that dyes might have on the wider pond community. There was no impact of dye on predation by either *Gammarus* or

Chaoborus but there are many other possible interactions that could be affected by a loss of algal nutrition and reduction in visibility.

7.2. Black pond dye and pond communities

In chapter 4 and 5, an experiment to measure the effect of dye on small mesocosms found that evenness was not impacted by the presence of the black pond dye. However, in chapter 4 biodiversity was significant lower in treated ponds. In the experiment in chapter 4, ponds were stripped of their fauna and equal numbers of a few species reintroduced. *Daphnia pulex*, lesser water boatmen (*Corixa punctata*), *Cyclops* sp. and coleopteran larvae were found in lower abundance in ponds with dye, particularly following the second application. By contrast, in the second pond study, where the dye was applied to the existing macroinvertebrate communities, no differences were detected between treated and untreated ponds with respect to biodiversity or abundance.

Daphnia was the macroinvertebrate with the highest abundance in the pond communities in both experiments (chapter 4 and 5). I might suggest that the impact on *Daphnia* by the application of the dyes in pond experimental 1 (chapter 4) could be an indirect factor in the biodiversity in ponds (Table 7.1). However, it might be possible that more than one macroinvertebrate have an impact on biodiversity and evenness. Previous studies showed that *Daphnia* and *Cyclops* could have a direct impact on tropic cascade which might affect biodiversity and macroinvertebrates. Wickham (1998) showed that *Daphnia* was reduced in number in the presence of *Cyclops* and the mean sizes of *Cyclops* were lower when *Daphnia* were present In similar studies, *Daphnia* populations have a direct effect over mosquito populations (Duquesne *et al*., 2011). At higher *D. magna* populations (population well established), mosquito oviposition was inhibited and no mosquito larvae were present; and in the absence of *D. magna*, larval biomass, oviposition and abundance of *Cx. pipiens* was higher (Duquesne *et al*., 2011).

The invertebrate community diversity and the number of species were similar in both studies. Thirteen taxa of macroinvertebrates were recorded during the sampling period in the first study after the reintroduction of the species (Table 7.1). This can be an indicator that ponds provide a niche for invertebrate taxa and can be refugia for biodiversity. Passive and active dispersers such as snails, *Cyclops* and water mites were able to colonize the ponds in both treatments in a relatively short space of time. The lack of any effect of the dye in the more established pond compared to the newly created pond is, to some extent, similar to the results with mosquitoes.

Table 7.1. A summary of the results presented in black pond dye and pond communities in both chapters 4 and 5.

	Pond experiment 1 (Chapter 4)			**Pond experiment 2 (Chapter 5)**		
	19 Weeks	**Summer**	**Autumn**	**19 weeks**	**Summer**	**Autumn**
Biodiversity	**↑P = 0.026**	P = 0.557	**P = 0.014**	↑P = 0.881	P = 0.874	P = 0.991
Evenness	↑P = 0.761	P = 0.912	P = 0.531	↑P = 0.778	P = 0.750	P = 0.945
Water flea: Daphnia	**↓P < 0.001**	**P < 0.001**	**P < 0.001**	↓P = 0.385	P = 0.362	P = 0.898
Diving beetle	**↓P =0.030**	**P = 0.035**	P = 0.312	↓P = 0.849	-	-
Water mite	-	-	-	↑P = 0.884	P = 0.824	-
Lesser water boatman	**↓ P <0.005**	**P < 0.001**	**P < 0.001**	**↓P < 0.001**	**P < 0.005**	P = 0.801
Pond snail	↓ P =0.093	**P <0.001**	**P < 0. 024**	**↓P < 0.001**	P = 0.088	**P < 0.001**
Pond olive	P = 0.29	P =0.524	P =0.366	P = 0.891	P = 0.707	P = 0.949
Cyclops	**↓P < 0.001**	**P < 0.001**	**P = 0.014**	↑P = 0.286	P = 0.366	**P < 0.005**
Mosquito larvae	P = 0.376	P = 0.314	P = 0.438	**↑P = 0.015**	**P = 0.010**	P = 0.173
Mosquito egg batches	-	-	-	↓P = 0.282	P = 0.137	-
Mosquito pupae	-	-	-	P = 0.105	↑P = 0.089	↓P = 0.655
Non-biting midge larvae	↓P = 0.059	P = 0.9	P < 0.001	↑P = 0.811	P = 0.80	-
Meniscus midge	P = 0.683	P = 0.26	↓P = 0.238	↓P = 0.292	-	-
Seed Shrimp: Ostracoda	P = 0.6	↓P = 0.80	P = 0.315	↑P = 0.665	P = 0.777	P = 0.176

↑ Increase of abundance of macroinvertebrates in treated ponds

↓ Decrease of abundance of macroinvertebrates in treated ponds

New ponds are important habitats in protecting freshwater biodiversity (Williams *et al.*, 1997) but colonizing them are adaptations by the species to a new environment. New ponds present different physicochemical environments in contrast to more mature ponds (Williams *et al.*, 2008). Presence of inorganic substrates, low vegetation cover and possible absence of predation by fish are some of the potential differences between new and old ponds (Williams *et al.*, 2008). Previous studies have shown that in the UK, rapid colonization of plants and animals in a new pond depends on the close association with existent wetlands (Williams *et al.*, 1997). Similar studies observed that colonization and macroinvertebrate diversity of taxa might be correlated with the connectivity between water bodies in urban areas (Hill *et al.*, 2015). We might conclude that after the reintroduction of the species in the ponds, the colonization of the new species might be as a result of the close interaction with the other mesocosms.

In Great Britain, small water bodies have been less studied, even though they are present in high densities in both urban and rural areas (Wood *et al.*, 2003). However, in the last 150 years, the number of ponds had started to decrease as a result of urban development and land fragmentation, threatening the wildlife present in these ecosystems (Wood *et al.*, 2003; Céréghino *et al.*, 2008). As a result, in the last few years, conservation of wetlands is driving the creation of new ponds and restoration of existing aquatic ecosystems (Medlock and Leach, 2015; Snow and Medlock, 2006b; Williams et al., 2010; Pond-Conservation, 2011; Hill et al., 2015).

In the last few decades, the diversity of flora and fauna in terrestrial and aquatic environments are at risk by anthropogenic factors caused by the human environmental changes. Aquatic and terrestrial species are in danger of extinction by habitat destruction and climatic changes, however, more surveys and monitoring of the ecosystems are needed to know the capacity of the freshwater habitats and taxa communities to respond to a variety of disturbances and global climatic change (Céréghino *et al.*, 2008). It is well documented that pond community is susceptible to disturbance, pollutants, climate and land disturbance, decreasing biodiversity, abundance and species richness. Few studies showed that rivers and streams are more sensitive to anthropogenic factors and climate change compared to lakes, affecting biodiversity (Sala *et al.*, 2000).

Over the past 60 years, the use of chemicals (pesticides and synthetic fertilisers) has increased worldwide (Isenring, 2010). The application of non-toxic dyes implemented to control algae in freshwater ecosystems growth has been documented (Martin et al., 1987; Tew, 2003). For example, another commercial dye called Aquashade has similar properties as Dyofix products in that the dye inhibits photosynthesis through shading (Aquashade, 2017). Previous studies showed that the use of Aquashade, rose bengal, methylene blue, zinc phtalocyaninetetrasulfonate (ZPS) and erythrosine can reduce algal growth (Martin *et al.*, 1987; Spencer, 1984; Tew, 2003) as well as decreasing oxygen in laboratory and in ponds.

Martin *et al.* (1987) observed a 43% reduction in oxygen production following treatment with rose bengal, 35% with methylene blue and 25% with zinc phtalocyaninetetrasulfonate and erythrosine in plastic trays under ambient conditions where samples of *L.majuscula* were added. Tew (2003) found that in six catfish ponds at Hebron State Fish Hatchery, Hebron, Ohio, survival of yearling catfish was not affected by the blue dye; however, the fish growth was affected by the low concentration of oxygen (3 mg/l). In contrast with the study, in chapter 2 and 3, the results showed that the survival of mosquito larvae was reduced in presence of the dyes. The reduction in mosquito survival could be by the presence of any component in the dyes.

In similar studies, Spencer (1984) observed that at higher concentrations of Aquashade and low light intensity not all species of algae were affected by the dye; *Pediastrum tetras* and *Ankistrodesmus falcatus* var. *acicularis* were the algae most affected. It is pure speculation to suggest first that Dyofix products might have an indirect effect in the concentrations of oxygen, as they decrease photosynthesis. The reduction of oxygen and the inhibition of algae growth could influence the low levels of biodiversity, evenness and the abundance of most of the macroinvertebrates collected in the ponds (Chapter 4). In chapter 5, it might be possible that concentrations of oxygen were reduced but not all algae present in the pond were controlled. For instance, ponds treated with dyes may have the presence of algae and oxygen produced by these algae.

Studies of *Daphnia magna* at different dissolved oxygen concentrations showed that fitness of *Daphnia* (growth and reproduction) was decreased at low oxygen concentrations (Hanazato, 1996). Nebeker *et al*. (1996) exposed embryos, larvae stages and pupal stages of the caddisfly *Clistoronia magnifica* (Limnephilidae) at different dissolved oxygen (DO) concentrations. He observed that egg hatch, larval development, moulting success, time of moulting, pupation, and adult emergence were reduced at low DO concentrations. However, more information is needed to identify if the Dyofix products decrease dissolved oxygen (DO) concentrations in ponds and lakes and therefore, these products decrease fitness of macroinvertebrates present in these ecosystems.

7.3. Conclusions

Despite the fact that dyes (black, blue and shadow) had a negative impact on the total number of adults emerging of wild *Cx. pipiens* mosquito, the results did not show a direct effect on the abundance of larvae and pupae collected in a more natural environment where interactions with other organisms could occur (chapter 2 and 3).

In small ponds that were controlled for initial macroinvertebrate species and numbers, biodiversity and evenness in pond communities was impacted by dye (positively) whereas the abundance of *Daphnia*, lesser waterboatmen, Coleoptera larvae, *Cyclops* and pond snail were negatively affected by the dye (chapter 4). However, in chapter 5, when the ponds were heterogeneous at the beginning of the experiment, no impact could be found on biodiversity, and evenness and almost no impacts were found on species abundance.

Predation and the functional response of both *Chaoborus* larvae *and G. pulex* under the presence of black dye, was not different from the treatments without dye. However, *Chaoboros flavicans* and *Gammarus pulex* exhibited an increment on consumption of mosquito larvae under a rising prey supply. This might be important because measuring the functional response, we can understand how *Chaoborus* sp and *Gammarus* sp can regulate mosquito populations.

7.1. Limitations

It was intended to continue with a study of the water butts which had matured with an interesting mix of invertebrates. However, many of my water butts were destroyed by workmen who had been employed to make some adjustments to the area and no one had consulted us. Another limitation was a problem with the adult mosquitoes in the laboratory colony. Work had begun with two laboratory colonies in addition to the wild mosquitoes but females did not feed or reproduce sufficiently to allow their use for the whole of 2016. As a result, we were only able to test blue dye in both laboratory strains: *Cx. molestus* and *Cx. quinquefasciatus,* but we could not test shadow dye. Finally, in the predation experiment, we would like to have had more results about the impact of dyes colours in larval consumption.

However, the company supplying the *Chaoborus* larvae were unable to provide larvae that survived the postage for several months.

7.2. Further Work

Dyes are new products to control algae blooms. Even that there is no evidence of acute toxicity in larval stages of mosquito, our results showed that there is an impact on diversity and abundance in macroinvertebrate in freshwater and on the survival of mosquito, having a low survivorship. Thousand of people are using dye colours to improve appearance ponds and lakes. Urban garden ponds are relatively close to humans; this could be a real problem in a future. The black colour pond is an attractant in mosquito oviposition, it might be a speculation that abundance and density will be higher as a result of the dye colours.

A possible long-term consequence can be observed from these dyes is the colonisation of freshwater ecosystems treated with dyes colours. In addition, it is important to continue the studies with these dyes and know the effects in biodiversity and abundance in macroinvertebrates. Dyofix products recommend in reapply 15% of the original amount of their products every month. Changes in biodiversity, evenness, abundance, survivorship can be analysed before and after the reapplication of the colour pond.

Chaoborus and *Gammarus* are mosquito predators but more studies are needed. These predators are present in the same mosquito breeding sites and both of them are predaceous animals. It is vital to know how mosquito populations can be controlled in field conditions by *Chaoborus* and *Gammarus.*

APPENDIX

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Pond dyes are *Culex* mosquito oviposition attractants

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ABSTRACT

Background. British mosquito population distribution, abundance, species composition and potential for mosquito disease transmission are intimately linked to the physical environment. The presence of ponds and water storage can significantly increase the density of particular mosquito species in the garden. *Culex pipiens* is the mosquito most commonly found in UK gardens and a potential vector of West Nile Virus WNV, although the current risk of transmission is low. However any factors that significantly change the distribution and population of *C. pipiens* are likely to impact subsequent risk of disease transmission. Pond dyes are used to control algal growth and improve aesthetics of still water reflecting surrounding planting. However, it is well documented that females of some species of mosquito prefer to lay eggs in dark water and/or containers of different colours and we predict that dyed ponds will be attractive to *Culex* mosquitoes.

Methods. Black pond dye was used in oviposition choice tests using wild-caught gravid *C. pipiens*. Larvae from wild-caught *C. pipiens* were also reared in the pond dye to determine whether it had any impact on survival. An emergence trap caught any adults that emerged from the water. Water butts (80 L) were positioned around university glasshouses and woodland and treated with black pond dye or left undyed. Weekly sampling over a six month period through summer and autumn was performed to quantified numbers of larvae and pupae in each treatment and habitat.

Results. Gravid female *Culex* mosquitoes preferred to lay eggs in dyed water. This was highly significant in tests conducted under laboratory conditions and in a semi-field choice test. Despite this, survivorship in black dyed water was significantly reduced compared to undyed water. Seasonal analysis of wild larval and pupal numbers in two habitats with and without dye showed no impact of dye but a significant impact of season and habitat. Mosquitoes were more successful, with significantly higher numbers of pupae, in the habitat where they had vegetation cover and shade.

Discussion. Our study has raised some interesting possibilities; one is that where used, pond dyes may be encouraging mosquitoes to breed in gardens in close proximity to people. Considering the concerns over potential future spread of disease in urban environments, this as well as shading of ponds and water butts, should inform future advice over reducing mosquito breeding and spread.

Subjects Ecology, Entomology

Keywords Urban ecology, British mosquito, *Culex pipiens*, Ponds, Oviposition, Pond dye, Behaviour, Disease, Mosquito control, Habitat

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INTRODUCTION

West Nile virus (WNV) is a positive-sense RNA virus belonging to the Flaviviridae family and is transmitted by mosquitoes, including *C. pipiens* complex mosquitoes. There have been several major outbreaks of WNV in Europe in recent years, affecting both humans and horses (*Calistri et al., 2010*; *Di Sabatino et al., 2014*; *Hernández-Triana et al., 2014*). A laboratory test of the vectorial competence of European *C. pipiens*, including the phenotypic and physiological variant *C. molestus*, demonstrated that both the *molestus* form and a hybrid between C. *pipiens* and C. *molestus* were capable of transmitting WNV (*Brustolin et al., 2016*). Both of these variants are found in Britain, raising the possibility that outbreaks of human or animal viral diseases could occur in Britain if conditions and climate permitted. Whilst the threat is likely to come from invasive species, more than 30 species of mosquito, including putative vectors of arboviruses, are native to the UK (*Blagrove et al., 2016*). To date there is no evidence of mosquito-borne virus transmission of public health concern in the UK (*Blagrove et al., 2016*). However, we know that mosquitoes are established in both rural and urban habitats and are often found in gardens (*Townroe & Callaghan, 2014*). Understanding and mitigating future threats requires detailed ecological knowledge of the putative vector species and prediction of how mosquito populations are influenced by anthropogenic activity.

In England, 80% of the human population lives in towns and cities which cover more than 7% of the land area (*Wilby & Perry, 2006*). Urbanisation changes the physical environment in a way which is known to alter habitat types, species numbers and the community composition of ecosystems (*McKinney, 2006*; *Sala et al., 2000*). These changes are likely to impact British mosquito populations and influence distributions, abundances, species composition, mosquito-host interactions, biting nuisance and the potential for mosquito borne disease to occur in the UK.

Gardens make up a large proportion of the urban area and provide a significant contribution to the green spaces within many UK cities providing areas of ecological value which may support diverse wildlife populations including mosquitoes (*Smith et al., 2005*). The creation of ponds is encouraged as a means of enhancing the biodiversity value of gardens, particularly in the face of a widespread decline of ponds in the wider rural landscape (*Gaston et al., 2005*). Although individually small ($\sim$2.5 m^2) and fragmented into small patches, urban ponds are distributed widely across the urban landscape and are likely to contain water all year round (*Gaston et al., 2005*). Where fish are not present, they are likely to provide a valuable breeding site for mosquitoes. Water butts also provide an ideal habitat for mosquitoes with a recent study recording five British mosquito species; *Anopheles claviger*, *An. plumbeus*, *Culesita annulata*, *C. pipiens* and *C. torrentium* (*Townroe & Callaghan, 2014*). Predicted future changes to the climate, with increased summer temperatures and more frequent heavy rainfall in winter, will continue to place pressure on water supplies and encourage domestic water storage (*Snow & Medlock, 2006*). This in turn is likely to increase populations of the most common species, *C. pipiens*, particularly in urban gardens.

C. pipiens is a potential enzootic (primary) vector of West Nile Virus—WNV (*Medlock & Leach, 2015*) and the species most likely to be directly affected by changes in water storage and pond formation (*Townroe & Callaghan, 2014*). The current risk of WNV transmission in the UK is considered low because the abundance of enzootic and bridge (non-primary) vectors is too low for sustained transmission (*Medlock & Leach, 2015*). Changes in climate, migration of mosquito species and longer flight seasons in dense urban areas creates conditions more conducive to high levels of human host biting and an increased risk of disease transmission. Therefore, any factors that significantly change the distribution and population of *C. pipiens* are likely to impact subsequent risk of disease transmission.

Pond dyes are a relatively new cosmetic product for garden ponds and lakes. They have proved to be popular at recent high profile garden shows such as Chelsea and Hampton Court. They stop the growth of algae by blocking the red end of the visible light spectrum (of wavelength 620–740 nm) from penetrating the water. The red end of the light spectrum is needed for photosynthesis, as peak absorption for photosynthetic pigments is approximately 650 nm (*Douglas, Raven & Larkum, 2003*). Although there is no evidence to suggest that these dyes are toxic to fish and invertebrates, the impact on invertebrate communities may well be behavioural. In this study, we investigate the impact that pond dyes have on oviposition and survival in *C. pipiens* mosquitoes. Previous studies have shown that the cues for oviposition are often visual and have demonstrated a preference for oviposition in dark containers and dark waters (*Beckel, 1955*; *Collins & Blackwell, 2000*; *Hilburn, Willis & Seawright, 1983*; *Hoel et al., 2011*; *Panigrahi et al., 2014*). We therefore predict that pond dyes will act as an attractant for mosquito ovipostion, with a potential impact to increase mosquito population densities in garden ponds. This is the first study to specifically look at pond dyes to see if they impact on mosquito breeding behaviour and success.

MATERIALS AND METHODS

Trapping wild gravid female mosquitoes

Wild gravid female Culicine mosquitoes were trapped using modified oviposition traps (*Reiter, 1987*; *Townroe & Callaghan, 2015*). A total of 10 traps were placed on the Whiteknights campus at the University of Reading, Berkshire, England (51.4419°N, 0.9456°W). Approximately 1,000 gravid female mosquitoes were caught in July and August 2014 and 2015. Most of the mosquitoes sampled belonged to the *Culex* genus although a few *Anopheles plumbeus* (<5) and *Culiseta annulata* (<5) were also trapped.

Oviposition preferences of wild mosquitoes

An oviposition choice experiment was performed by releasing 200 of the trapped gravid mosquitoes into a tent (245 × 145 × 95 cm) placed outdoors on campus (51.4419°N, 0.9456°W). Mosquitoes were allowed to freely oviposit in one of 14 2 L plastic containers placed randomly in the tent: seven with 1.2 L tap water and seven with 1.2 L tap water treated with pond dye at the concentrations recommended by the manufacturer (Dyofix, Leeds, UK). After seven days, the containers were taken to the laboratory to count egg batches laid in each container. The experiment was performed three times with freshly trapped females and a choice between tap water and black colour dye.

The choice experiment was repeated with wild-caught gravid females under laboratory conditions (25 °C, 16:8 light:dark). Five groups of 20 gravid females were chosen randomly and each group transferred into a cotton net cage 25 × 25 × 25 cm per treatment set. In each cage, two 200 ml plastic bowls were filled with 150 ml of either tap water or dye water. The choice experiments were repeated in normal rearing conditions (16:8 light:dark) and also in the absence of light (black bags were used as a cover in each cage during the experiment).

Emergence study

A modified emergence trap (*Hamer et al., 2011*) was used to measure the impact dye had on mosquito survival. Eggs from the oviposition experiment were hatched in the laboratory (25 °C, 16:8 L:D) and reared in tap water through to 2nd instar, fed with pelleted rabbit food. One hundred were then transferred to each of 18 11 L plastic bins (23 × 28 cm) containing 10L tap water or 10L tap water and dye (Dyofix, Leeds, UK). Food was added to each bin (1.2 g guinea pig food) which was capped with a conical fabric mesh to trap emerging adults. The bins were placed outdoors in the area used to trap the females.

Traps were monitored daily for emerging adult mosquitoes. These were captured using a manual aspirator, transferred into small tubes and stored at −20 °C for identification (*Snow, 1990*).

Wild population numbers in dye treated and untreated artificial containers

Thirty two 80 L water butts (44.5 cm × 58.5 cm, *Townroe & Callaghan, 2014*) were placed around the secure area behind the School of Biological Sciences Harborne building on Whiteknights campus in the summer of 2014. Each container was filled with 60 L of tap water and 8 g of ground oak leaves. Bins were placed in pairs with the second bin additionally containing black pond dye added according to manufacturers instructions (DyoFix, Leeds, UK). For each treatment, eight replicates were organized in each habitat: woodland (51°26′12.8″N; 0°56′39.7″W) and glasshouses (51°26′13.2″N; 0°56′31.2″W). Bins pairs were several metres apart. Containers were sampled weekly for 26 weeks in 2014. Sampling was carried out using a device adapted from *Onyeka (1980)* and *Townroe & Callaghan (2014)*. The device included three sections of drain pipe (4 cm high, 0.4 cm thick and 8 cm diameter) bolted together in line with fine mesh net glued to the bottom of each ring and a flexible wire handle attached to the outer edge of the furthest two rings. The device was lowered into the container and allowed to rest on the bottom for 5 min before being drawn swiftly up through the water to collect animals. This method was carried out once per container per sampling event. The number of larvae and pupae collected were recorded and larvae were replaced in the container. All pupae were taken to the laboratory for rearing to adult then frozen at −22 °C. Adults were identified using a 10–40× magnification microscope using the key of *Cranston et al., (1987)*.

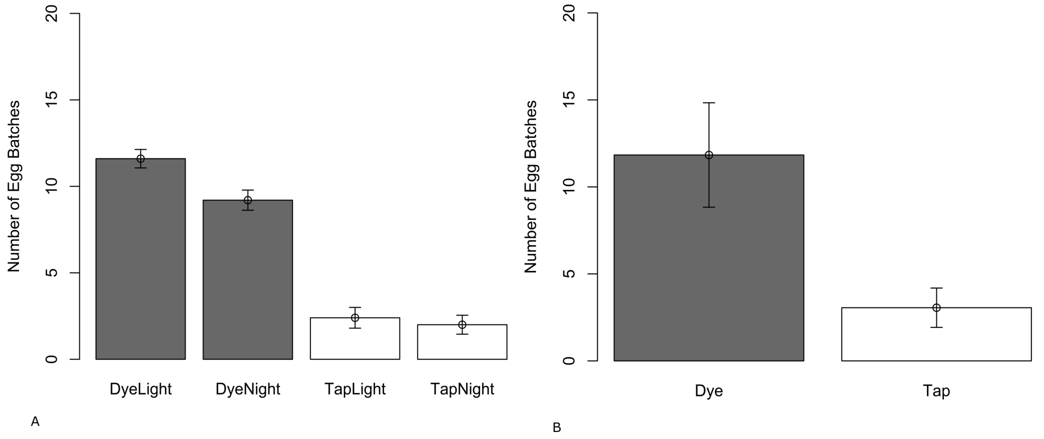

Figure 1 **Mean number of egg batches (±SE) laid by wild-caught *C. pipiens* in paired choice tests in (A) the laboratory with a 16:8 Light/Dark plus or minus a blackout cover and (B) semi-field conditions (tent).**

Statistics

All statistical analyses were performed using R version 3.2.2 (*R Core Team, 2015*). Data were tested for normality using a Shapiro–Wilk normality test. Where data were normally distributed, parametric statistics were used and oviposition data were analysed using a paired t-test and one-way analysis of variance (ANOVA). Oviposition in the tent was not normally distributed and a nonparametric Mann–Whitney U-test was performed. Differences in adult emergence between treatments were analysed using a generalised linear model binomial test. Abundance data in water butts were Log $(x+1)$ transformed and the relationship between mosquito abundance, treatment and location was analysed using 2 way repeated measures ANOVA.

RESULTS

Mosquito oviposition selection in laboratory conditions and oviposition selection in the tent

In the laboratory experiment, wild gravid females laid significantly more egg rafts in dye water compared with tap water ($t = 5.4928$; $df = 8$; $P < 0.001$) (Fig. 1A). Similar results were observed in the tent; wild gravid females laid significantly more egg rafts in the dye water compared to the tap water ($W = 250$; $P < 0.001$) (Fig. 1B). Females laid significantly fewer eggs in the dye treatment when there was a reduction in light ($t = 3.0358$; $df = 8$; $P = 0.016$). Light had no significant effect on numbers of eggs laid in tap water ($t = 0.49237$; $df = 8$; $P = 0.6357$). Even though females laid fewer eggs when light was reduced, they still preferred to lay in dye water rather than tap water ($t = 9$; $df = 8$; $P \leq 0.001$).

Wild population numbers in dye treated and untreated artificial containers

Larval and pupal numbers were analysed by season; summer (June–August) and Autumn (September–November), treatment and habitat (Fig. 2). No significant differences were observed in larval or pupal densities between dye and tap water in the summer (larval

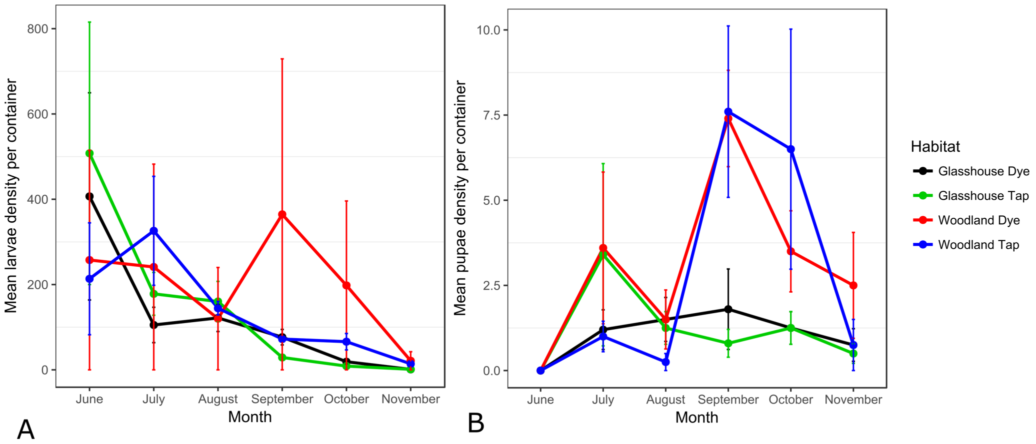

Figure 2 Mean (±SEM) number of *C. pipiens* larvae (A) and pupae (B) sampled in dye and tap water in woodland and glasshouse habitats.

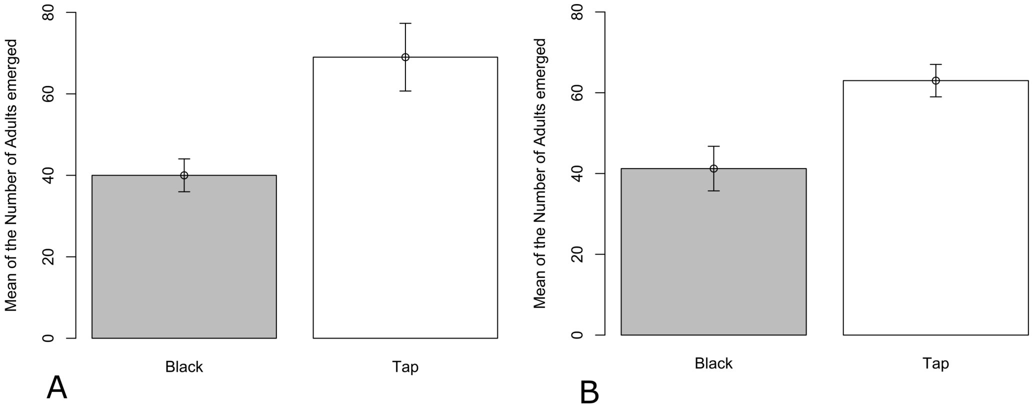

Figure 3 Mean number (±SEM) of *C. pipiens* adults emerging from bins containing tap water or tap water and dye in (A) Summer and (B) Autumn.

$F_{124} = 0.062$; $p = 0.8048$; pupal $F_{124} = 0.034$; $p = 0.856$) or in the autumn (larval $F_{124} = 0.162$; $p = 0.691$; pupal $F_{124} = 0.002$; $p = 0.962$). Habitat impacted on larval numbers, with higher numbers in the glasshouse in the summer ($F_{124} = 4.488$; $p = 0.045$) and higher numbers of pupae in the woodland in the autumn ($F_{124} = 4.240$; $p = 0.049$). However, there were no habitat differences in larval densities in the autumn ($F_{124} = 0.130$; $p = 0.722$) or in pupal densities in the summer ($F_{124} = 0.002$; $p = 0.969$).

Emergence study: field conditions

The total number of adults emerging from different treatments varied significantly in both summer ($Z = -11.800$, $P < 0.001$) and autumn ($Z = -9.172$; $P < 0.001$) (Fig. 3). In each season, fewer adults emerged from the black dye.

DISCUSSION

Urban garden ponds represent an abundant and reliable network of aquatic resources within which juvenile mosquitoes may develop and adults move across the landscape. Adding pond dyes might influence the attractiveness of ponds as breeding sites to mosquitoes. This is important since the exploitation of human domestic habitats has facilitated mosquito-borne human disease outbreaks in other parts of the world, such as the WNV outbreaks in North America (*Patz et al., 2004*).

It was not unexpected to find that *C. pipiens* females prefer to lay eggs in water with the black dye. It is well known that female mosquitoes have preferences for oviposition in containers of different colours and previous studies have demonstrated oviposition choice in dyed water although no work has been undertaken on pond dyes (*Collins & Blackwell, 2000*; *Li et al., 2009*; *Oliva, Correia & Albuquerque, 2014*; *Beehler & DeFoliart, 1990*; *Beehler, Millar & Mulla, 1993*; *Isoe et al., 1995*). A possible explanation for this is that mosquitoes choose to oviposit in dark containers as it indicates depth and therefore a lower threat of desiccation before juveniles develop. It might also indicate a higher concentration of organic matter providing nutrition (*Hoel et al., 2011*; *Williams, 1962*). Another suggestion is that the dark water mimics shading of the water body (*Vezzani et al., 2005*).

Visual cues seem to have some importance. Covering the adult cages with black plastic in the laboratory oviposition experiment significantly reduced oviposition in black dye containers, although oviposition remained significantly higher than in the control suggesting that either some light was leaking in or that other factors were in play.

Although the black dye was an oviposition attractant, it had a significant negative impact on the survival of mosquitoes through to adults. Adult mosquitoes still emerged from the dye-treated water but the breeding success of the female was almost halved by the low survivorship. The results of the breeding experiment were repeated with the same significant reduction in emergence in dyed water. Laboratory tests have found no evidence of acute toxicity of dyes to *Culex* larvae over 48 h that would explain this result. The poor survival of mosquitoes is therefore unlikely to be related to dye toxicity. It is also unrelated to the algal-killing property of the dye. If mosquitoes were in a natural environment where algae were a significant part of their diet, we might hypothesise that dye would impact survival by killing the algae. However in this artificial system larvae were given a supply of food and no algae were present in either treatment.

It is well known that mosquito larvae and pupae dive in the water column in response to threat, relying on visual or mechanical cues (*Awasthi, Wu & Hwang, 2012*). This requires considerable amounts of energy and constant or deep diving is associated with increased mortality (*Lucas et al., 2001*). It is pure speculation to suggest that the dye changes the behaviour of *C. pipiens* but in fourth-instar *Anopheles gambiae* growing in murky water columns deep diving increased significantly compared to clear water columns (*Tuno et al., 2004*).

Monitoring of wild population numbers in dye treated and untreated artificial containers were undertaken in two habitat types. The greenhouse habitat represented one in full sun where undyed water would reflect light presenting a large contrast between water treatments and the woodland habitat would have potentially less of a contrast since there was a lower light level.

Habitat type was found to be far more important than dye in determining the number of larvae and pupae, with the darker woodland habitat producing significantly more pupae compared to those in the brighter greenhouse area and the greenhouse habitat producing significantly more larvae. There is little information on the impact of shade on British mosquitoes but this result agrees with that of *Fischer & Schweigmann (2004)*, who found that seasonal patterns of abundance of *C. pipiens* in urban Argentina showed positive relationships with vegetation cover. A further study on container breeding mosquitoes in an Argentinian cemetery found that the numbers of both *C. pipiens* and *Aedes aegypti* immatures were higher in shaded containers than in containers in full sun (*Vezzani & Albicocco, 2009*; *Vezzani et al., 2005*). Clearly it is likely that temperatures were higher in Argentina and shaded, cooler, containers have higher adult mosquito production rates because of a negative effect of high temperatures (*Vezzani et al., 2005*). In our study the greenhouse habitat produced significantly more larvae, although this did not translate into more pupae, possibly indicating larval mortality.

The fact that pond dye treatment had no impact on wild mosquito numbers can be explained by two possibilities. One is a balancing of oviposition preference against survival. If more eggs are laid and yet fewer mosquitoes emerge because of the dye, the net effect could well be neutral in terms of numbers of mosquitoes produced by the habitat. The second is that although pond dye is an important factor for *Culex* female oviposition in artificial environments, there are many factors in play that will influence the success of mosquitoes in a natural habitat, including temperature and shading.

We undertook the wild population experiment using water butts rather than ponds for two reasons. The first was to limit the number of factors that might interfere with the experiment such as competing mosquito species and predators to allow us to determine whether dye was an important factor in mosquito breeding success in a more natural setting. The second is that water butts are an important urban habitat for *C. pipiens* mosquitoes and an estimated 60% of UK garden water butts are colonised (*Townroe & Callaghan, 2014*). Our results demonstrate that the dyes do influence both mosquito behaviour and survival but there is no evidence that this translates into a significant difference in mosquito numbers.

Populations of *C. pipiens* are expected to increase with future changes to the landscape and climate, and it has been suggested that towns and cities represent some of the highest risk areas for potential transmission of bird-related mosquito-borne disease (*Snow & Medlock, 2006*). The ornithophagic habit of *C. pipiens* limits its potential as a bridge vector but seasonal abundance and other eco-behavioural characteristics predispose this species to serve as a potential enzootic vector of WNV, capable of maintaining cycles among bird populations, in the UK (*Medlock, Snow & Leach, 2005*). It is important to understand environmental factors that might impact on mosquito population success in urban habitats, particularly if these factors are anthropological in nature.

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The authors declare there are no competing interests.

Author Contributions

- Natali Ortiz Perea conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Amanda Callaghan conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as a Supplementary File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3361#supplemental-information.

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RESEARCH ARTICLE

The effect of pond dyes on oviposition and survival in wild UK *Culex* mosquitoes

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Abstract

British *Culex pipiens* complex [*Culex pipiens* sensu lato) mosquito distribution, abundance, and potential for disease transmission are intimately linked to their environment. Pond and lake dyes that block light to restrict algal photosynthesis are a relatively new product assumed to be an environmentally friendly since they are based on food dyes. Their use in urban garden ponds raises questions linked to mosquito oviposition, since coloured water can be an attractant. *Culex (*mostly *pipiens)* is commonly found in UK gardens and is a potential vector of viruses including the West Nile Virus (WNV). Any factors that significantly change the distribution and population of *Cx pipiens* could impact future risks of disease transmission. A gravid trap was used to catch female *Cx pipiens* mosquitoes for use in oviposition choice tests in laboratory and semi-field conditions. Two types of pond dye, blue and shadow (which looks slightly red), were tested for their impact on oviposition and survival of wild caught *Cx pipiens*. There were no significant differences in the number of egg batches laid when gravid mosquitoes were given a choice between either blue dye and clear water or shadow dye and clear water indicating that these dyes are not attractants. Larvae hatched from egg batches laid by wild-caught gravid females were used to measure survival to adulthood with or without dye, in a habitat controlled to prevent further colonisation. The experiment was run twice, once in the summer and again in the autumn, whereas the dyes had no impact on emergence in the summer, there were highly significant reductions in emergence of adults in both dye treated habitats in the autumn. Containers with or without shadow dye were placed outside to colonise naturally and were sampled weekly for larvae and pupae over a 6 month period through summer and autumn. There was a significant negative effect of shadow dye on pupal abundance in a three week period over the summer, but otherwise there was no effect. It is likely that population abundance and food was a more powerful factor for mosquito survival than the dye.

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Introduction

The last 15 years have seen an unprecedented change in the status of vector-borne disease in Europe as a result of multiple and complex environmental changes influencing mosquito

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populations [1, 2]. There are many examples throughout history, in temperate and tropical countries worldwide, of how changes to human activities, e.g. deforestation, agricultural practices and urbanisation, alter the distribution, ecology or behaviour of a disease vector and create the environmental conditions conducive to disease transmission [2]. In the UK, changes to land use, climate change and human activities in adaption to that change, are likely to affect mosquito populations. This provides a compelling rationale to investigate how we impact mosquito ecology and behaviour, especially considering their potential as vectors of diseases. It is against this backdrop that we have been studying urban artificial containers (such as water butts) and small ponds which are ideal habitats for a number of mosquito species [3].

In previous studies, we investigated mosquito populations in water butts in both urban and rural habitats [3, 4] where we found a marked difference in mosquito species composition and abundance, with *Culex pipiens* (*pipiens*) dominating urban habitats [3]. We concluded that the storage of water in domestic gardens was increasing urban populations of *Culex pipiens*, a potential vector of West Nile Virus (WNV). Water butts are not the only artificial water bodies in gardens; many gardens have ponds. In the UK domestic gardens are estimated to contain 2.5–3.5 million ponds [5], forming important reservoirs for taxa and helping to sustain aquatic biodiversity [6]. Where ponds are developed as wildlife refugia, the lack of voracious fish predators means that mosquitoes can reach high densities. Understanding factors that will impact on mosquito numbers is important information in the bank for any future issues with mosquito control.

Despite their potential importance for wildlife, domestic ponds are often managed for aesthetic purposes and difficulties arise in maintaining normal ecosystem function whilst retaining desirable aesthetic qualities [7]. An example is the current fashion for using pond dyes to improve reflection and reduce algal growth. Pond and lake dyes are a relatively new commercially available product, sold as an environmentally friendly way to stop the growth of algae through the disruption of photosynthesis [8]. They have proved to be popular at recent high profile garden shows such as Chelsea and Hampton Court. One such product on the market is produced by DyoFix who state that their pond dyes are a blend of European food approved colour dye. The mode of action explained by the manufacturer is that it acts as a light filter, stopping colours on the red end of the spectrum from penetrating the water. Since the plant pigment chlorophyll *a*, which is crucial to photosynthesis, absorbs red light at 662nm, the theory is that addition of a red dye filter will prevent red light from reaching algae below the surface of the water, thereby inhibiting photosynthesis.

The concept of using dyes to limit algal growth by surface inhibition has been around for many years with an early example being aniline dye [9]. Whilst effective at reducing blue-green algal growth it was a particularly hazardous chemical and was never intended for practical use. Modern pond dyes, however, claim to be environmentally friendly with manufacturers stating that they can be used not only at a domestic level in residential ponds, but also have commercial application, being able to work on large bodies of water such as lakes. The manufacturers of pond dyes are confident that they are environmentally friendly since they meet European Food Additive regulations, although very little actual toxicity information is available [8]. A few studies exist that look at the impact of wavelength- blocking pond dyes on algal growth, with mixed results. One found no significant impact on phytoplankton growth, with no difference in chlorophyll a concentrations at the concentrations of dye used (Aquasure, 4ml/m3) [10] whilst another found no reduction in microalgae growth until dye was applied at a high concentration (Aquashade, 5ml/m3 [11]

However there are even fewer studies investigating the use of the product on freshwater fauna, and no investigations into any secondary non-lethal effects the dye may have on organisms, with the exception of a PhD thesis [12] where a dye (Crystal Blue-Ocean) had no impact on catfish survival and yield. Incidentally this study also failed to find any difference in algal growth between dye treated and untreated water.

The use of pond dyes in domestic and ornamental gardens raises questions linked to mosquito oviposition, since coloured water can be an attractant [4]. *Culex pipiens* is commonly found in UK gardens and is a potential vector of viruses including the West Nile Virus [13, 14]. Whilst currently there is no evidence of disease transmission in the UK, any factors that significantly change the distribution and population of *Cx pipiens* could impact future risks of disease transmission. Our previous work demonstrated that *Cx. pipiens* females prefer to lay eggs in black dye water compared with the control in the laboratory and semi-field conditions. It was also observed that survival of larvae through to adults was significantly reduced in dyed water, suggesting that there is some form of chronic toxicity [4]. These results suggest that the dyes are in fact not as environmentally friendly as previously suggested. It also raises the possibility that pond dyes could attract mosquitoes to lay eggs in garden ponds. Studies have reported that *Culex* sp. females use water reflection, darkness, temperature, pheromones and kairomones as part of the cues to choose an oviposition site [15]. There is also evidence that mosquito oviposition is influenced by water body or container colour, type and size [16–19].

Blue pond and shadow lake dyes (red colour) are products similar to the black pond dye which blocks the red end of the visible light spectrum penetrating the water. These dyes were created to be more natural than the black colour pond when applied to the water and are less reflective [20]. Pond blue is the most popular dye used and the most economic although lake shadow is a popular product because it is a colourless dye in the water [20].

Our previous work demonstrated that Dyofix black dye was an attractant to gravid mosquitoes with a significant impacts on survival (emergence) but no measureable impact on mosquito numbers in a semi-natural habitat [4]. The lack of an impact in a natural habitat was explained by a balance between higher oviposition but reduced survival in a black dye treated habitat. Based on this hypothesis, two further pond dyes were studied to determine whether the impact was one found generally or whether the impact of pond dyes on mosquito numbers varied depending on the dye.

Methods

Trapping wild gravid females

Wild gravid female *Culex* were collected using Reiter ovitraps [21] modified by [3] (Fig 1A). Attracted to the bait infusion, females are pulled into a duct connected to the collection chamber by use of a fan located in the upper portion of the trap. The fan is connected to a valve-regulated lead-acid battery that produces a negative air pressure inside the box, allowing for mosquito collection (Fig 1B). The ovitrap consisted of two parts; a lightweight upper portion (a modified toolbox containing a fan, battery and trap for the adults) and a lower portion (5 litre tray) which contained the attractant infusion. Infusions were prepared by fermenting 1 lb of freshly cut grass, 1 lb of hay, 5 g of brewer's yeast and 60 L of tap water. The mixture was fermented in an 80 L black waste bin (44.5cm x 58.5 cm) outdoors for 7 days at the University of Reading. Prior to use the infusion was filtrated using a metallic ring that at the bottom presents a net to remove the grass and the hay.

In total, 10 traps were used for this study, placed in the glasshouse area of the Whiteknights campus of the University of Reading, Berkshire, England (51.4419˚ N, 0.9456˚ W). Gravid female mosquitoes were collected in summer (July to August) in 2014 and 2015. Approximately 1000 gravid female mosquitoes were collected through the sampling period in 2014 and 2015. Most of the mosquitoes sampled belong to the *Culex* genus. However, a few number of *Anopheles plumbeus* (<5) and *Culiseta annulata* (<5) were also present throughout the trapping period.

Pond dyes

Two pond dyes (SGP Blue and SGP Shadow (Dyofix)) were used in this study, supplied as odourless solutions. Pond Blue has a pH of 5–6 at 10g/L water and a rat oral LD50 of 2g/Kg and fish LD50 of >100mg/L, and Lake Shadow has a pH of 7–8 at 10g/L water, a rat oral LD50 of 2g/Kg and fish LC50 of >100mg/L [22].

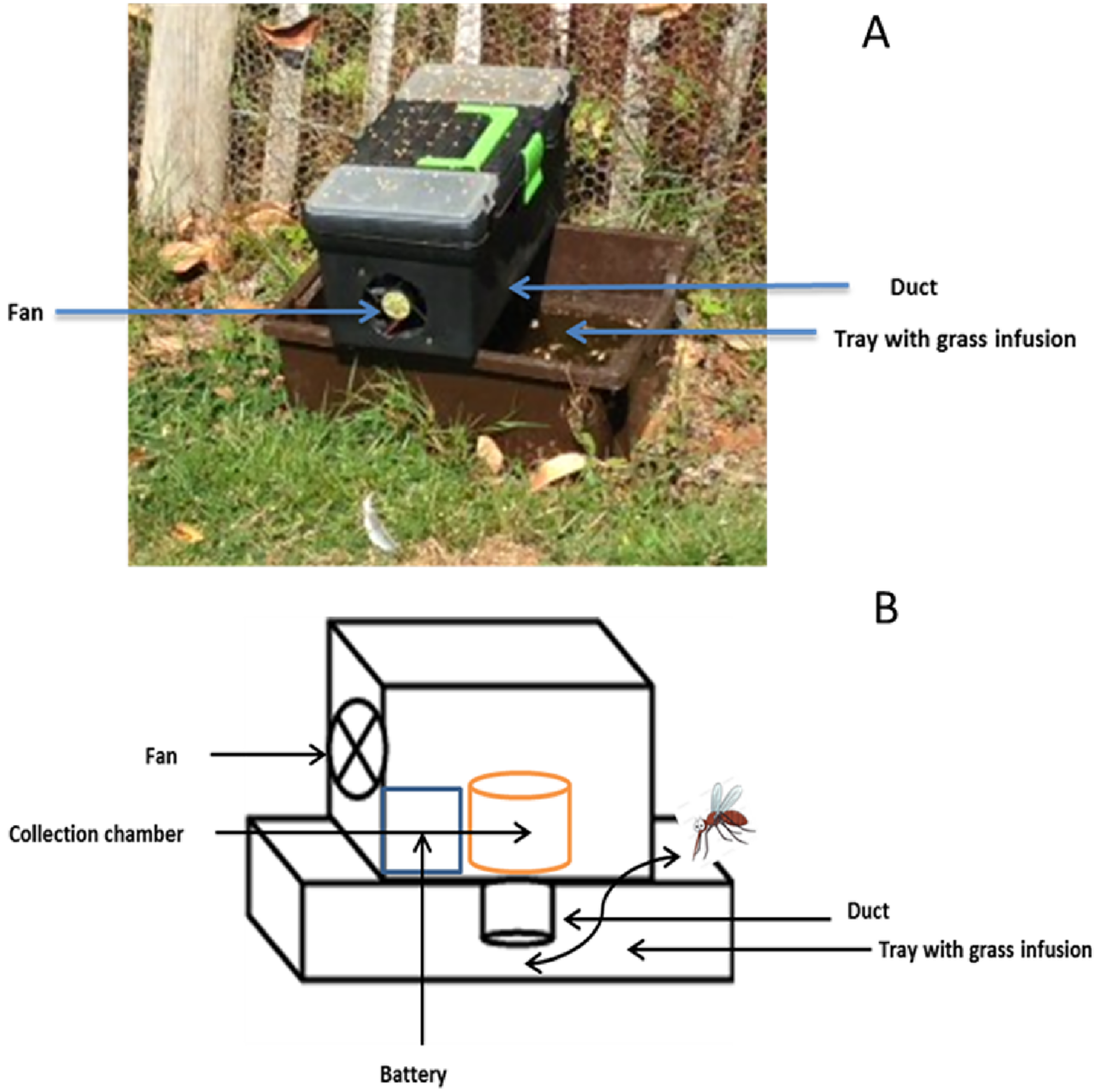

Fig 1. A modified emergence trap [3] based on Reiter's gravid box trap design. A. A diagram of the ovitrap. **B**. Ovitrap used during the experiments.

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Oviposition preferences of wild mosquitoes

A choice experiment was performed between July to September 2014 and again in 2015. A tent (245 x 145 x 95 cm) was placed adjacent to a wooded area in the same location as the gravid traps. Approximately, two hundred gravid mosquitoes were collected from the modified traps and transferred into the tent 24 h post collection. Adult females were provided with a 10% sucrose solution. The tent contained 14 2 L plastic containers (14 length x 21width x 10 height cm): 7 with 1.5 L tap water and 7 with 1.5 L water treated with either blue or shadow pond dye [8]. A randomised block design was used to remove edge effects. After seven days, the containers were taken to the laboratory to count egg batches laid in each container. The experiment was repeated two times and with two types of dye: blue and shadow. Treatments were tap water, 1.5 μl of blue or 1.5 μl of shadow Dyofix pond dye/ 1.5L.

The choice experiment was repeated with wild-caught gravid females in laboratory conditions (25 ± 2˚C) and normal light/dark photoperiod (16:8 h). Twenty gravid females were chosen randomly and transferred into each of 5 cotton net cages 30 x 30 x 30 cm per treatment set. Each cage contained a 10% sucrose solution and two 200 ml transparent plastic cups (12 x 5 cm) filled with 150 ml of either tap water or dye water (10 μ1 blue or 10 μl shadow). The plastic cups in each cage were rotated 90˚ daily to eliminate positional effect and collection of eggs was begun the day after the experiment set up. Eggs were removed from the plastic cups in each cage daily to eliminate oviposition effect. Treatments were tap water and blue and shadow Dyofix pond dye. 10 μl of blue or 10 μl of shadow were dissolved in 1L of tap water and then transferred to the 5 200 ml plastic cups.

The choice experiments were repeated as above but in the absence of light; black bags were used as a cover in each cage during the experiment.

Emergence study

A modified emergence trap [23] was used to measure the impact dye had on survival (Fig 2). Traps were made from lidded 11 litre cylindrical plastic bins (23 x 28 cm). The surface of each lid was removed, keeping the peripheral edges (ring) connected to the bin. Four holes were punched on each ring where two metallics cables were glued to create a conical structure. The conical structure was covered with a white net with an opening in the apex to remove adults. Each bin (9 replicates per treatment) was filled with 10 litres of tap water; a hundred wild larvae (above 2nd instar) and 1.2 g of guinea pig food (3mm pellets). The wild larvae were obtained from egg batches collected from wild mosquito females. Treatments were tap water, 10 μl of blue or 10 μl of shadow of liquid Dyofix pond dye in 10L water [22]. The bins were placed in 9 sites at the glasshouse area (51˚26'13.2"N; 0˚56'31.2"W).

Traps were monitored daily for adult mosquitoes. These were captured using a manual aspirator and transferred into small plastic tubes. The emergence experiment was repeated twice. The first experiment was performed on 27th August—8 th September (Summer) and the second experiment was set up on 29th September—16 th November 2015 (Autumn).

Natural colonisation of containers with or without dyes

Eighteen 10 L bins (26 cm x 26 cm) were filled with 8 L of tap water or 8 L of tap water and shadow dye and 4 g of oak leaves tree collected from the Harris Garden at the University of Reading. For the dye treatment, 8 μl of dye was added to each container (i.e. 1μl concentrate/L water). The bins were placed in pairs in the experimental grounds of the School of Agriculture at the University of Reading (51.4419˚N, 0.9456˚W).

Containers were sampled weekly from August 11th 2014 until 12th November 2014. Sampling was carried out using an aquarium fish net (6 x 12 cm; 1 mm mesh). The net was

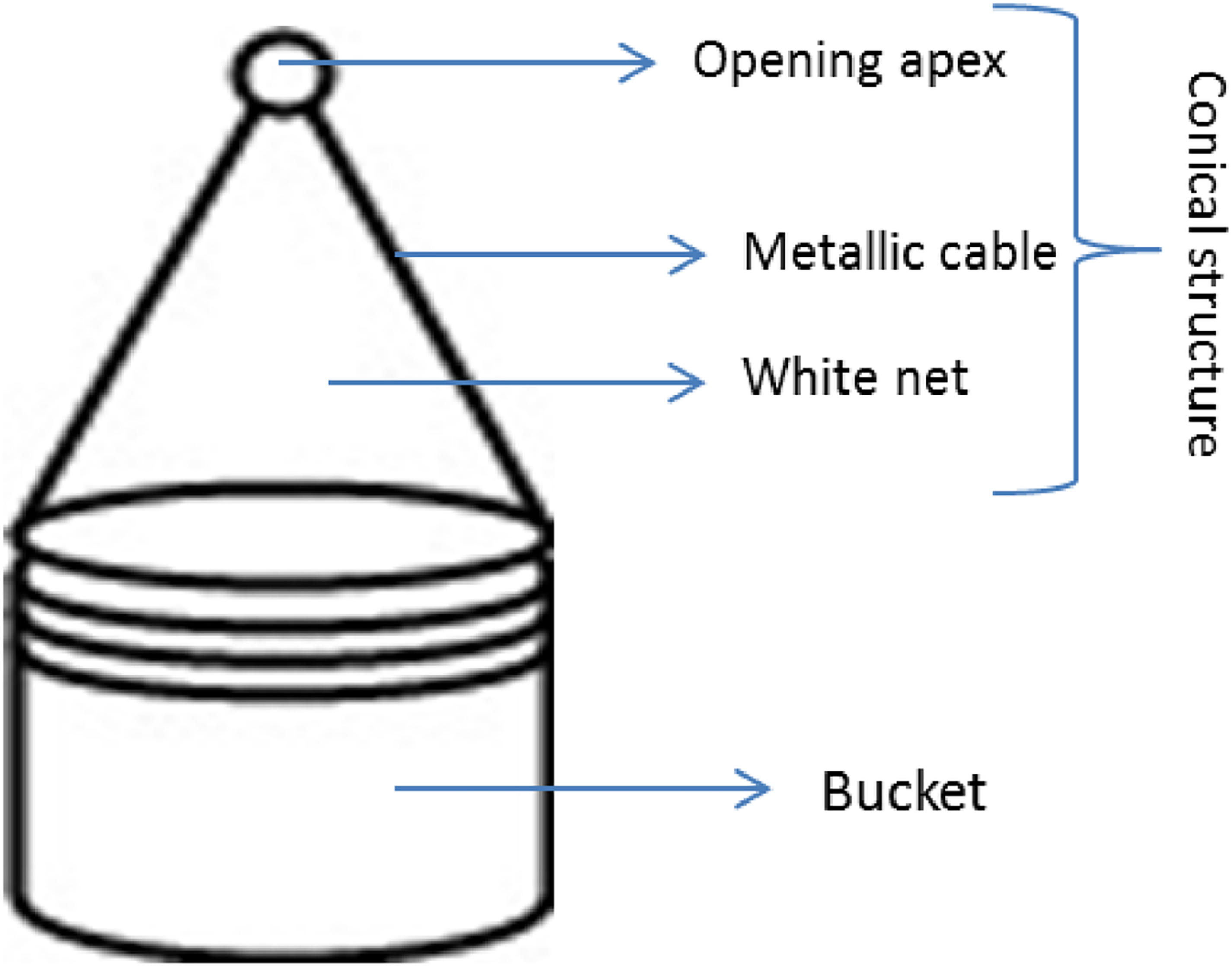

Fig 2. A modified emergence trap.

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dropped into each container and moved in circles from the top to the bottom for 10 seconds before removal. The larvae and pupae collected were transferred to a tray where they were counted; the larvae were returned to the container. Pupae sampled with the net were placed in plastic tubes using plastic pipettes and taken to the laboratory to complete their development to adults. In addition to the net sampling, a visual search for pupae was performed to remove all pupae in each container.

Statistical analysis

Data were tested for normality using a Shapiro-Wilk normality test. Where data were normally distributed a paired t-test and one-way analysis of variance (ANOVA) was used. To observe the difference between the number of egg batches laid by gravid females and the preference in colour a paired t-test was performed. One-way analysis of variance (ANOVA) was used to compare egg batches laid in darkness and light. Oviposition in the tent was not normally distributed and a nonparametric Mann–Whitney *U*-test was performed to assess differences in the number of egg batches laid by blood feed mosquito in semi-field conditions.

Differences in adult emergence between treatments were analysed using a generalised linear model binomial test. Abundance data in water butts were Log (x+1) transformed and the relationship between mosquito abundance and treatment analysed using a 2 way repeated measures ANOVA (Analysis of variance). Mosquito abundance among untreated and treated bins was analysed through the period of time and across the season (summer and autumn). We performed all statistical analysis using R version 3.3.1 [24].

Results

Oviposition selection in a tent

The total number of eggs batches laid by the ovipositing female mosquitoes were 593; 386 in (65.1%) treated treatments and 207 in (34.9%) in untreated treatment (S1 Table). Despite the difference in numbers, statistical analysis found no preference for either the blue or shadow dye compared to the tap water (Shadow W = 126.5; *P* = 0.185 (Fig 3A); Blue W = 135; P = 0.093 (Fig 3B).

Oviposition behaviour in laboratory

Culex spp. showed no preference for oviposition in blue or shadow colour dye in normal light/dark (blue t = -1.776; df = 8; *P* = 0.114; shadow t = -0.919; df = 8; *P* = 0.385) or in darkened conditions (blue t = 0.219; df = 8; *P* = 0.832 (Fig 4A); shadow t = -0.888; df = 8; *P* = 0.400 (Fig 4B, S1 Table)).

Emergence study: field conditions

The total number of adults emerging from different treatments was not significant in the summer (Z = -1.259, *P* = 0.208) but varied significantly in the autumn (Z = -4.049, *P* < 0.001) (Fig

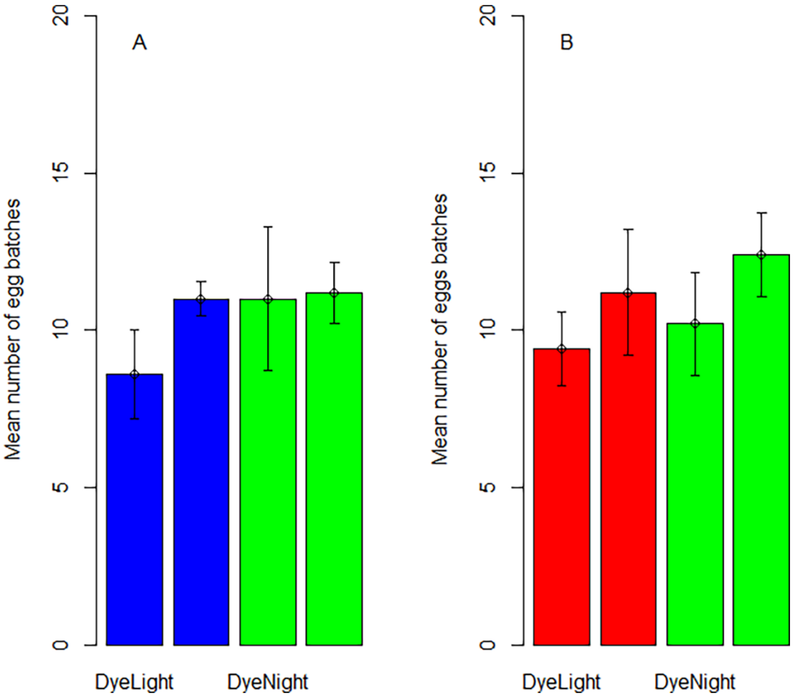

Fig 3. Mean number of egg batches (±SE) laid by *Culex* spp. in semi-field conditions. A. Differences between blue colour dye and tap water. **B.** Differences between shadow colour dye and tap water.

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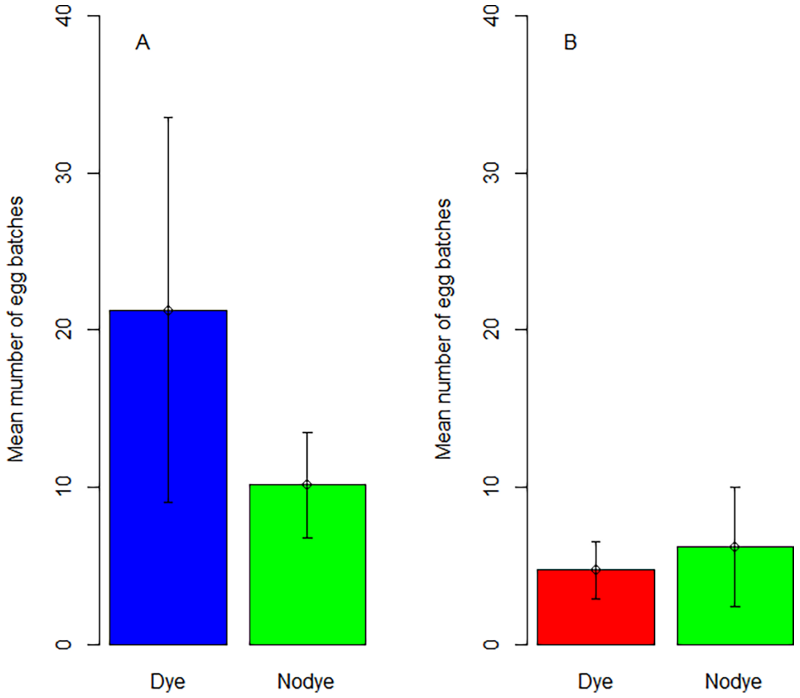

Fig 4. Mean number of eggs batches (±SE) laid by *Culex* wild mosquito in temperature control room in normal and darkened conditions. A. blue colour dye and tap water. **B.** shadow colour dye and tap water.

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5). A Tukey post-hoc analysis found significant differences between the shadow dye and untreated bins and between the blue dye and untreated bins in the autumn season (Table 1, S2 Table).

Natural colonisation of containers

The only species recorded breeding in the small experimental bins were *Culex pipiens (sensu lato)*. Larval and pupal numbers were analysed by season; summer (August-Middle of September) and autumn (Middle to September to October). No significant differences were observed in overall larval and pupal densities between treatments (larval $F_{1\ 32} = 0.318$, $P = 0.576$; pupal $F_{1\ 33} = 2.611$ $P = 0.116$) (Fig 6). However untreated bins showed overall higher pupal densities in summer season compared with treated bins ($F_{1\ 16} = 6.317$, $P = 0.023$) (Fig 7A). This was because pupal density varied significantly in weeks 5, 6 and 7 between treated and untreated bins ($F_{1\ 16} = 5.254$, $P = 0.036$). The total number of larvae varied significantly between seasons, with higher numbers in the summer ($F_{1\ 32} = 14.528$, $P < 0.001$) (Fig 7). However, pupal abundance did not vary between seasons ($F_{1\ 32} = 1.861$, $P = 0.172$).

Discussion

We previously demonstrated that gravid female *Culex* mosquitoes preferred to lay eggs in black dyed water [4]. However we show here that blue and shadow (colourless) pond dyes had no impact at all on oviposition in either laboratory tests or in the semi-field study in the tent. Given these results, it would seem that the black dye colour does have more attractive or stimulant properties than either the blue or shadow dyes. Possible explanations for these results are that mosquitoes choose to oviposit in black water because i) it indicates depth and therefore a lower threat of desiccation before juveniles develop, ii) it might indicate a higher concentration

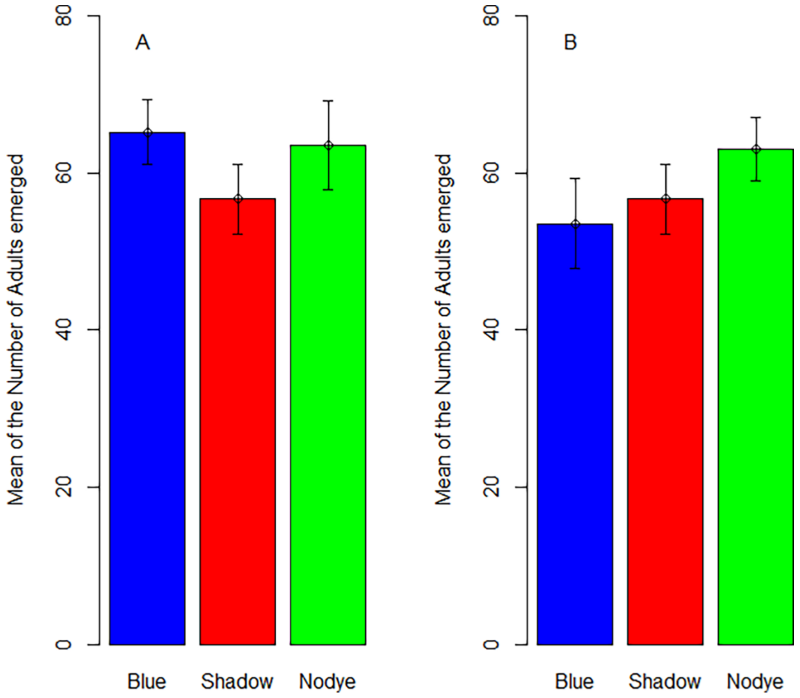

Fig 5. Mean number (±SE) of adults emerged from the three treatments (tap, blue and shadow) in A. summer and B. autumn.

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of organic matter providing nutrition [25, 26], iii) it mimics shading of the water body [27] and iv) black dyed water holds heat longer than undyed and mosquitoes may be able to visually sense near-infrared radiation (700 to >900 nm) [23].

Although there was no sustained effect on egg laying, blue and shadow dyes had an impact on *Culex* sp mosquito survival. For both dye treatments, significantly fewer adults emerged from containers that had been placed outside and covered to prevent colonisation by other mosquitoes or macroinvertebrates. These results are similar to those previously reported by Ortiz and Callaghan [4] (using an identical experimental design) where the number of adults emerging from a black dye treatment were significantly lower than the control. This was not easily explained since a toxicological assay found no significant larval mortality following exposure to any of the dyes, at various concentrations over a 48 hour period. If the experiment had been in a treated natural pond full of algae, and if that algal population was impacted by the treatment, then an explanation of the results might be a reduction in the availability of food, since algae form a significant proportion of the larval diet. However, mosquito larvae are not discriminatory and their diet will consist of detritus and microorganisms as well as algae [28]. In this particular experiment, tap water was used with guinea pig food (and, potentially,

Table 1. Tukey post doc tests comparing adult emergence from the three treatments (tap, blue and shadow) in summer and autumn.

Interaction	Summer		Autumn	
	Z	*P*	Z	*P*
Blue-Shadow	0.738	0.741	-1.327	0.38
Blue-Tap	-1.262	0.417	-4.056	<0.001
Shadow-Tap	-2.001	0.112	-2.738	0.017

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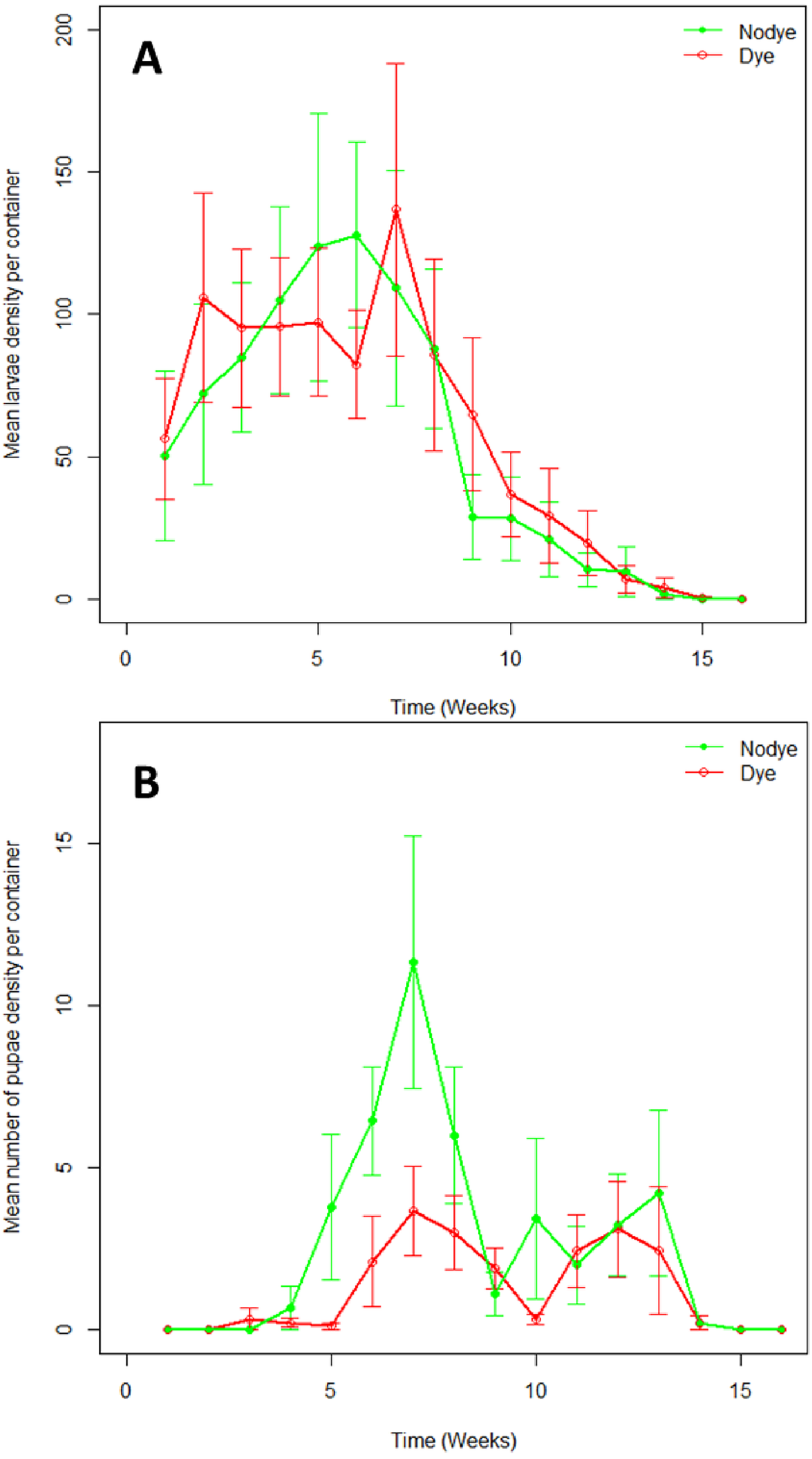
A
Nodye
Dye
Mean larvae density per container
0
50
100
150
200
0
5
10
15
Time (Weeks)
B
Nodye
Dye
Mean number of pupae density per container
0
5
10
15
0
5
10
15
Time (Weeks)

Fig 6. Mean (±SEM) *Culex pipiens* abundance in untreated and shadow dye-treated small bins across the sampling period. A. larvae. **B.** pupae.

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resulting microorganisms) for larvae to eat. Therefore algae should not have been a limiting factor.

Our previous work on the effect of black pond dye failed to detect any measureable impact on mosquito abundance when the experiment was conducted in a naturally colonised container rather than under controlled conditions [4], despite a very strongly significant impact on survival and oviposition under controlled conditions. However, in contrast, the shadow dye treatment had a significantly negative impact on pupal abundance in a naturally colonised container in late summer. The containers all had high abundances of *Culex pipiens* mosquitoes which generates competition and can have a significant impact on the development rate [29–31] and survival of conspecific mosquitoes [17, 19, 32]. It is likely that, since these dyes are not oviposition attractants, a lack of difference between treatments (apart from the impact on pupae during one season) is more related to overcrowding and competition having a greater impact than the dye. The relationship between mean larval numbers converted into mean pupal numbers shows a very low survival rate (between 1% minimum and 7% maximum) compared to the emergence rates in the controlled experiment (around 60%).

Vision is a long-range cue used for oviposition site location by many mosquito species [33]. A number of studies have looked at the behavioural ecology of oviposition choice, including colour, for a variety of mosquitoes of the genera *Aedes*, *Culex*, *Anopheles*, and *Toxorhynchites* [16, 18, 25, 34–37]. Black, blue and red colours all seem to be attractive to species including *Aedes albopictus*, *Culex annulirostris*, *Aedes albopictus* and *Aedes aegypti* [25, 35]. However many of these studies result from laboratory studies, which whilst instructive, may not accurately reflect the cues that are used in the field. Whilst laboratory studies that measure the total number of mosquito eggs (or egg batches) laid in test versus control conditions can provide useful information on oviposition stimulants and repellents, these studies can say little about the impact of these chemicals on oviposition in nature. This has certainly been the case in our experimental research.

It has been suggested that the terminology used in laboratory and field studies should be clarified, so that oviposition attractants or repellents are terms used when mosquitoes are using long- to middle-range cues resulting in a reorientation of flight direction [38]. In the case of short-range or contact cues, such as those used in laboratory studies such as ours, Day [35] suggests that the term stimulant or deterrent would be more accurate.

Conclusion

These results show that pond dyes have an impact on mosquito behaviour and survival. Although the blue and shadow dyes had no impact on oviposition (unlike black dye [4]), the emergence of adults in dyed water was significantly impacted. These results do imply that the dye is in some way toxic to the mosquitoes over a long period of time, although it is not clear what is happening.

Populations of mosquitoes are likely to change as landscape and climate changes, and it has been suggested that towns and cities represent some of the highest risk areas for potential transmission of bird-related mosquito-borne diseases [39]. The ornithophagic habit of *Cx. pipiens* limits its potential as a bridge vector but seasonal abundance and other eco-behavioural characteristics predispose this species to serve as a potential enzootic vector of WNV, capable of maintaining cycles among bird populations, in the UK [40]. It is important to understand environmental factors that might impact on mosquito population success in urban habitats,

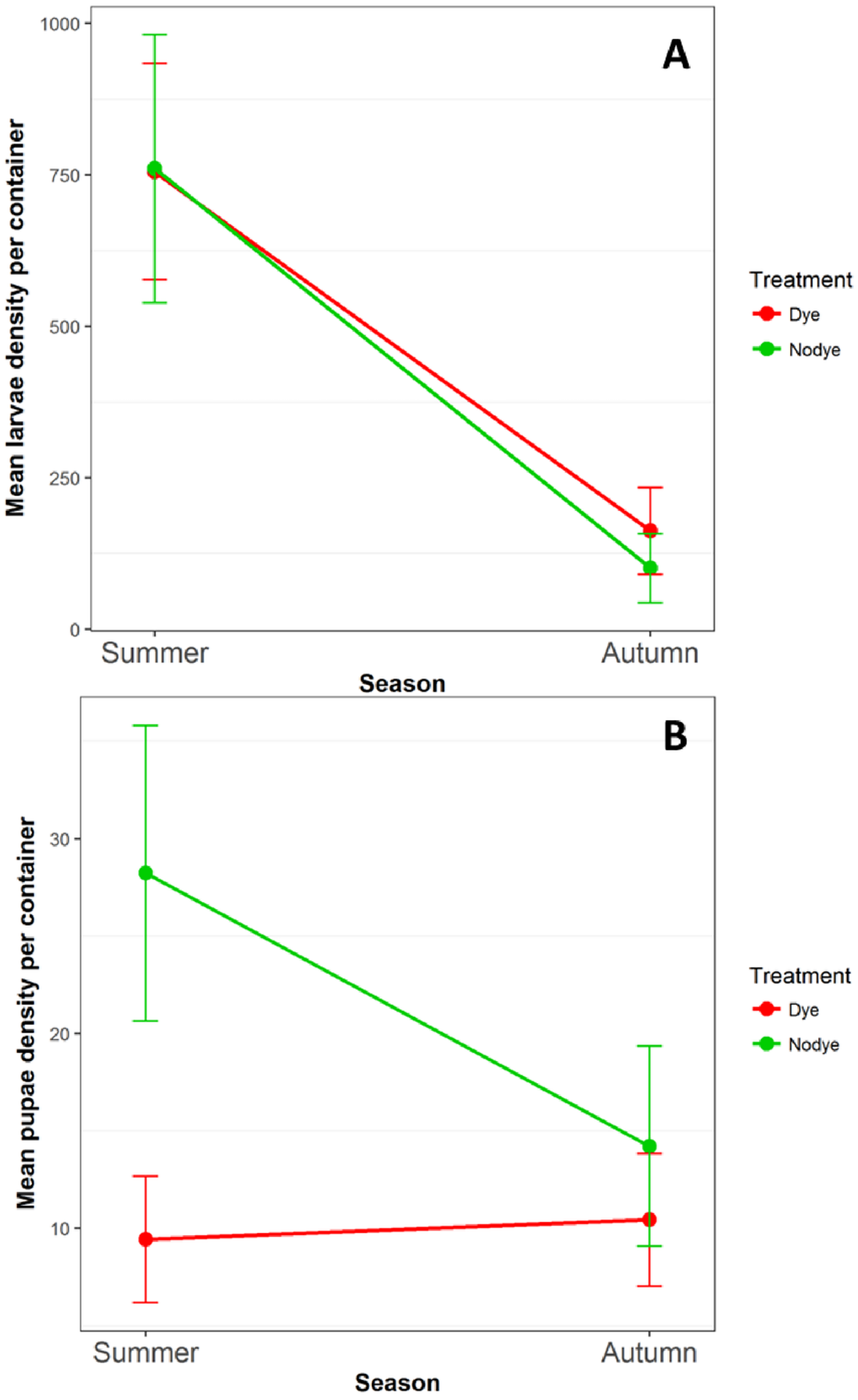
A
Mean larvae density per container
1000
750
500
250
0
Summer
Autumn
Season
Treatment
Dye
Nodye
B
Mean pupae density per container
30
20
10
Summer
Autumn
Season
Treatment
Dye
Nodye

Fig 7. Mean (±SEM) abundance between untreated and treated small bins. **A**. larvae. **B**. Pupae.

https://doi.org/10.1371/journal.pone.0193847.g007

particularly if these factors are anthropological in nature. The results presented here and in our previous work show that dyes are not totally neutral and can reduce fecundity as well as act as attractants [4]. Mosquito larvae are normally one member of a freshwater ecosystem that includes other macroinvertebrates. We know that these interact with each other and so the next stage will be to look at mosquito populations in dyed ponds containing whole communities.

Supporting information

S1 Table. Raw data for experiment to measure number of egg batches laid by *Culex* spp. in semi-field conditions and in light or dark conditions in the laboratory.
(XLSX)

S2 Table. Raw data for experiment to measure adult emergence from the three treatments (tap, blue and shadow) in summer and autumn.
(XLSX)

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Investigation: Natali Ortiz-Perea, Rebecca Gander, Oliver Abbey.

Supervision: Amanda Callaghan.

Writing – original draft: Natali Ortiz-Perea.

Writing – review & editing: Amanda Callaghan.

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