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Effects of container colour on the oviposition of Culex pipiens complex mosquitoes (Diptera: Culicidae) and indicators of wastewater quality

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EFFECTS OF CONTAINER COLOUR ON THE OVIPOSITION OF *CULEX PIPIENS* COMPLEX MOSQUITOES (DIPTERA: CULICIDAE) AND INDICATORS OF WASTEWATER QUALITY

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A research report submitted to the Faculty of Science,
University of the Witwatersrand,
In partial fulfilment of the requirements for the degree of Master of Science

Johannesburg

7 December 2019





DECLARATION

I declare that this research report is my own, unaided work and was carried out under the supervision of Prof A. Gardiner, Dr C. Graf, and Dr D. Woodford. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



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ABSTRACT

Mosquitoes are important potential vectors for the spread of various diseases. Constructed wetlands (CW) are breeding sites for mosquitoes especially when used to treat sewage water. Sewage water is high in bacteria and organic material but has low oxygen and light levels. This means few mosquito larvae predators can survive but the larvae can feed and their populations grow unchecked.

This study investigated the influence of container colour, water dissolved oxygen, electrical conductivity, and temperature on mosquito oviposition and breeding success. Sets of ten black and ten white containers were set up in various locations for a period of 15 days. Water quality measures and egg raft counts were taken from the fifth up to the 15th day. After the 15th day of each trial all larvae (except for the first instar and pupa) were collected and identified.

There were more rafts and larvae, and higher temperatures, found in the black containers. The white containers had higher dissolved oxygen levels and lower conductivity levels, which suggests that white containers had a positive effect on dissolved oxygen levels in water. To minimize the breeding of *Culex* mosquitoes in a CW it is therefore suggested that white containers be used to minimize mosquito breeding.

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Completing a master's degree and writing a research report is hard work. One curious question leads to a path of self-discovery, theory discovery, and discovering what people mean to you in life. Statistics is still not a friend but with the patient assistance of Dr Cleo Graf, Prof Alan Gardiner, and Dr Darragh Woodford I could see myself growing as a scientist. The importance of time became once again apparent, life is short and if you want to get some where you need to take small steps. There were many people that assisted in these small steps before and during the study process. I would like to thank Tammy van Tonder and the Wits team for their assistance with the administration, Dr Chevonne Reynolds and the APES lecturers for their information and inspiration.

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LIST OF SYMBOLS

ALU	–	Applied Learning Unit – Part of the Southern African Wildlife College
BAS	–	Biologically Activated Sludge
CW	–	Constructed Wetland
DDT	–	Dichlorodiphenyltrichloroethane
df	-	Degrees of freedom
DO	–	Dissolved Oxygen
H	-	Kruskal-Wallis H test – Test statistic
KCl	–	Potassium chloride
n	-	Number of observations
NS	-	Non Significant
p	-	Significance
s.l.	–	Senso lato – in the broad sense
SAWC	–	Southern African Wildlife College
Sp	–	One species
Spp	–	Two or more species
Std. Dev.	–	Standard deviation

1 INTRODUCTION

The human population in Sub-Saharan Africa, including South Africa, is growing and with that comes the need for clean water and sanitation (Dos Santos *et al.*, 2017). Various South African communities do not have regular access to clean water and use natural sources such as rivers to fulfil their needs. The South African government recognized this need for clean water and aim to provide safe drinking water, at a reasonable price, to all households by 2030 (Johnson, 2017). Unfortunately rivers are becoming more and more polluted as a result of raw sewage flowing directly into the ecosystem (Obi *et al.*, 2002). This is because of failing, insufficient and non-existing sewage treatment facilities (Barlow and Clarke, 2002; Dos Santos *et al.*, 2017). These conditions could have detrimental impacts on human and environmental health (Isife, 2012).

There are alternative methods to clean water. In America and Europe, artificial wetlands are widely used to improve water quality by cleaning wastewater (Vymazal, 1996). In South Africa, artificial wetlands are being used for mine drainage remediation (Vymazal, 2019). In Australia artificial wetlands are also used for clean various levels of polluted water. This increased the mosquito populations, especially with the use of open wetlands because of the increase in favourable habitat (Webb and Russell, 1999; Webb, 2013).

1.1 WETLANDS AND CONSTRUCTED WETLANDS

Wetlands are important in natural systems for a number of different ecosystems services. They act as natural filters improving water quality (Hammer and Bastian, 1989; Mitsch and Gosselink, 2000), assist in flood control, stabilize river banks, recharge ground water, and provide food and habitat for wildlife (Reddy *et al.*, 2000), even small wetlands are important for biodiversity (Semlitsch and Bodie, 1998). It is the combination of substrate, flow, and vegetation in wetlands responsible for the various valuable tasks (Vymazal, 2005).

Natural wetlands can filter various natural and anthropogenic contaminants out of the water. In too large concentrations, contaminants can lead to wetland pollution, degradation and biodiversity loss (Oberholster *et al.*, 2008). Such damage can be prevented by treating wastewater, such as sewage, before it goes into a natural system using constructed wetlands (CW) (Stefanakis *et al.*, 2014). CW remove

pollutants from water mimicking processes found in natural wetlands (Kadlec and Knight, 1996).

CW designs are based on two main types, namely free water surface wetlands and sub-surface flow wetlands (Vymazal, 2010). Compared to traditional wastewater treatment plants CW's are effective filters that are affordable to install, operate, and maintain (Vymazal, 1998). The free water surface CW is the cheaper of the two to build as no gravel is needed (Vymazal, 2010). As with natural wetlands, a well-constructed CW can become a hotspot for biodiversity such as birds and aquatic insects (Worrall *et al.*, 1997; Longcore *et al.*, 2006). The biodiversity increase and a well-designed CW may even attract people to use it as a focus for leisure activities such as running, picnics, and bird watching (Webb, 2013). Unfortunately, mosquito population are still breeding in the ultimate open water CW.

Sub-surface flow CW would be more effective in minimizing mosquito populations. These systems are set up like open water systems and then filled with a substrate such as gravel through which the water flows. Sub-surface CW is effective at treating wastewater but is expensive to set up and can clog easily when incorrectly managed (Vymazal, 2010). This makes open water systems more attractive to use for water treatment. However, mosquitoes prefer these open water sites with thick vegetated areas (Russell, 1999; Thullen *et al.*, 2002) and shallow, stagnant water (Knight *et al.*, 2003; Thangamathi *et al.*, 2018) rich in nutrients (Webb and Russell, 1999).

Theoretically, a shallow CW with pre-treated wastewater trickling through thick stands of vegetation flowing into large, shallow, open ponds will treat the water the best (Webb, 2013). However, these create ideal conditions for mosquito breeding, whereas deep, sparsely vegetated pools with steep sides and fast flowing water create favourable conditions for mosquito larvae predators such as fish and dragonfly larvae (Russell, 1999; Webb, 2013; Segev *et al.*, 2017).

The design of CW should be done with care to ensure effective water treatment. A system with fast flowing water limits the capture of impurities in the sediment. Too little plant biomass will limit their water cleaning potential. Overly deep pools will limit the effect of the sun and plant roots on the impurities in the water. These factors can result in the decline of mosquito predators (Russell, 1999; Knight *et al.*, 2003; Webb, 2013). Some of these factors may limit mosquito breeding but not effective in terms of treating water (Russell, 1999; Knight *et al.*, 2003). A good

balance between effective purification and limiting mosquito habitat is important. Polluted water and mosquitoes are both a potential health risk to humans and livestock. These health risks are higher in warm areas with more mosquito species that are potential vectors for human and livestock disease.

The ideal constructed wetland as designed by the SAWC

To address the lack of sanitation in rural areas of South Africa, and to effectively clean its own wastewater, the Southern African Wildlife College has a vision to create a cheap, robust and modular constructed wetland. The system is a version of a surface water flow system comprising of multiple, interlinked, and small (700lt) open tanks with floating reed mats on them. Vegetation in the containers assists in removing contaminants and filtering the water (Webb, 2013). Because of the small size of the containers, the plants' leaves can be easily removed which is important to increase dissolved oxygen (DO), pH and temperature (Alvarez and Becares, 2008). This is important in the process of water treatment. Less vegetation also means an increase in photolysis helping with water treatment and the killing of pathogens (Alvarez and Becares, 2008). However, small open containers with vegetation make this ideal mosquito-breeding habitat, while the poor water quality makes it unlikely that mosquito larvae predators will survive (Winkster, 2018).

The modular container-based system can easily be reconfigured as wastewater volume changes. Maintenance is also easier because the pipes are easy to reach and the vegetation within reach. It can easily be harvested which is necessary to prevent clogging and remove carbon form the system. Experiments are still ongoing to understand the setup and limit mosquitos, and this research project specifically examined container colour as a factor in oviposition.

1.2 MOSQUITOES

Mosquitoes all fall under the family Culicidae with the most important genera in disease transmission being *Aedes, Anopheles,* and *Culex* (Dale and Knight, 2008; Onchuru *et al.*, 2016). Apart from being vectors of disease, *Culex* species are also know to become a nuisance in areas with stagnant and nutrient rich water (Webb and Russell, 1999; Webb, 2013). In wetland systems mosquitoes fulfil a biologically important role as filter feeders, feeding on micro-organisms, organic detritus particles, and bacteria (Mazzacano and Black, 2013; Onchuru *et al.*, 2016). Mosquito larvae and adults are an important food source for predators including

hydras, flatworms, spiders (Ndava *et al.*, 2018), insects, copepods, fish, birds (Mazzacano and Black, 2013; Collins *et al.*, 2019) and bats (Gonsalves *et al.*, 2013). Mosquito larvae can however be used as biological indicators as mosquitoes include ecological generalists and specialists that interact with the terrestrial and aquatic fauna of wetlands (Schafer *et al.*, 2004).

The mosquito lifecycle includes four stages (Figure 1). It can take as little as five days for the lifecycle to be completed depending on temperature and species (Dale and Knight, 2008). Eggs are laid either on damp soil (*Aedes, Ochlerotatus and Psorophora* species*)*, as single eggs in the water (*Anopheles, Toxorhynchites, Sabethes and Wyeomyia* species) or, egg rafts (*Culex, Coquillettidia, and Culiseta* species*)* on the water surface (Schafer *et al.*, 2004; Dale and Knight, 2008; Webb, 2013), or eggs are attached to vegetation under the water (*Mansonia)* depending on the species (Silver, 2008). Eggs hatch between 24 and 36 hours after laying for *Culex* species, and between 48 and 72 hours for *Aedes aegypti* (Bartlett-Healy *et al.*, 2012)*.*

Female mosquitoes carefully select their site for oviposition. They use chemical cues to hone in to the breeding site. At the site they use a combination of tactile (Bentley and Day, 1989)**,** olfactory cues (Afify and Galizia, 2015), and colour (Torrisi and Hoback, 2013) to select the breeding site. Each mosquito species has different niche habitats in which their larvae can survive. They therefore need to take care and use all the sensors they have before making their valuable egg deposit.

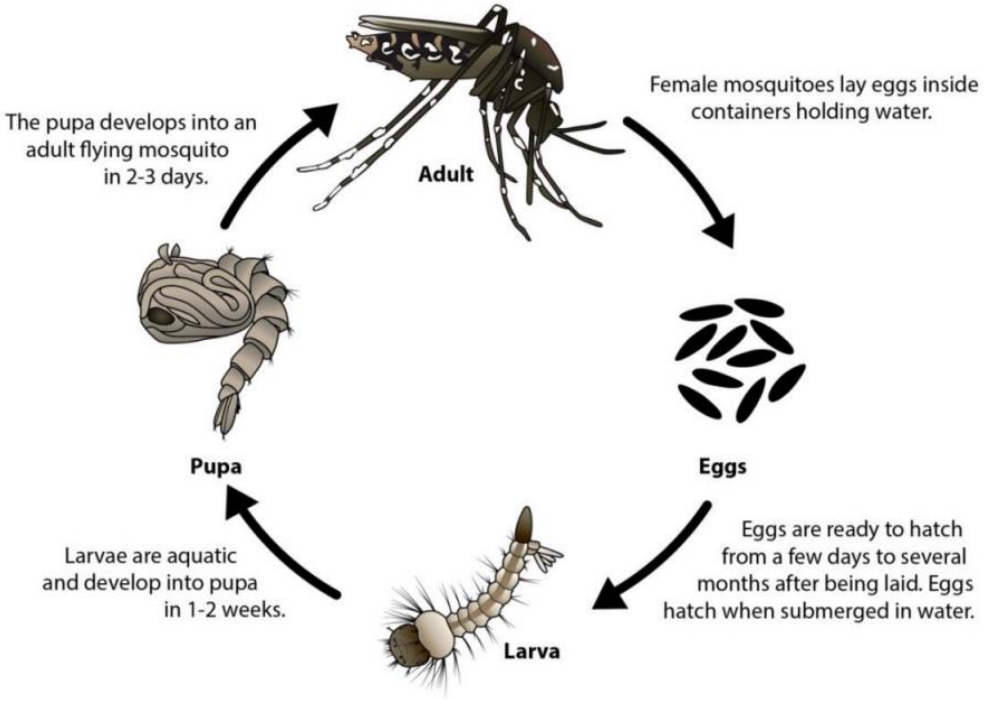


Figure 1: *The life cycle of a mosquito as presented by the Centre for Disease Control and Prevention (CDC, 2014).*

The larval stage consists of four instars that are dependent on water for food and development (Day, 2016). They use a siphon to breath at the water surface and do not need any dissolved oxygen in the water (Dale and Knight, 2008). Development, once again, depends on temperature. In optimal constant temperature of 25 degrees Celsius, it takes *Culex pipiens* eight days from first instar larvae to adult, while lower and higher temperatures increase development time to a maximum of 20 days (Loetti *et al.*, 2011). In optimum conditions, it takes *Aedes albopictus* five to seven days to develop into adults while *Anopheles* mosquitoes need between ten and 15 days (Schafer *et al.*, 2004; Stresman, 2010). In colder conditions, it can take up to 25 days for *Anopheles* mosquitoes to become adults (Collins *et al.*, 2019). Development is therefore highly species and temperature dependent.

1.2.1 Method of feeding

Female mosquitoes need the protein in a blood meal to assist in the development of eggs (Jupp, 2005). With their chemical sensors, found on the antennae, they hone in on the lactic acid and carbon dioxide released by their food source. As they get closer to their target, or blood source they use movement and heat to find uncovered skin. Their proboscis then pierces through the skin in search of a vein

or arteriole. Once found the mosquito injects an anti-coagulant into the host's body to prevent blood clotting enabling them to suck up the blood. The anti-coagulant causes skin irritation, carries disease into the host, and could lead to death (Jupp, 2005). Blood meals are taken from either humans, mammals, or birds depending on the mosquito's specialisation (Cornel *et al.*, 2018).

The larvae are filter feeders feeding on bacteria. Some of these bacteria stay in their gut up to adult stage. The bacteria has a significant effect on adult mosquito development and egg production (Gaio *et al.*, 2011). Adult *Anopheles gambiae* with limited gut micro biota is more susceptible to *Plasmodium* infections, suggesting that gut bacteria could defend the adult mosquito from pathogens (Onchuru *et al.*, 2016).

1.2.2 Mosquitoes as vectors of disease

Mosquito management is important, as they are effective transmitters of disease, and pathogens (Jupp, 2005; Kilpatrick *et al.*, 2008; Onchuru *et al.*, 2016). There are 22 mosquito borne diseases found in South Africa of which ten effect human health. Of the ten diseases, four are medically important, of which malaria the most well-known. Other disease known but rarely found in South Africa include Chikungunya, Yellow fever, Sindbis, West Nile virus, Rift valley virus, and Dengue (Russell, 1999; Rey *et al.*, 2006; Nguyen *et al.*, 2012; Onchuru *et al.*, 2016).

The *Anopheles gambiae* complex (s.l.) are the main vectors for malaria in South Africa (Coetzee *et al.*, 2000; Sinka *et al.*, 2012; Collins *et al.*, 2019). There are eight different species recognised today of which three, namely *Anopheles gambiae*, *Anopheles culozzi*, and *Anopheles arabiensis* (Lindsay *et al.*, 1998; Collins *et al.*, 2019) are identified as vectors of medical importance to South Africa.

Culex mosquitoes are, in comparison, not considered as important a vector for disease. This could change as temperatures change creating more habitats for *Culex* mosquitoes. In South Africa mosquitoes are considered potential vectors for human disease pathogens (Bhattacharya and Basu, 2016). Globally *Culex* mosquitoes carry various disease of which West Nile virus is the only disease considered important to human health (Morales *et al.*, 2006; Hamer *et al.*, 2008; Loetti *et al.*, 2011).

Aedes mosquitoes are carriers of various diseases such as chikungunya, yellow fever, and dengue (Jupp *et al.*, 1993). *Aedes aegypti* is an urban host for dengue

fever in South Africa (Jupp 2005). Dengue fever is mainly spread to livestock, but if there is a human around, the vector can infect humans as well (Jupp, 2005).

1.2.3 Management of mosquitoes

Understanding mosquito ecology and biology is important in managing their numbers. Changes in ecology would affect different mosquito species differently as they have different habitat preferences. Changes in water quality such as increases in phosphates and ammonium, and changes in local climate has a positive effect on vector mosquito breeding and therefore on disease transmission (Onchuru *et al.*, 2016). Other factors that affect mosquito breeding success include nitrogen content, temperature, pH, dissolved oxygen (DO), turbidity, and salinity (Minakawa *et al.*, 2000; Mwangangi *et al.*, 2007; Mala and Irungu, 2011; Onchuru *et al.*, 2016).

For example, a healthy wetland system has a higher diversity of mosquitoes, which has shown to decrease the total number of mosquitoes breeding at the site. The possible reasons is competition for habitat, food (Mazzacano and Black, 2013; Webb, 2013; Carver *et al.*, 2015), and species preying on one another (Ruybal *et al.*, 2016). Urbanisation, through poor waste and sanitation management, has increased available habitat and therefore the distribution of vector mosquitoes (Okogun *et al.*, 2005; Okiwelu *et al.*, 2012; Cuthbert *et al.*, 2018). The result is an increased occurrence and spread of diseases such as malaria (Rani *et al.*, 2018).

Historical methods used to control malaria outbreaks was indiscriminate spraying of DDT (Dichlorodiphenyltrichloroethane). South Africa banned the general use of DDT in 1974, but still used it to combat malaria in the northeastern parts of the country. Instead of DDT, Pyrethroid deltamethrin was used to spray in and around homesteads. It was regarded to be just as effective as DDT but less harmful to humans. The limited spraying stopped for the period 1995 until 2000 (Wells and Leonard, 2006) when the spraying of DDT was resumed. This was a direct result of the increase in malaria cases in the period 1995 until 1999 (Jupp, 2005). Spraying of houses continues in known malaria areas during mosquito outbreaks to decrease the chance of malaria infections (Jupp, 2005).

Fish is a well-known biological control method to control mosquito larvae numbers in waterbodies (Segev *et al.*, 2017). Alternative large-scale methods being

investigated as biological control for mosquito larvae are calanoid copepods (Cuthbert *et al.*, 2018). Another biological method being investigated is called Sterile Insect Technique (SIT) to reduce mosquito populations (Chung *et al.*, 2018). Infertile mosquito males are introduced into an area. They then mate with the females that as a result produce infertile eggs (Vaníckova *et al.*, 2017; Chung *et al.*, 2018).

A personal precaution to limit transmission of disease from mosquitoes to humans is by covering skin at night, use mosquito nets while sleeping, use repellents, and adding screens to entrances of houses (Jupp, 2005). There is a difference in when mosquitoes are most active. *Anopheles* and *Culex* species are active after dark while *Aedes aegypti* bite in the day and at dusk (Jupp, 2005).

1.3 MOSQUITO ECOLOGY AND BIOLOGY

Culex

Culex pipiens complex is the focus of this study as they are a potential vector for disease. In South Africa they are mainly considered nuisance mosquitoes as their numbers can increase rapidly (Webb and Russell, 1999). The modular waste water-treating constructed wetland setup at the Southern African Wildlife College (SAWC) was found to be a productive breeding area for various mosquito species (Winkster, 2018). This could be a result of high nutrient levels in the sewage water (Vymazal, 2010). Their eggs are not drought resistant (Jupp, 1967). The species survive the winter as dormant larvae and pupa in permanent water and as adult females (Jupp, 2005).

Culex mosquito adults can breed in most qualities of water, but prefer water with high nutrient content that serves as food for the larvae. Habitats created by humans such as containers, drains, gutters, and sewage treatment make important breeding sites (Subra, 1981). Drains lack predators and have high levels of nutrients, which make them attractive breeding sites for *Culex* mosquitoes (Arana-Guardia *et al.*, 2014). *Culex* mosquito larvae dart to the bottom of the water body with any predator like disturbance above the water. They feed under the water surface unlike *Anopheles* mosquitoes that feed closer to the water surface (Onchuru *et al.*, 2016). A female *Culex pipiens* complex mosquito takes blood from humans or birds. They then lay a single egg raft of an average of 155 eggs. These eggs develop into larvae in about 24 to 36 hours after laying (Day, 2016). These

larvae develop at a rapid speed but males develop at a faster rate than female mosquitoes (Subra, 1981). The rate of larvae development depends on nutrient availability and temperature (Subra, 1981).

Aedes

Aedes mosquitoes are black and white mosquitoes active during the day as well as at dusk (Jupp, 2005). They lay single eggs that can survive for several years as long as they are in an area with a high humidity (Jupp 2005). They can therefore use a wide range of habitats to breed in such as tyres, containers, tree holes, rock pools, and in the soil of dry pans waiting for the rain (Gargan *et al.*, 1988; Reiskind and Zarrabi, 2012). Females do spread the risk by laying a few single eggs in multiple locations. This behaviour is known as skip-oviposition (Oliva *et al.*, 2014). However, they are more specific in site selection than Culex species (Onchuru *et al.*, 2016). It was found that *Aedes aegypti* breed in locations with higher ammonium and sulphate levels than what *Culex* and *Anopheles* species would use for oviposition (Onchuru *et al.*, 2016).

1.3.1 Breeding site selection and colour

Colours are used by biologists to attract specific insects such as flies (Diclaro, II *et al.*, 2012), aquatic insects (Kriska *et al.*, 2006), and diurnal insects (Long *et al.*, 2011; Vrdoljak and Samways, 2012) for research and population monitoring. As with flies and diurnal insects, mosquitoes are also influenced by colour. Lab studies have indicated that *Aedes aegypti* prefer darker colours when choosing a breeding site. They chose red, blue, and black containers to breed in rather than white and yellow (Yap *et al.*, 1995). Various other studies were done that indicate mosquitoes are attracted to darker rather than white colours using cloth (Brett, 1938), paper, light (Hoel *et al.*, 2011), coloured water (Beehler *et al.*, 1992), and containers (Mayhew and Raman, 1999; Collins and Blackwell, 2000; Torrisi and Hoback, 2013). A large amount of these studies focussed on *Aedes spp.* and their attraction to different colour traps. Derraik and Slaney (2005) have performed some lab studies on *Culex spp* indicating that they are attracted to darker colours. Therefore, colour may be an important consideration when choosing any open water containers in areas with mosquito populations, especially in areas where medically significant mosquito species occur.

Container colour can also play a role in the water temperature and the chemical composition of water. Darker colours absorb more energy from the sun (Chapin *et al.*, 2011), therefore water in darker containers can become warmer than light coloured containers. Dissolved oxygen is very sensitive to temperature change (Cao *et al.*, 2019). With the increase in temperature DO levels decrease, which then makes it difficult for mosquito predators to live in as they use dissolved oxygen to breathe (Russell, 1999). Sunlight also kills some bacteria, such as *Escherichia coli* and *Enterococcus faecalis*, and influences biochemical processes in water (Tadesse *et al.*, 2004; Kadir and Nelson, 2014; Koumaki *et al.*, 2015; Bidin *et al.*, 2017). Using white containers will reflect sunlight deeper into the container increasing its effect on biochemical processes. The increased sunlight in these containers makes it possible for photosynthetic biofilm organisms to grow on the inside, which then has a further effect of increasing DO during the day (Glud *et al.*, 1992, Tadesse *et al.*, 2004). The modular wastewater constructed wetland setup of the SAWC could therefore be more effective if white or light coloured containers, instead of black or dark coloured containers are used. This project thus took a mesocosm experiment approach to see whether the colour of containers used for treating wastewater could affect mosquito oviposition.

2 AIM

Above ground, constructed wetlands have the potential to be important water purifying systems in rural areas. They are constructed using large plastic containers (710l). However, mosquitos use these systems as breeding sites and it is therefore important to understand them. My aim is to see if container colour influences:

- *Culex* mosquito site selection for oviposition,
- *Culex* larval survival, and
- Water quality parameters

2.1 HYPOTHESES

Hypotheses 1: There will be more mosquitoes breeding in black containers than white containers.

1a) Black containers will have more rafts than white containers.

1b) Black containers will have more larvae than white containers.

1c) Larval survival will be higher in containers with higher average temperature

Hypothesis 2: There will be a significant relationship between wastewater quality and container colour

2a) Container colour effects water quality (pH, DO, EC and water temperature)

2b) White containers will have lower average temperature than black containers

3 METHODS

3.1 STUDY AREA

The Southern African Wildlife College (SAWC) (Figure 2) situated close to the Orpen rest camp was used as the research site (Figure 3). The SAWC is surrounded by Kempiana Nature Reserve and adjacent to the Kruger National Park, Mpumalanga Province, South Africa. These parks host a diversity of fauna that could be potential sources of blood meals for mosquitoes (Onchuru *et al.*, 2016).

The site was in the Savanna biome part of the Lowveld bioregion and classified by Mucina and Rutherford (2006) as Granite Lowveld. The elevation of the SAWC is around 480 meters above sea level. The area is in a summer rainfall region (Mucina and Rutherford, 2006, p. 492). The actual measures taken at the SAWC for the last seven years (1 October 2012 – 30 September 2019) were as follows. The maximum and minimum temperatures were 41.8 and 7.1 degrees Celsius respectively with an average of 22 degrees Celsius. The average rainfall for the same period was 429.3 mm per annum. The maximum and minimum temperature for the experimental run (February, March, April) was 38.2 and 16.1 degrees Celsius respectively with an average of 24 degrees Celsius for the three months. Rainfall was evenly spread over the three months with an average of 53 mm per month. This is below the average of 61mm for the last seven years (*SAEON weatherstation data*, 2019).



Figure 2: *The star on the insert indicates the position of the college in the Greater Kruger National Park (Peace Parks Foundation, 2018).*

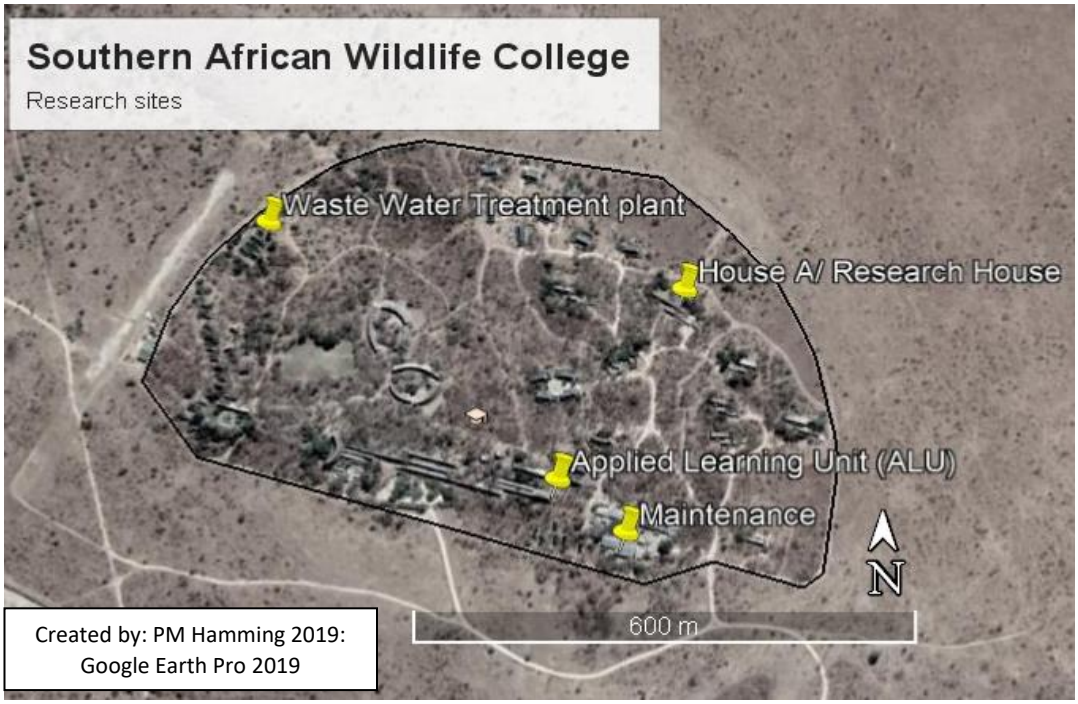


Figure 3: *A map of the Southern African Wildlife College indicating where the study sites were in comparison to each other (S 24° 32,536; E 31° 20,019).*

3.2 EXPERIMENTAL DESIGN

Wastewater from the SAWC water treatment plant was used as an oviposition attractant in the study. The wastewater was a mixture of domestic wastewater from staff housing, industrial laundry and canteen kitchen water, dog unit wastewater, and water from the student rooms. The wastewater was collected after being treated in a septic tank and BAS tank (Biologically Activated Sludge). On the first day of the experimental run, wastewater was collected and transported to each of the experimental sites.

The wastewater coming from the BAS tank had some solids and mosquito larvae already present. To avoid introduction of either into the experimental setup the collected wastewater was filtered through a cheesecloth (Grade 50) before being used in the experiment. To minimize the influence of other water insects, the experimental sites were situated about a 100m away from the college CW. The only other insect that found in the containers were chironomids.

Experimental containers were used to mimic the ideal modular CW at the SAWC. The smaller containers for the study was chosen to make set up easier. It was also

easier to control variables in the small containers compared to the ideal modular system of the SAWC. Three experimental runs were performed to increase the sample size. The third experimental run was late in the mosquito breeding season. A fourth run would not have contributed to the study. The three sites used for the experiment were HouseA (Site 1), Applied Learning Unit Building (ALU) (Site 2), and Maintenance (Site 3) (Figure 3).

The containers at each site were placed under a roof to limit contamination with leaves, dilution through rain, and evaporation. Wastewater was filled up to the first rim of each container (Figure 4). The roof and open side of the experimental site resulted in a gradient of light shining onto the containers as the sun moved through the sky. Containers deeper under the roof were exposed to less sunlight than the containers closer to the side of the roof structure over the period of the experiment.

The largest influence of container colour on oviposition was found by Torrisi and Hoback's (2013) between light and dark colours. Black and white containers were therefore chosen for the experimental setup because they have the highest contrast.

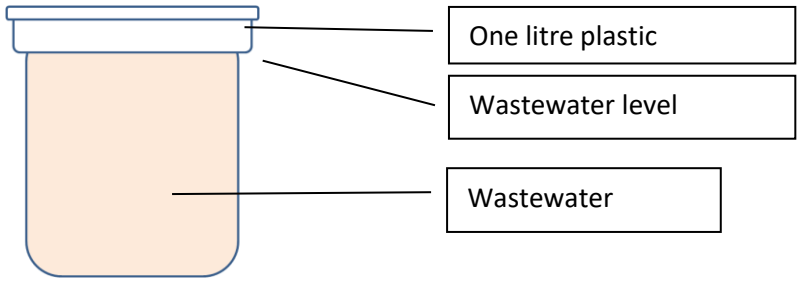


Figure 4: *All the containers in the experiment were filled with wastewater to the first rim of the container.*

Experimental Run 1

In the first experimental run 15 white and 15 black one litre containers (Figure 5) were randomly arranged in three rows of ten. Containers were spaced a container width apart between rows and columns. All containers were filled with wastewater up to the first ridge in the container (Figure 4).

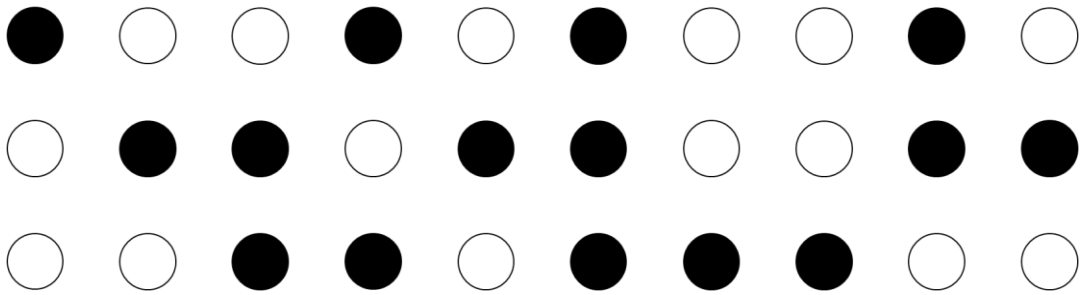


Figure 5*: The first experimental run was set at site one. Three rows of ten containers were set equally spaced. An equal amount of black and white containers was randomly set using the flip of a coin.*

Experimental Run 2

In the second experimental run, two rows of ten one litre containers were set at two sites. Ten black and ten white containers were randomly set as in the first experimental run but in two different locations (Site 1 and Site 2 - Figure 3). The number of containers were limited to 20 per site to make setup and measurements less time consuming. A second site was chosen to get more replication in a shorter period of time.

Experimental Run 3

In the third experimental run 20 containers were place in two rows in two different locations (Site 3 and Site 1). The site one was used throughout the study but the second site had to be moved because of external factors. The black-and-white container setup was changed to a checkerboard (Figure 6) configuration rather than randomized setup, based on concerns raised about potential bias in the original experimental design used in the first two runs. A checkerboard setup was thought to better take the light gradient of the sun into consideration. The

randomized setup from the first two runs created clumps of white and black that could have influenced oviposition. A second set of 20 containers, covered in a mosquito cloth to prevent oviposition, were placed in the same location and in the same pattern as a control for water quality.

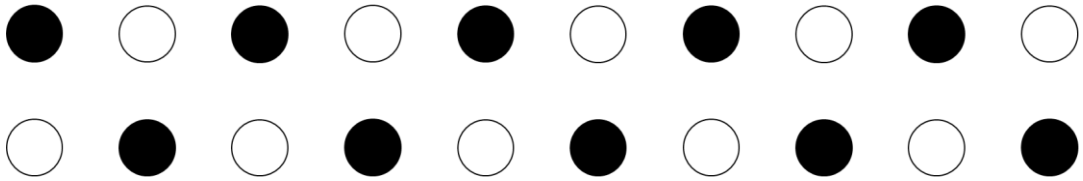


Figure 6: *A checkerboard setup of black and white containers was used in the third period of the experimental run at Site 1 and Site 3.*

3.3 MEASUREMENTS

A daily raft count was done for ten days, starting on the fifth day of each of the three, 15-day experimental runs. The larvae were collected on day 15 of each study and placed into 25 ml glass containers with push in caps. The containers were filled with formalin and transported to the Applied Learning Unit lab, at the SAWC, for further identification under the microscope.

Water quality tests were done *in situ* for each of the containers. Water tests were done every second day for the last ten days of each experimental run. DO, electric conductivity (EC), pH and temperature were measured using a HACH HQ40D multi water meter. The water temperature measure used for analysis was the average between the temperatures measured by the EC and DO probe. The pH probe broke before the first study started and was replaced just before the third experimental run started. The first two experimental runs therefore, have no pH measurements. Each measure was taken around 15h00 which was the average warmest period of the day as measured by the onsite weather station operated by SAEON (Figure 7).

Inserting the probes into the water disturbed the surface and made counting rafts more difficult as they sometimes broke into pieces therefore, raft counts were done before the water measures. There were three or four occasions where rafts stuck to the probe and were accidentally removed from the study. To minimize contamination between the different containers the probes were rinsed with clean water after each measurement.

Rafts counted were left in the containers to hatch. An effort was made not to recount rafts, but it was difficult to distinguish between old and new rafts. New rafts were slightly glossier in appearance than the old rafts. The absence or presence of larvae was also noted before water quality measurements, using a flashlight to inspect all dark areas in the containers.

Counts and measurements commenced on the fifth day of study to give mosquitoes time to find the containers and ended on day 15 preventing larvae from developing into adults. The ultimate goal was to find a period long enough for eggs to hatch and develop into fourth instar larvae but short enough that they did not emerge as adult mosquitoes and fly away before being counted and identified. The identification guide used was developed to identify third and fourth instar larvae (Day *et al.*, 2002) and so it was hard to identify younger larvae than this.

Initially the larvae were grouped into *Aedes* and *Culex* according to their siphons and characteristic seta on the tail. To confirm that the species was correctly identified a microscope was used as this makes identifying and counting the seta easier (Day *et al*., 2002).

Under controlled conditions Ciota *et al.* (2014) found that the optimum ambient temperature for *Culex pipiens* complex mosquitoes to breed in is 25 degrees Celsius. Under these conditions it takes larvae ten days to develop from first instar to adult mosquito (Loetti *et al*., 2011). Since the study setup has natural temperature fluctuations and higher temperatures (Figure 7), and considering that it takes eggs between 24 and 36 hours to develop (Day, 2016), it was decided to use 15 days as experimental run. The 15-day period gives a margin of error for less favourable ambient temperatures, egg development time, and time for female mosquitoes to find the water containers.

On day 15 of each of the three experimental runs, the study was ended and larvae collected. Second, third, and fourth instar larvae were caught using pipette and forceps, put in formalin in 25ml tubes, and transported to the ALU lab for identification (Oberholster *et al.*, 2008). Each instar was grouped according to size. The first instar larvae were very small and could not be accurately identified therefore, they were excluded from the study entirely.

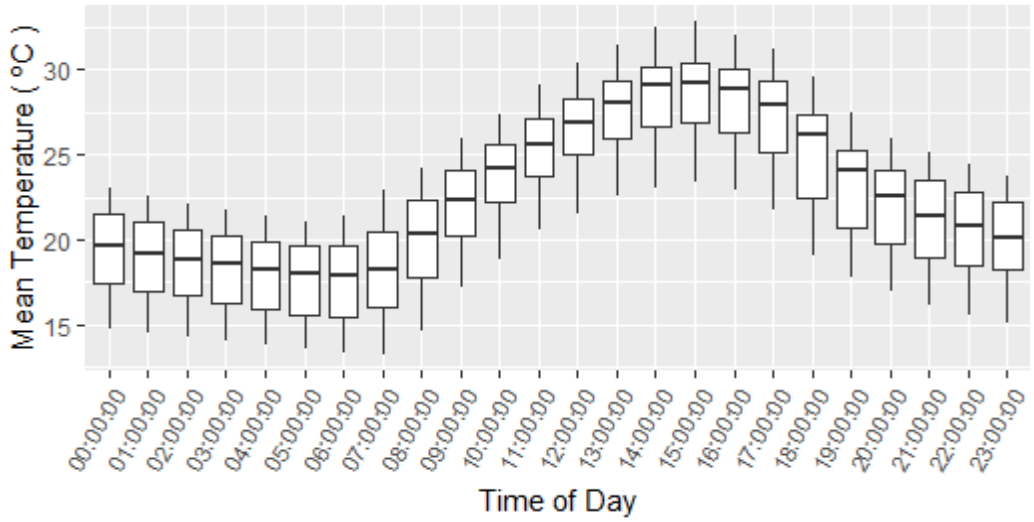


Figure 7: *The mean daily temperature at the Southern African Wildlife College for 12 months of 2018 used to estimate which time of day would be the warmest. Temperature plays a role in water quality measures, therefore the warmest period (15:00) of each day was used to standardise measurements (SAEON weatherstation data, 2019).*

3.4 EQUIPMENT

The containers used for the experimental design were black and white as they are most contrasting. Colours such as green and blue has also been used in other studies but was excluded to simplify this experimental design. The two trials done before the commencement of the study indicated that there was a larger difference between the black and white containers than any of the other containers. These trials were not included into this document to minimize confusion that it created.

A HACH HQ40D multi water meter was used to measure pH, temperature, dissolved oxygen levels (DO), and electrical conductivity (EC). Each of the probes were calibrated as per user manual and confirmation by a HACH technician.

- pHC201 – The probe was used to measure pH and was calibrated with pH4, pH7, and pH10 standards as provided by HACH.
- LDO101 – The probe was designed to measure dissolved oxygen and temperature.
- CDC401 – The EC probe was calibrated with the 1413 potassium chloride (KCl) standard. The multi meter was configured to compensate for temperatures higher or lower than 25 degrees Celsius using the built in factor of 1.9% per degree Celsius change in temperature.

A Zeiss Stemi DV4 microscope was used to count and identify the larvae. It has a magnification power of 8x up to 32x. The highest magnification was mainly used to identify the mosquito larvae.

3.5 STATISTICAL ANALYSIS

The Shapiro-Wilks test was used to test for normality of the data. The *Culex* rafts data had a skewness of 8.977 (Kurtosis 84.173; df = 0.199) and the data for the total amount of larvae counted for each period had a skewness of 3.589 (Kurtosis = 15.150; df = 38.856) which all indicated non-normal distribution of data. As the data were not normally distributed, a log transformation was done and the results re-tested for normality. As the data were still not normally distributed and the data having only two variables, the Mann-Whitney U test was used to describe the data. The non-parametric Kruskal-Wallis test was chosen to compare the total egg rafts and larvae counts per container between the three experimental runs and the container position within each trial. The test was followed by a post-hoc pairwise analysis with a Bonferroni correction. The Bonferroni correction took the type 1 error into consideration for the level of significance.

The reason for the non-normal data distribution is most probably a result of *Culex* species raft and larvae data with a large amount of absence data (zero counts). Using non-parametric testing was therefore the only option for data analysis (Childs, 2018).

All statistical analysis was done using SPSS version 25 (IBM Corp, 2017) and R programming (R Core Team, 2019). The graphs were produced using base R and the ggplot2 package (Wickham, 2016).

4 RESULTS

Over the period of the study, 2341 larvae were sampled of which 2016 were identified as part of the Culex pipiens complex. A total of 153 rafts were found in 54 of the 110 containers. Each of the sites were tested separately. The difference in egg rafts and larval counts for the black and white containers was first analysed for each of the three experimental runs. These data were analysed again but this time for each of the five trials separately. The outcomes of significance differed to the extent that it was decided to use the trial data for further analysis and the discussion. A good example is the second experimental run. The difference in egg raft counts between the two colours was significant for the second run (U=71, n=40, df=1, p=0.003). When the trials were analysed the run for site one was not significant (U=25.5, n=20 df=1, p=0.611) while site two was significant (U=10.5, n=20, df=1, p=0.002).

For each container there was an average of five measures taken over the period of the trial for DO, EC and temperature. The mean measure of each container was used in the analysis of the five trials and three experimental periods. There was only one count per container for larvae and egg rafts so the total was used. The mean measures taken was used in the Mann Whitney U test with continuity correction.

Rafts

There were too few rafts counted over the course of the study to have a mean higher than zero. The black containers in the first experimental run were the only containers with some data that could be used to compare the mean (Figure 8). For the other sites and containers, the count was too low. The Mann-Whitney U test gave clearer results. It indicated that there was a significantly larger amount of *Culex species* egg rafts counted in the black containers than in the white containers for two of the five experimental runs (Run 1, Site 1 – U= 22.5, n =30, df=1, p<0.001; Run 2, Site 2 – U=10.5, n=20, df=1, p=0.002; Table 1). The physical sites also had a significant effect on the amount of rafts in the containers. For run two, the Mann-Whitney U test indicated that there was a significant overall difference between the black and white containers (U=71, n=40, df=1, p =0.003) and there was a significant difference in the number of rafts between sites (Site 1 – U= 25.2, n=20,

df=1, p=0.611; Site 2 – U=10.5, n=20, df=1, p=0.002; Table 1). There were no significant differences between containers or sites for run three (Table 1).

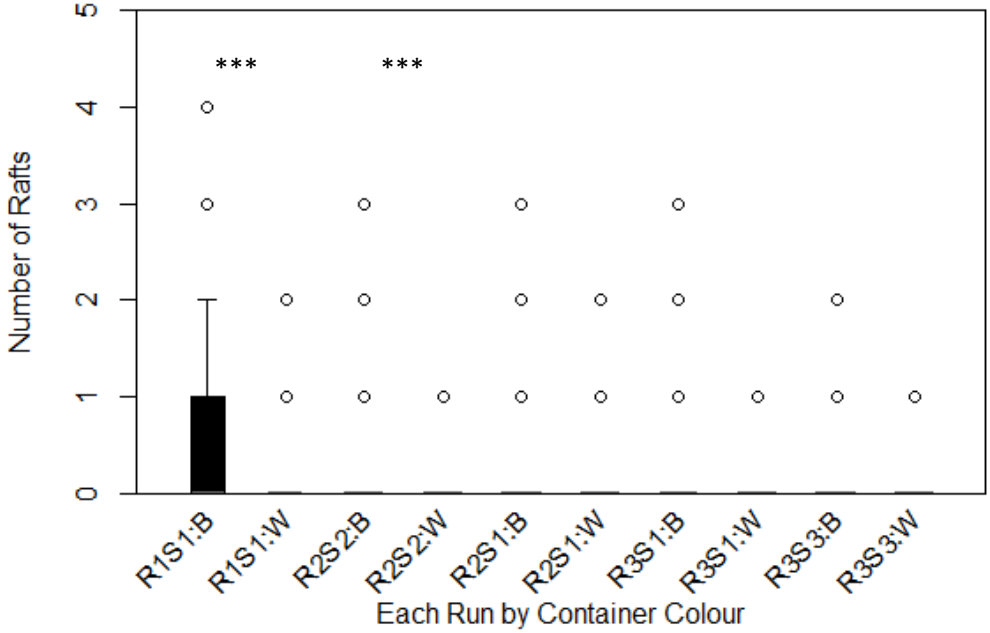


Figure 8: *Boxplot illustrating the total number of rafts found per container, grouped by colour and trial. (Codes: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code: *** p<0.0001).*

Table 1: *A summary of the amount of rafts found for each trial and their significance using the Mann-Whitney U test. (Sig. code: **p=0.01-0.001;***p=0.001-0.0001; NS=p>0.05)*

Location/ Experiment	Number of containers	Rafts Counted		Mann-Whitney U		Significance (p)
		Black	White	U	p	
Run1_Site1	30	45	6	22.5	0.0001	***
Run2_Site1	20	34	3	25.2	0.611	NS
Run2_Site2	20	29	9	10.5	0.002	**
Run3_Site1	20	16	3	28	0.066	NS
Run3_Site3	20	6	2	49	0.957	NS

Larvae

The difference between larvae found in the black and white containers for the whole study was significant (U=2279.5, n=110, df=1, p<0.001) and there were more larvae found in the black containers that in the white containers (Figure 9). The first and third experimental run had higher counts of larvae in the white containers that in the black containers. It was only a few white containers that contributed to the large count. The larvae counts in the black container were more evenly distributed. The difference between these experimental runs (Run 1: U=78.5, n=30, df=1, p=0.147; Run 3: U=152.5, n=40, df=1, p=0.350) and trials (Run 3: Site1 – U= 54, n=20, df=1, p=0.670; Site3 – U=55, n=20, df=1, p=0.368; Table 2) were not significant. The second experimental run had significantly more larvae in the black containers that in the white containers for both sites (Run 2: Site1 - U=22.5, n=20, df=1, p=0.02124; Site2 – U=25, n=20,

At the end of the first experimental run a number of dead larvae were noted on the water surface. The dead larvae were only noticed in two containers and not taken into consideration for statistical analysis.

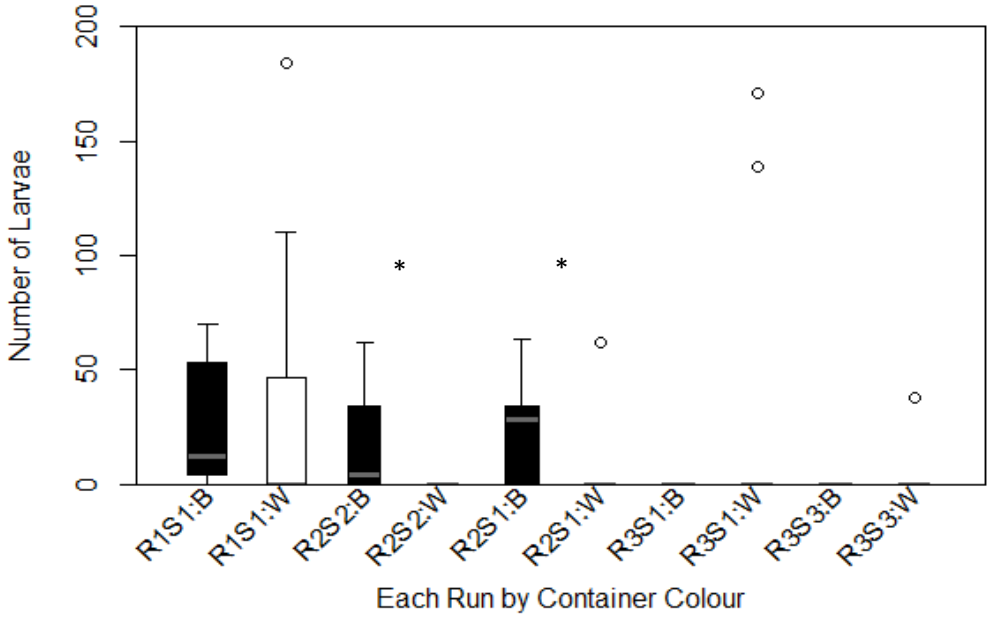


Figure 9: *Boxplot indicating the decline in mosquito larva over the course of the study. The mean, indicated in grey, shows the higher amount of larvae found in the black containers in comparison to the white containers for run one and two. (Code: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site1 and B/W indicates the colour; Sig. code * p=0.01-0.05)*

Table 2: *The table gives a summary of the larvae found for each run and site of the study. It further illustrates that the only trials with a significant difference in larvae between the black and white containers was in the second run. (Sig. code: NS=p> 0.05; *p = 0.01 – 0.05)*

Location/ Experiment	Number of containers	Larvae Counted		Mann-Whitney U		Significance (p)
		Black	White	U	p	
Run1_Site1	30	388	479	78.5	0.1471	NS
Run2_Site1	20	270	62	22.5	0.0212	*
Run2_Site2	20	177	0	25	0.0149	*
Run3_Site1	20	253	310	54	0.6704	NS
Run3_Site3	20	0	38	55	0.3681	NS

4.1 WATER QUALITY AND CONTAINER COLOUR

The Mann-Whitney U test indicated that there was a significant difference between the water quality measures from the black containers versus the white containers. The water quality constituents that were measured included DO, EC, pH, and water temperature.

Dissolved oxygen (DO) levels were only significant in colour at site two (U=100, n=20; df=1, p=0.0002) in the third trial and site three in the fifth trial (U=96, n=20, df=1, p=0.0006; Figure 10). The measures were not significant for all the trials at site one (Run1: Site1 – U=159, n=30, df=1, p=0.056; Run2: Site1 – U=55, n=20, df=1, p=0.734; Run3: Site1 – U=34, n=20, df=1, p=0.241)

EC levels were also significantly influenced by colour with black containers having higher EC readings than the white containers when looking at the first and second experimental run (Run1: U=38, n=30, df=1, p=0.0021; Run2: U=58.5, n=40, df=1, p=0.0001365; Figure 11). On further test on the two trials in the second run separately indicated that only site one had a significant difference (Run2: Site1 - U=2, n=20, df=1, p=0.0003; Site2 - U=31, n=20, df=1, p=0.162). There was no significant difference in the EC levels between the container colours for the third experimental run (U=140.5, n=40, df=1, p=0.110). There was neither a significant

difference for EC between the two container colours when each site was analysed separately (Run3: Site 1 – U=28, n=20, df=1, p=0.1038; Site3 – U=38, n=20, df=1, p=0.3847).

The mean water temperature was slightly higher in the black containers compared to the white containers (Figure 12). Between the three experimental runs only the third one was significant (U=121.5, n=40, df=1, p=0.03477). All the trials were also compared for each of the experimental runs. Only site two in the second experimental run was significant (U=21, n=20, df=1, p=0.0312).

Last of the water quality measures that were taken was pH. Unfortunately, it could only be taken for the last experimental run of the study as the first pH probe broke just before the experiment started. The pH levels between the white and black containers were significantly different for both the study sites (Run 3: Site 1 - U=15, n=20, df=1, p=0.008979; Site 3 - U=96, n=20, df=1, p=0.0005828; Figure 13). The mean was higher for the black container in site one and for the white container in site two. For the control, the mean of the white contianers were higher but it was only significant for site three (Control Run 3: Site1 - U=60, n= 20, df=1, p=0.4727; Site 3, U=83, n=20, df=1, p=0.01402).

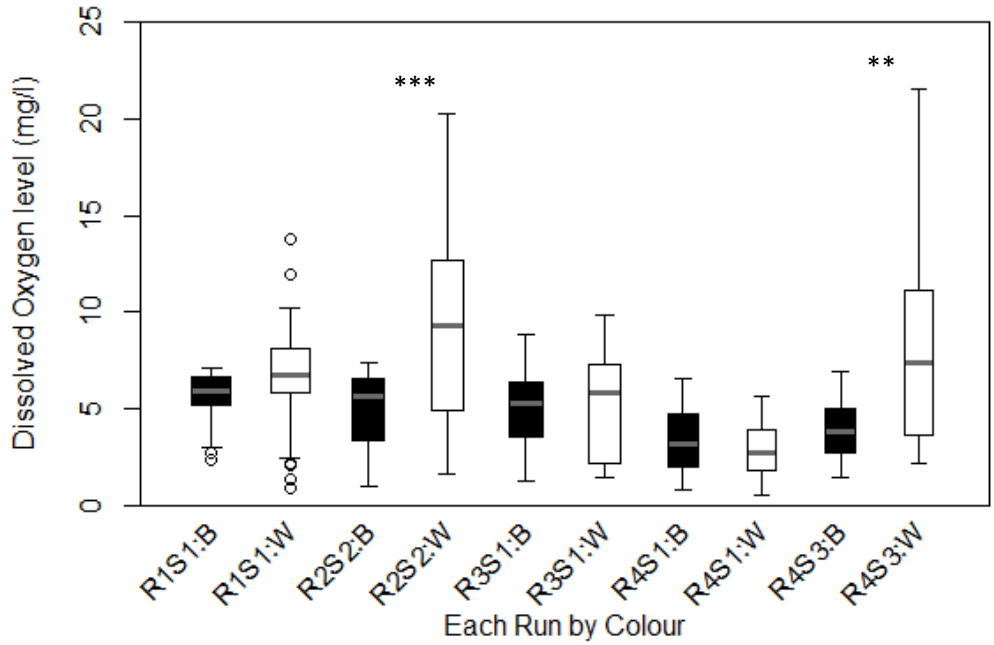


Figure 10: *There was a significant difference between the DO levels for R2S2 and R4S3 as indicated with an asterisk. (Code: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code: ** p=0.01-0.001;*** p<0.001).*

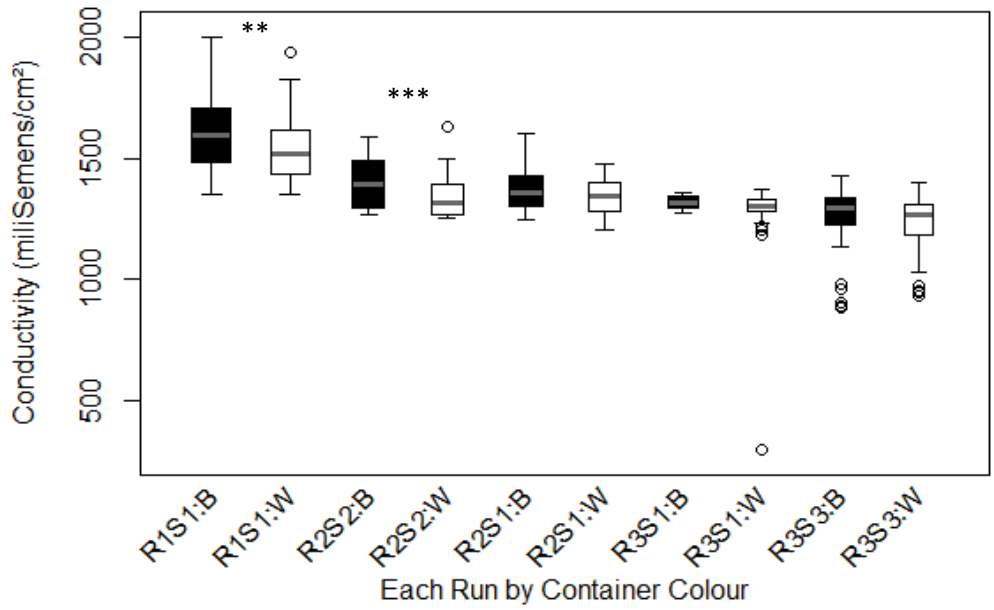


Figure 11: *There was a decline in the mean EC over the periods of the experiment. (Code: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code** p=0.01-0.001;*** p<0.001).*

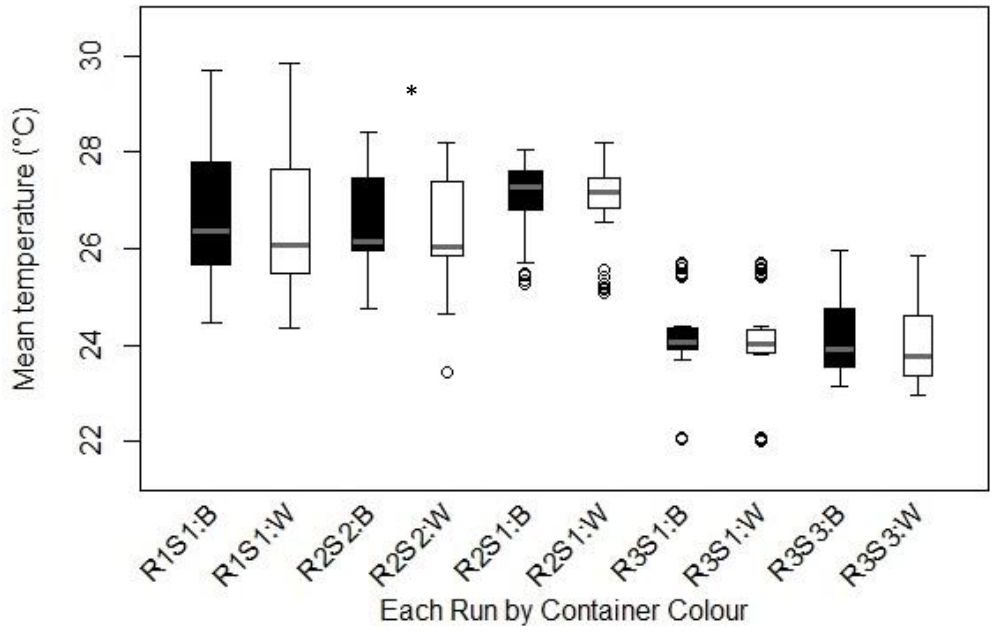


Figure 12: *The mean water temperature decline over the duration of the study. The temperatures in the containers did not differ significantly (Code: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code:* p= 0.01-0.05).*

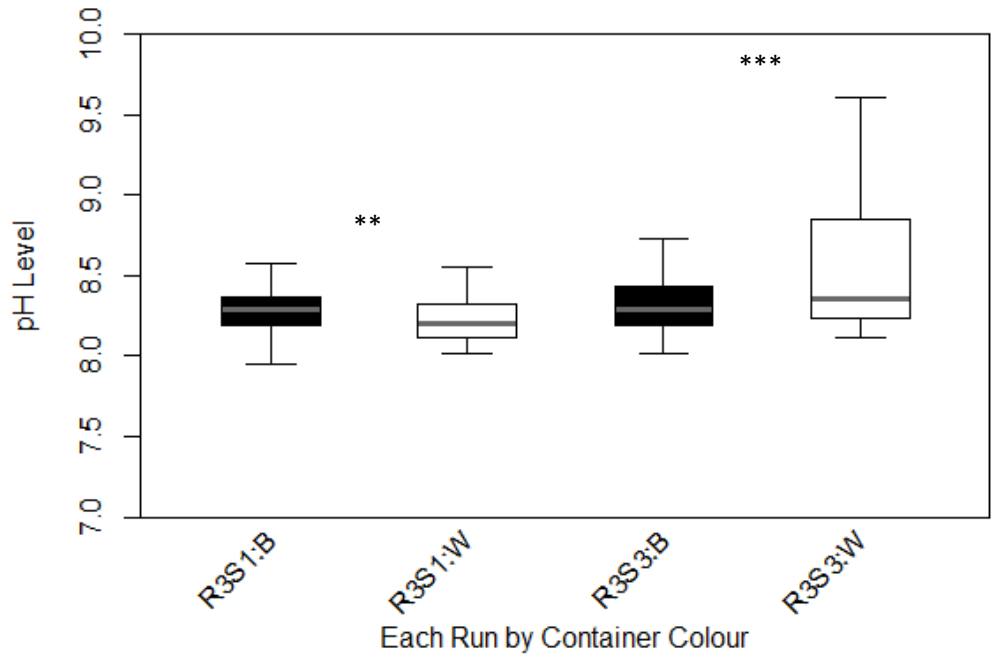


Figure 13: *The difference in pH levels for the third run. The pH levels of the white containers at site three was higher than that of the black containers (Code: R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code: ** p = 0.01-0.001; *** p<0.001).*

4.2 CHANGES THROUGH TIME

The mean EC, DO, and water temperature as well as the totals of raft and larvae counts of each container was used to compare the different periods using the Kruskal-Wallis test. The test was used to compare the changes in EC (H=85.247, n=110, df=2, p<0.0001), DO (H=28.691, p<0.0001, n=110, df=2), rafts numbers (H=10.480, p=0.005, n=110, df=2), larvae numbers (H=14.519, p=0.001, n=110, df=2), and water temperature (H=76.039, p<0.0001, n=110, df=2) between the three time periods. The Kruskal-Wallis test was followed by a post hoc pairwise analysis with a Bonferoni adjustment to see how each of the variables related to each of the three experimental runs.

The test indicated that EC was significantly different between all three off the experimental runs (All experiments: p<0.0001, df=2). As with EC, DO levels were significantly different between all three the experimental runs (All experiments: p<0.0001, df=2). This outcome lead to analysing each of the trials on their own using a Mann-Whitney U test.

There was a significant difference in the amount of rafts found in each trial over the experimental period (H=16.114, p=0.001, df=2) with the most rafts counted in the first period of the study. The follow-up post hoc test indicated a significant difference between the first and third (H=18.458, p =0.029, df=2), and the second and third period (H=19.363, p =0.01, df=2) of the study. There was no significant difference in raft counts between the first and second period of the study (p=1, df=2).

The Kruskal-Wallis test indicated that there was a significant difference in larvae numbers between the periods. After the pairwise post hoc test was run it was found when adjusted with a Bonferoni only the first and third period differed significantly (H=24.029, p>0.001, df=2). There was no significant difference between the second and third (H=10.613, p=0.207, df=2) and between the first and second period (H=13.417, p=0.100, df=2). This means that there was only significantly more larvae found in the first period compared to the third period than between any of the other periods.

For the mean water temperature the same adjusted analysis indicated a significant difference between the first and the last or third period of the study (H=52.4, p<0.0001, df=2) as well as between the second and third period (H=56.950, p<0.0001, df=2). There was no significant relationship between the temperature of the first and second period. There was a strong correlation between air and water temperatures when temperatures were compared with the Pearsons Correlation test in R (t=4.8069, n=23, df=21, p=0.00009, Figure 14). The decline in ambient temperature will therefore have an impact on water temperature. The strong relationship between temperature and larvae survival or production can clearly be seen on Figure *15*15 where the number of larvae fluctuated as the average temperature fluctuated.

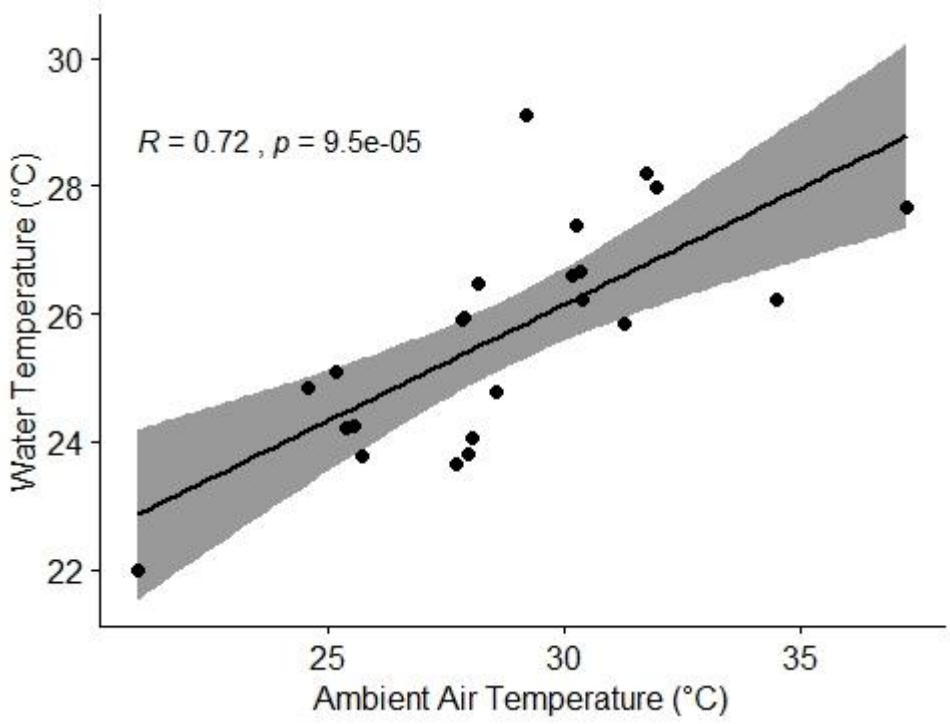


Figure 14: *There was a significant correlation between water temperature in the containers and ambient temperature (t=4.8069, n=23, df=21, p=0.00009). The mean day temperature between 15h00 and 17h00 was used for ambient temperature and the daily mean water temperature of all containers were used for the correlation.*

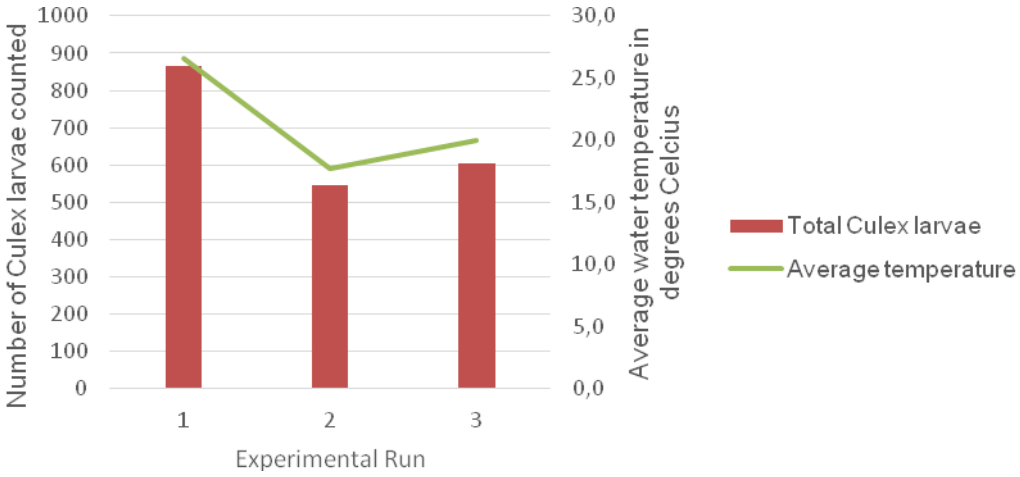


Figure 15: *A comparison between Culex pipiens complex larvae survival and water temperature for the duration of the study.*

4.3 CONTAINER POSITION

The significance of the positions (1-10) of the containers were tested for all five the different trials over the three experimental runs using a Kruskal-Wallis test. The variables that were significant for some of the trials were EC and temperature ($p<0.05$ indicated in bold, Table 3). For the rest of the containers there was no significance detected ($p>0.05$). The significance that was detected was not relevant once the pairwise post-hoc test was corrected for type 1 error using the Bonferroni correction.

Table 3: *Each of the variables for each run (R) and site (S) was tested to see if there was difference between containers in relation to their position under the building roof. The Kruskal-Wallis (H) test statistic and significance (p) was tabulated to easily compare each variable for each site. (Sig. Code: *p=0.01-0.05)*

	R1S1 n=30; df=9	**R2S1 n=40; df=9**	**R2S2 n=40; df=9**	**R3S1 n=40; df=9**	**R3S3 n=40; df=9**
Rafts	H=9.242 p=0.415	H=4.156 p=0.901	H=2.179 p=0.988	H=5.694 p=0.551	H=12.456 p=0.189
Larva	H=8.136 p=0.521	H=3.676 p=0.931	H=6.416 p=0.698	H=7.837 p=0.551	H=9 p=0.437
DO	H = 6.196 p=0.720	H=7.857 p=0.549	H=7.2 p=0.616	H=5.286 p=0.809	H=4.943 p=0.839
EC	H=20.166 *** p=0.017**	H=13.886 p=0.126	H=8.571 p=0.478	H=8.863 p=0.551	H=17.771 *** p=0.038**
Mean Temp	H=19.531 *** p=0.021**	H=17.683 *** p=0.039**	H=9.464 p=0.396	H=5.96 p=0.921	H=13.738 p=0.132
pH	N/A	N/A	N/A	H=3.846 p=0.921	H=3.457 p=0.943

5 DISCUSSION

Oviposition and Container colour

Colour is known to affect the oviposition of mosquitoes, be it the colour of water (Beehler *et al.*, 1992), paper, light (Hoel *et al.*, 2011), and containers (Mayhew and Raman, 1999; Collins and Blackwell, 2000; Allan and Kline, 2004; Torrisi and Hoback, 2013; Oliva *et al.*, 2014)**.** Most of these studies focused on how the colour of oviposition traps influenced oviposition of *Aedes* mosquitoes (Yap *et al.*, 1995; Torrisi and Hoback, 2013).

Oviposition and experimental design

In the study, identical one litre containers made from either black or white plastic were used. This meant the inner surfaces of the containers only varied in colour, and not in texture or surface area, or any other way, that may have unintended consequences. *Culex* and *Aedes* mosquito larvae and chironomids were found across all experimental runs and sites. As hypothesised, black containers had a larger amount of *Culex* species egg rafts than white containers in at least some of the experimental runs (Figure 8). This concurs with laboratory studies on *Aedes* mosquitoes where darker colours attracted significantly more females than yellow and white (Brett, 1938; Beehler *et al.*, 1992; Derraik and Slaney, 2005; Hoel *et al.*, 2011). These results also concur with a study done in Florida where gravid *Culex* species mosquitoes chose green and black containers above the lighter containers for oviposition (Allan and Kline, 2004).

Colour and larvae

Colour did play a role in the amount of *Culex* larvae counted at the end of experimental run. Like the egg rafts, the number of *Culex* spp. larvae found in black containers was significantly more abundant than in the white containers. Although gravid female mosquitoes take great care in selecting a site for oviposition (Day, 2016), and regards black containers more favourably than white ones, conditions may change, influence the survival of larvae (Silver, 2008). The first experimental run of the study had more rafts in general. This was most probably the result of favourable weather conditions. During the second run there was a slight decline and the third run a rapid decline in larvae numbers. The water temperature and

water quality measures were taken into consideration for the duration of the study to support the effect colour has in the successful breeding of *Culex* mosquito populations.

Water Quality

The container colour not only had an influence on the amount of rafts and larvae numbers, but also on water temperature, EC, and DO measurements. The EC measures were lower in the white containers. EC is an indicator of the amount of ions found in the water and can be used to calculate total dissolved solids. The amount of dissolved solids is an indication of the pollution in the water (Miller *et al.*, 1988). Survival of mosquito larvae depends on various factors contributing to a larvae's feeding and physiological niche such as bacteria, fungi (Pavlovich and Rockett, 2000), organic matter, and maximum temperatures (Luhken *et al.*, 2015). Changes in water quality would therefore have an influence on mosquito larvae. The lower EC levels in the lighter containers could be a result of more sunlight in the liquid which speeds up biochemical processes in water such as the formation of hydrogen peroxide (Cooper and Zika, 1983; Bidin *et al.*, 2017), decreasing the amount of ions found in the water. With the decrease in EC fewer processes use oxygen, therefore higher oxygen levels were observed.

There could be an additional explanation for the increase in dissolved oxygen in the white containers. All the containers had a thin layer of biofilm, the white containers closest to the edge of the roof received more sun and had green biofilm. The biofilm in the other containers looked browner or were absent. Biofilm is the collective name for a group of bacteria, fungi, algae, and protozoa that form on surfaces submerged in water (Singh *et al.*, 2006). The green algae in the biofilm could have increased DO levels through photosynthesis (Glud *et al.*, 1992; Tadesse, *et al.,* 2004). The white containers allowed light through the container walls and reflected more of the light into the water. This is beneficial for the group of organisms in biofilm that can photosynthesise and the possible explanation for the difference in biofilm colour.

The significant relationship between EC, DO, and container colour was not true for all sites in the study. This indicates that location also played a role in changes in water quality measures. Each site has its own microclimate which we could not control. This could have effected EC and DO levels as they are both sensitive to

changes in temperature (Barron and Ashton, 2007). Further, changes in pH has an effect on DO (Cao *et al.*, 2019). They all effect one another which in the end has an impact on larvae survival. This complex interrelationship makes it difficult to pinpoint the main drivers for mosquito larva health in this study.

The differences between the container temperatures were not significant but showed a trend that black containers were warmer than white containers. Should these temperatures be above 30 degrees larvae will die (Loetti *et al.*, 2011). The higher temperatures decrease DO and make the water less attractive to potential mosquito predators. Lower levels of DO thus limits the risk of predation and competition for mosquito larvae as long as the temperatures stay below the threshold of 30 degrees Celsius (Silberbush *et al.*, 2015). Since mosquitoes have the ability to use atmospheric oxygen to survive it was thought that they do not need DO. Silberbush *et al.* (2015) however indicated that the very low levels of DO slows *Culex pipiens* larval development down and leads to death. This was, however, always observed with other stress factors such as higher temperatures, overcrowding, and lack of nutrients (Subra, 1981; Clements, 1992). To make breeding sites less successful, DO levels can either be limited to limit larva growth or elevated to give mosquito predators a chance of survival. Low levels of DO and high temperatures could therefore be a possible explanation for the observations of dead larvae in containers with many larvae and high temperatures.

Climate, oviposition, and larvae

For the period February, March, and April a decrease in average ambient temperatures, and rainfall, was measured at the SAWC weather station. A decreasing trend in rafts counted and larvae present was also noted between the first, second, and third run of the study as the mean temperature changed. Earlier studies also found a strong link between mosquito oviposition, larvae survival, temperature (Madder *et al.*, 1983; Rueda *et al.*, 1990; Loetti *et al.*, 2011; Ruybal *et al.*, 2016), and rainfall (Silver, 2008, p. 100). It is especially the first instar larvae that are very sensitive to climatic changes (Collins *et al.*, 2019). This is in line with the findings of Nguyen *et al.* (2013) where mosquito numbers and oviposition responded to seasonal temperature change. Apart from influencing survival rates, temperature also has a great effect on adult characteristics such as fecundity, body size, longevity, and competence (Clements, 1992; Mottram *et al.*, 1994; Loetti *et al.*, 2011). On a global scale, temperature change can therefore influence the

geographic spread and rate of disease occurrence (Kovats *et al.*, 2001; Loetti *et al.*, 2011; Ruybal *et al.*, 2016). The larger amount of oviposition in warmer weather could result in more rafts, which may result in higher levels of oviposition in white containers to avoid competition in the black containers. Spacing experimental runs throughout the year may give a broader understanding on the relationship between container colour, oviposition, and temperature. Wind was considered a limiting factor for oviposition in the third experimental site and needs to be considered in future study setups (Webb, 2013). On the practical side, mosquito management must take seasonality and climatic events such as rainfall into consideration to prevent large outbreaks of mosquitoes. As the conditions for mosquito survival becomes more favourable their potential as vectors for disease increases (Bhattacharya and Basu, 2016).

Evaporation was taking place throughout the study and was not compensated for by topping up the containers with more water. This may have resulted in more larvae per volume of water which could have increased competition between the larvae and therefore increased mortality. Apart from the rate of evaporation, container size can influence population makeup and density found in the containers (Bartlett-Healy *et al.*, 2011). A study in Italy found that *Culex pipiens* prefer larger containers (100-200 litres) and *Aedes albopictus* smaller containers with the two species overlapping in containers sizes of 10-50 litres (Carrieri *et al.*, 2003). They claim that the difference in size preference was connected to evaporation and the duration of development of the species. Only one litre containers were used in this study and both *Aedes aegypti* and *Culex pipiens* complex were present. Although evaporation and indirectly, container size might have had an influence on available habitat for the larvae, *Culex* species were found in 53 of the 110 containers. The small containers size did not seem to have an influence in site selection for the *Culex* mosquitoes. It would be a consideration for a future study to compare the influence of different container sizes and colour on oviposition.

Overcrowding, evaporation, and temperature are not the only environmental factors that influence population size. During the experiment there were two observations where a larger larvae killed a smaller larvae. The one event was while collecting the larvae at the end of the experimental run. Both larvae were placed in a small container with little water when the larger *Culex* species attacked the

smaller *Culex* individual. In another instance a fourth instar *Aedes* species larvae killed a second instar larvae. This suggested that cannibalism and predation, apart from overcrowding could have decreased the number of larvae found in the study. Koenraadt *et al.* (2004) studied *Anopheles gambiae* mosquitoes and found that limited space increases interactions between larvae which increases predation and cannibalism. Ruybal *et al.*, (2016) also found cannibalism in *Culex* species. However, predation and cannibalism may not be a result of food scarcity; Muturi *et al.* (2010) found that third instar *Anopheles gambiae s.s.* and *Culex quinquefasciatus* larvae predated on first instar larvae. In this study food was plentiful and not the reason for predation behaviour.

The extent of larvae lost to predation and cannibalism is not known but it was possible as different instar sizes were found in one container. In this study observations were made that *Aedes* species develop much faster than *Culex* species. The reason for the observation is that *Aedes* species convert food to biomass at a faster rate than other species which then results in faster growth (Carrieri *et al.*, 2003). Their larger size could therefore make it possible to predate on the slower developing *Culex* species. In the first experimental run third and first instar larvae were found in the same container. Based on the difference in size, cannibalism could also have influenced the outcome of larvae counted on the last day of the experiment.

The observations of evaporation, large number of larvae, and the influence of temperature is in line with other studies as the main cause of larval death (Subra, 1981).

Limitations and future considerations

Site consideration and setup is important in any study. In this study the setup was changed from a randomized design to a checkerboard setup to take the influence of light on water quality into consideration. The change in setup further complicated the analysis process. The row of ten containers was used to mimic the SAWC on-site water treatment system. Since water quality and oviposition was considered, other setups such as a Latin square design could have been used to better understand the influence of light on the setup.

There was a limited amount of suitable sites with a protective wall and a roof within the SAWC campus. Moving sites also added variables to the study such as wind. The study took place under rooves to limit evaporation and keep conditions as natural as possible. This approach did work but for future studies it compensation for evaporation, by filling the containers on a daily base to keep water levels constant, should be considered. When there are concerns about open sky or trees dropping leaves into the containers influencing oviposition, sites can be set under a tarp to try keeping other variables constant.

Although Culex species are the only group that lay eggs in rafts, counting the rafts was harder than expected. The complexity was a result of the methodology to leave old rafts in the container to hatch. Differentiating between the old and new rafts was a bit more difficult than first anticipated. New rafts were a bit more shiny than older rafts. Some of the older rafts quickly disappeared while others stayed afloat for longer. Black containers did have higher levels of oviposition but in order to prevent over estimations rafts can be counted every third day. This will give time for eggs to hatch and can then be removed to prevent double counting (Blaustein *et al.*, 2005; Nguyen *et al.*, 2012). Some of the eggs may not yet have hatched and will then result in removal of possible larvae. In warm periods, eggs hatch within 48 hours. The objective was to indicate which colour had the most larvae surviving relative to the other colour. Therefore it is argued that removing eggs from all containers on a three day cycle will not have a significant influence on the difference in survival between the containers.

Water quality also had some challenges as not all the equipment functioned as the HACH experts predicted. The DO probe had readings of higher than 22mg/l which is the upper limit of the probe and a DO saturation of more than 150%. The technology used to measure the presence of oxygen in the water is called Luminescent Dissolved Oxygen (LDO) technology. The device emits a blue light to an oxygen sensitive membrane which than reacts with the luminescent light and returns a red light. The time delay and intensity of the red light is measured and used to determine the dissolved oxygen levels in the water (HACH, 2019). It was therefore thought that the physical colour of the container absorbed or reflected the light emitted differently and therefore gave inaccurate DO readings. A 200 ml white plastic cup was inserted in the containers with the probe to minimize the

effect. There was no need as Picot *et* al. (1992) and Richmond (1986) measured super saturations of DO in water with algae during periods with high light intensity. All the DO measures taken during this study could therefore be used. A future consideration is to use multiple DO probes or a probe that does not use light to confirm or deny the effect of container colour.

6 CONCLUSION

It is important to human and animal health that mosquito populations are controlled as some of them are vectors of disease (Jupp, 2005). Both adults and larvae are targeted in population management strategies. All species need water for the larvae to be able to morph into adult mosquitoes (Day, 2016) therefore the water is a good starting point for mosquito control. Water scarcity is unfortunately not recognised by everyone. They waste and pollute the fresh water that is available (Lewin *et al.*, 2007). This could be a result of poor maintenance or a lack of infrastructure (Barlow and Clarke, 2002; Dos Santos *et al.*, 2017). The polluted water with high nutrient content results in waterbody eutrophication. This results in the loss of mosquito predators (Isife, 2012). It further creates favourable conditions for mosquito larvae development (Nguyen *et al.*, 2013).

Constructed wetlands are seen as a cheap alternative for secondary water treatment (Shutes, 2001). Their design can vary from large pond system down to small container system for cleaning wastewater (Vymazel, 1998) to use for crop irrigation (Emongor and Ramolemana, 2004; Kurniadie, 2011; Al-Gheethi *et al.*, 2018). Direct sunlight, wind, and plants assist in treating waste water and killing bacteria (Webb, 2013). In the right balance these factors together with water quality also have an effect on mosquito breeding (Dale and Knight, 2008). Gravel can be used to prevent mosquitoes from reaching the water but is expensive and prone to clogging (Vymazal, 1996) which makes gravel less than idealfor rural communities.

Understanding mosquito biology and ecology, therefore, becomes important in the design process of a CW. In this study there were higher levels of oviposition in black containers. Although there were a few white containers with large numbers of larvae it was only black containers that had a significant impact on larvae numbers in some of the trials. The use of light colours could therefore be one of the design solutions to limit oviposition and larval development by making the habitat less favourable. Colour on its own may not be the ultimate solution for decreasing mosquito numbers as they also use chemical and tactile cues to select a breeding site (Bentley and Day, 1989).

The colour of the container can be changed by using paint (Collins and Blackwell, 2000). Unfortunately paint does not attach easily to plastic and it reflects light rather

than increasing light penetration through transparent walls. The paint may also influence mosquito oviposition as they use chemical cues for site selection. If colour can limit habitat selection in *Culex pipiens* complex species, it could be successful for many other species. The only research found that used light colours for attracting insects was to attract pollinator species (Clare *et al.*, 2000; Vrdoljak and Samways, 2012)

Water temperature and weather played a role in oviposition with raft and larvae numbers declining as the experimental run moved closer to winter. Should global temperatures increase, mosquito numbers may also increase and so also the spread of disease (Bhattacharya and Basu, 2016). On the other hand, constant high temperatures above 25 degrees Celsius (Ciota *et al.*, 2014) could also so the growth or kill off larvae and decrease population sizes.

Related to temperature, DO is affected by container colour with white containers having much higher DO levels than in the black containers. From these results it is difficult to directly link DO levels with larva survival or death. In the long term higher DO levels will result in more favourable conditions for mosquito predators. There was a study done that indicated that larva start dying at very low DO levels (Silver, 2008). High temperatures result in lower DO levels which make it more difficult for mosquito predators to survive. Temperature and DO is highly correlated and their influence on mosquito larvae can therefore not be seen in isolation.

The conditions in the black containers were good for mosquito oviposition and larval growth. Warm water temperatures, low DO levels and sewage water did stimulate oviposition and assisted in larval growth. Colour also had a direct and indirect influence on the breeding success of the mosquitoes. However, there is a need to further investigate the interrelationship between container colour, water quality, temperatures and how they separately and as a whole influence mosquito reproduction. Further factors that can be considered is the role of direct sunlight, container aperture, and size has on the oviposition choice of various mosquito species (Derraik and Slaney, 2005).

There is a great need for treating wastewater using cost effective biological methods while minimizing the risk of breeding mosquitoes. There is still a lot of

research needed to understand the physical qualities that attracts a female mosquito to lay her eggs (Reiskind and Zarrabi, 2012). This study highlights the complexity of mosquito breeding biology and how one variable such as colour can affect egg laying and water quality. There is potential to minimize mosquito population growth by changing the colour of the containers used to above ground CW's. Container colour will not only change mosquito behaviour but also the biochemical process in the wastewater. Minimizing mosquitoes in constructed wetlands while effectively treating the wastewater is more complex and will need to find a solution looking deeper into mosquito biology, water biochemistry and wetland design.

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