

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

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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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169 **LIST OF SYMBOLS**

170	ALU	–	Applied Learning Unit – Part of the Southern African Wildlife College
171	BAS	–	Biologically Activated Sludge
172	CW	–	Constructed Wetland
173	DDT	–	Dichlorodiphenyltrichloroethane
174	df	-	Degrees of freedom
175	DO	–	Dissolved Oxygen
176	H	-	Kruskal-Wallis H test – Test statistic
177	KCl	–	Potassium chloride
178	n	-	Number of observations
179	NS	-	Non Significant
180	p	-	Significance
181	s.l.	–	Senso lato – in the broad sense
182	SAWC	–	Southern African Wildlife College
183	Sp	–	One species
184	Spp	–	Two or more species
185	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

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

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

230 Sub-surface flow CW would be more effective in minimizing mosquito populations.
231 These systems are set up like open water systems and then filled with a substrate
232 such as gravel through which the water flows. Sub-surface CW is effective at
233 treating wastewater but is expensive to set up and can clog easily when incorrectly
234 managed (Vymazal, 2010). This makes open water systems more attractive to use
235 for water treatment. However, mosquitoes prefer these open water sites with thick
236 vegetated areas (Russell, 1999; Thullen *et al.*, 2002) and shallow, stagnant water
237 (Knight *et al.*, 2003; Thangamathi *et al.*, 2018) rich in nutrients (Webb and Russell,
238 1999).

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

245 The design of CW should be done with care to ensure effective water treatment. A
246 system with fast flowing water limits the capture of impurities in the sediment. Too
247 little plant biomass will limit their water cleaning potential. Overly deep pools will
248 limit the effect of the sun and plant roots on the impurities in the water. These
249 factors can result in the decline of mosquito predators (Russell, 1999; Knight *et al.*,
250 2003; Webb, 2013). Some of these factors may limit mosquito breeding but not
251 effective in terms of treating water (Russell, 1999; Knight *et al.*, 2003). A good

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

256 **The ideal constructed wetland as designed by the SAWC**

257 To address the lack of sanitation in rural areas of South Africa, and to effectively
258 clean its own wastewater, the Southern African Wildlife College has a vision to
259 create a cheap, robust and modular constructed wetland. The system is a version
260 of a surface water flow system comprising of multiple, interlinked, and small (700lt)
261 open tanks with floating reed mats on them. Vegetation in the containers assists in
262 removing contaminants and filtering the water (Webb, 2013). Because of the small
263 size of the containers, the plants' leaves can be easily removed which is important
264 to increase dissolved oxygen (DO), pH and temperature (Alvarez and Becares,
265 2008). This is important in the process of water treatment. Less vegetation also
266 means an increase in photolysis helping with water treatment and the killing of
267 pathogens (Alvarez and Becares, 2008). However, small open containers with
268 vegetation make this ideal mosquito-breeding habitat, while the poor water quality
269 makes it unlikely that mosquito larvae predators will survive (Winkster, 2018).

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

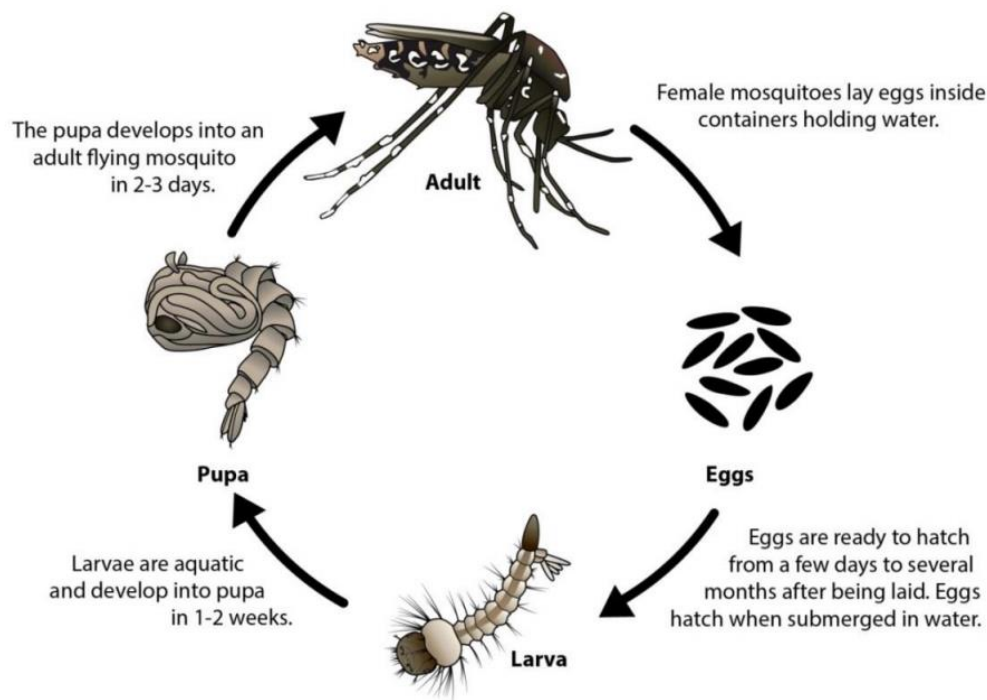
276 **1.2 MOSQUITOES**

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

285 hydras, flatworms, spiders (Ndava *et al.*, 2018), insects, copepods, fish, birds
286 (Mazzacano and Black, 2013; Collins *et al.*, 2019) and bats (Gonsalves *et al.*,
287 2013). Mosquito larvae can however be used as biological indicators as
288 mosquitoes include ecological generalists and specialists that interact with the
289 terrestrial and aquatic fauna of wetlands (Schafer *et al.*, 2004).

290 The mosquito lifecycle includes four stages (Figure 1). It can take as little as five
291 days for the lifecycle to be completed depending on temperature and species (Dale
292 and Knight, 2008). Eggs are laid either on damp soil (*Aedes*, *Ochlerotatus* and
293 *Psorophora* species), as single eggs in the water (*Anopheles*, *Toxorhynchites*,
294 *Sabethes* and *Wyeomyia* species) or, egg rafts (*Culex*, *Coquilleltidia*, and *Culiseta*
295 species) on the water surface (Schafer *et al.*, 2004; Dale and Knight, 2008; Webb,
296 2013), or eggs are attached to vegetation under the water (*Mansonia*) depending
297 on the species (Silver, 2008). Eggs hatch between 24 and 36 hours after laying for
298 *Culex* species, and between 48 and 72 hours for *Aedes aegypti* (Bartlett-Healy *et*
299 *al.*, 2012).

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



307

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

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

322 **1.2.1 Method of feeding**

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

328 or arteriole. Once found the mosquito injects an anti-coagulant into the host's body
329 to prevent blood clotting enabling them to suck up the blood. The anti-coagulant
330 causes skin irritation, carries disease into the host, and could lead to death (Jupp,
331 2005). Blood meals are taken from either humans, mammals, or birds depending
332 on the mosquito's specialisation (Cornel *et al.*, 2018).

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

339 **1.2.2 Mosquitoes as vectors of disease**

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

347 The *Anopheles gambiae* complex (s.l.) are the main vectors for malaria in South
348 Africa (Coetzee *et al.*, 2000; Sinka *et al.*, 2012; Collins *et al.*, 2019). There are eight
349 different species recognised today of which three, namely *Anopheles gambiae*,
350 *Anopheles culozzi*, and *Anopheles arabiensis* (Lindsay *et al.*, 1998; Collins *et al.*,
351 2019) are identified as vectors of medical importance to South Africa.

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

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

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

363

364 **1.2.3 Management of mosquitoes**

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

374 For example, a healthy wetland system has a higher diversity of mosquitoes, which
375 has shown to decrease the total number of mosquitoes breeding at the site. The
376 possible reasons is competition for habitat, food (Mazzacano and Black, 2013;
377 Webb, 2013; Carver *et al.*, 2015), and species preying on one another (Ruybal *et al.*,
378 2016). Urbanisation, through poor waste and sanitation management, has
379 increased available habitat and therefore the distribution of vector mosquitoes
380 (Okogun *et al.*, 2005; Okiwelu *et al.*, 2012; Cuthbert *et al.*, 2018). The result is an
381 increased occurrence and spread of diseases such as malaria (Rani *et al.*, 2018).

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

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

394 investigated as biological control for mosquito larvae are calanoid copepods
395 (Cuthbert *et al.*, 2018). Another biological method being investigated is called
396 Sterile Insect Technique (SIT) to reduce mosquito populations (Chung *et al.*, 2018).
397 Infertile mosquito males are introduced into an area. They then mate with the
398 females that as a result produce infertile eggs (Vaníckova *et al.*, 2017; Chung *et al.*, 2018).
399

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

405 **1.3 MOSQUITO ECOLOGY AND BIOLOGY**

406

407 ***Culex***

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

417 *Culex* mosquito adults can breed in most qualities of water, but prefer water with
418 high nutrient content that serves as food for the larvae. Habitats created by humans
419 such as containers, drains, gutters, and sewage treatment make important
420 breeding sites (Subra, 1981). Drains lack predators and have high levels of
421 nutrients, which make them attractive breeding sites for *Culex* mosquitoes (Arana-
422 Guardia *et al.*, 2014). *Culex* mosquito larvae dart to the bottom of the water body
423 with any predator like disturbance above the water. They feed under the water
424 surface unlike *Anopheles* mosquitoes that feed closer to the water surface
425 (Onchuru *et al.*, 2016). A female *Culex pipiens* complex mosquito takes blood from
426 humans or birds. They then lay a single egg raft of an average of 155 eggs. These
427 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).

431

432 ***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).

444 **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.

461 Container colour can also play a role in the water temperature and the chemical
462 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
463 coloured containers. Dissolved oxygen is very sensitive to temperature change
464 (Cao *et al.*, 2019). With the increase in temperature DO levels decrease, which
465 then makes it difficult for mosquito predators to live in as they use dissolved oxygen
466 to breathe (Russell, 1999). Sunlight also kills some bacteria, such as *Escherichia coli* and *Enterococcus faecalis*, and influences biochemical processes in water
467 (Tadesse *et al.*, 2004; Kadir and Nelson, 2014; Koumaki *et al.*, 2015; Bidin *et al.*,
468 2017). Using white containers will reflect sunlight deeper into the container
469 increasing its effect on biochemical processes. The increased sunlight in these
470 containers makes it possible for photosynthetic biofilm organisms to grow on the
471 inside, which then has a further effect of increasing DO during the day (Glud *et al.*,
472 1992, Tadesse *et al.*, 2004). The modular wastewater constructed wetland setup
473 of the SAWC could therefore be more effective if white or light coloured containers,
474 instead of black or dark coloured containers are used. This project thus took a
475 mesocosm experiment approach to see whether the colour of containers used for
476 treating wastewater could affect mosquito oviposition.
477
478

479

480

481 2 AIM

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

- 487 • *Culex* mosquito site selection for oviposition,
- 488 • *Culex* larval survival, and
- 489 • Water quality parameters

490 2.1 HYPOTHESES

491

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

494 1a) Black containers will have more rafts than white containers.

495 1b) Black containers will have more larvae than white containers.

496 1c) Larval survival will be higher in containers with higher average
497 temperature

498

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

501 2a) Container colour effects water quality (pH, DO, EC and water
502 temperature)

503 2b) White containers will have lower average temperature than black
504 containers

505

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.

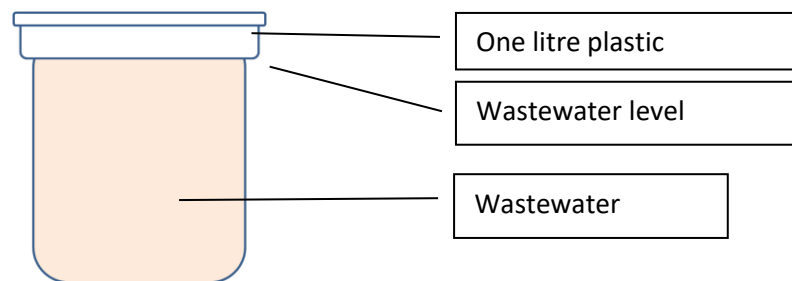
Experimental containers were used to mimic the ideal modular CW at the SAWC. The smaller containers for the study was chosen to make set up easier. It was also

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

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

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

564



565

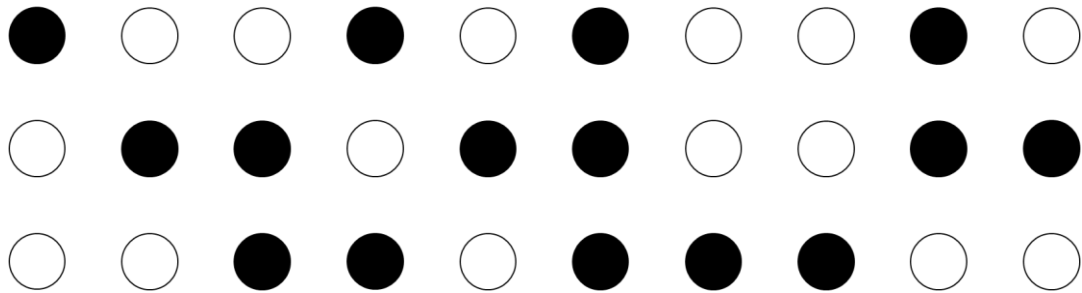
566 **Figure 4:** All the containers in the experiment were filled with wastewater to the first rim of
567 the container.

568

569 **Experimental Run 1**

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

574



575

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

579

580 **Experimental Run 2**

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

587

588 **Experimental Run 3**

589 In the third experimental run 20 containers were placed in two rows in two different
590 locations (Site 3 and Site 1). The site one was used throughout the study but the
591 second site had to be moved because of external factors. The black-and-white
592 container setup was changed to a checkerboard (Figure 6) configuration rather
593 than randomized setup, based on concerns raised about potential bias in the
594 original experimental design used in the first two runs. A checkerboard setup was
595 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.

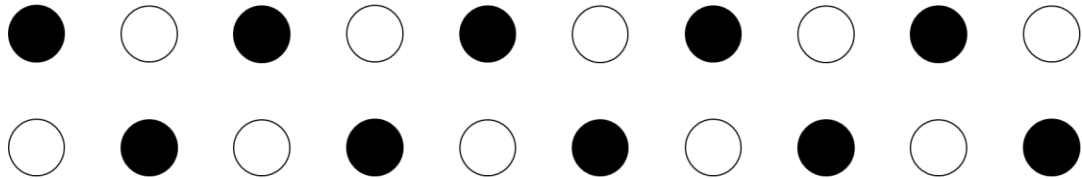


Figure 6: A checkerboard setup of black and white containers was used in the third period of the experimental run at Site 1 and Site 3.

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.

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

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

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

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

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

656

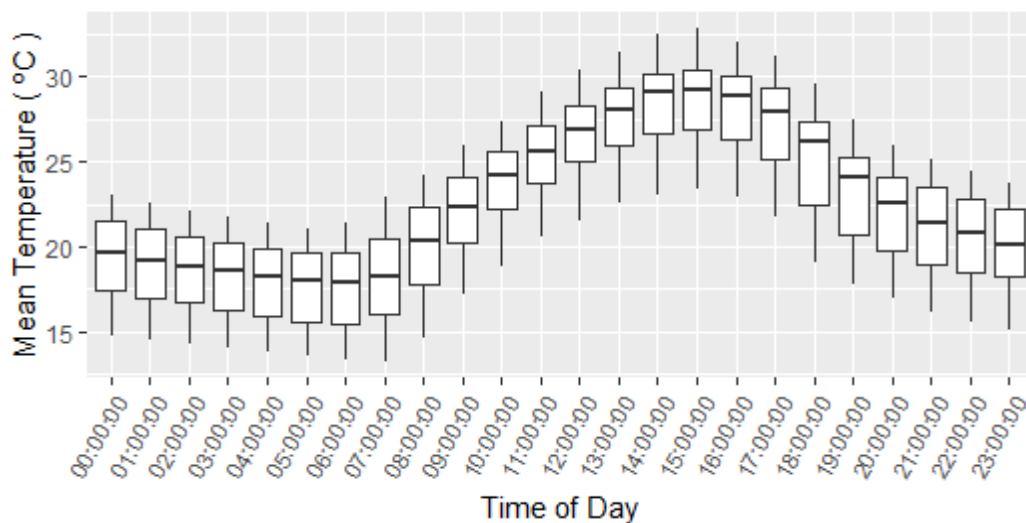


Figure 7: The mean daily temperature at the Southern African Wildlife College for 12 months of 2018 used to estimate which time of day would be the warmest. Temperature plays a role in water quality measures, therefore the warmest period (15:00) of each day was used to standardise measurements (SAEON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

- pH201 – 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.

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

684 **3.5 STATISTICAL ANALYSIS**

685 The Shapiro-Wilks test was used to test for normality of the data. The *Culex* rafts
686 data had a skewness of 8.977 (Kurtosis 84.173; df = 0.199) and the data for the
687 total amount of larvae counted for each period had a skewness of 3.589 (Kurtosis
688 = 15.150; df = 38.856) which all indicated non-normal distribution of data. As the
689 data were not normally distributed, a log transformation was done and the results
690 re-tested for normality. As the data were still not normally distributed and the data
691 having only two variables, the Mann-Whitney U test was used to describe the data.
692 The non-parametric Kruskal-Wallis test was chosen to compare the total egg rafts
693 and larvae counts per container between the three experimental runs and the
694 container position within each trial. The test was followed by a post-hoc pairwise
695 analysis with a Bonferroni correction. The Bonferroni correction took the type 1
696 error into consideration for the level of significance.

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

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

704

705 4 RESULTS

706 Over the period of the study, 2341 larvae were sampled of which 2016 were
707 identified as part of the *Culex pipiens* complex. A total of 153 rafts were found in
708 54 of the 110 containers. Each of the sites were tested separately. The difference
709 in egg rafts and larval counts for the black and white containers was first
710 analysed for each of the three experimental runs. These data were analysed
711 again but this time for each of the five trials separately. The outcomes of
712 significance differed to the extent that it was decided to use the trial data for
713 further analysis and the discussion. A good example is the second experimental
714 run. The difference in egg raft counts between the two colours was significant for
715 the second run ($U=71$, $n=40$, $df=1$, $p=0.003$). When the trials were analysed the
716 run for site one was not significant ($U=25.5$, $n=20$, $df=1$, $p=0.611$) while site two
717 was significant ($U=10.5$, $n=20$, $df=1$, $p=0.002$).

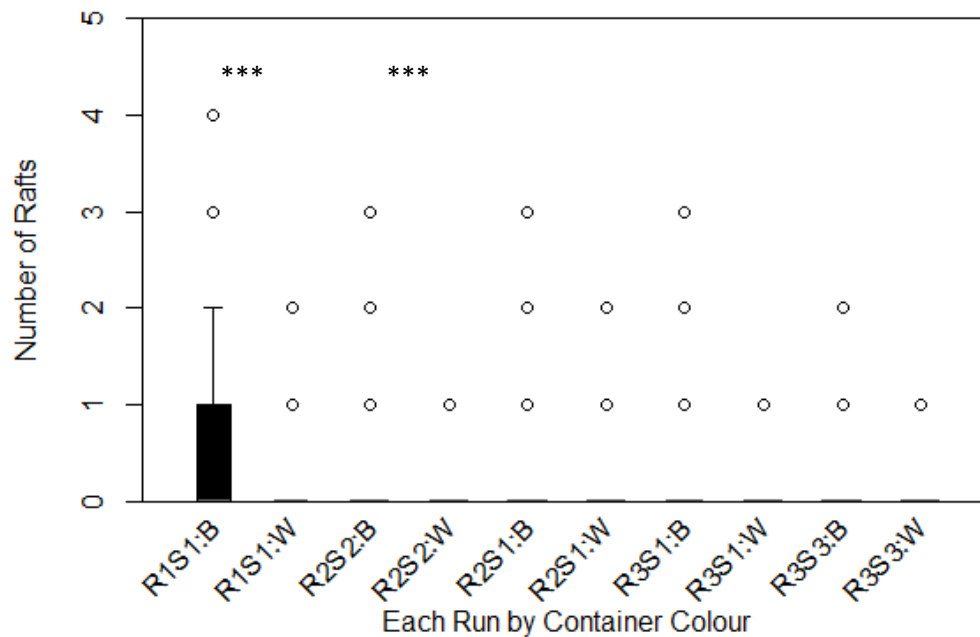
718 For each container there was an average of five measures taken over the period
719 of the trial for DO, EC and temperature. The mean measure of each container
720 was used in the analysis of the five trials and three experimental periods. There
721 was only one count per container for larvae and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

724

725 ***Rafts***

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

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



740

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

744

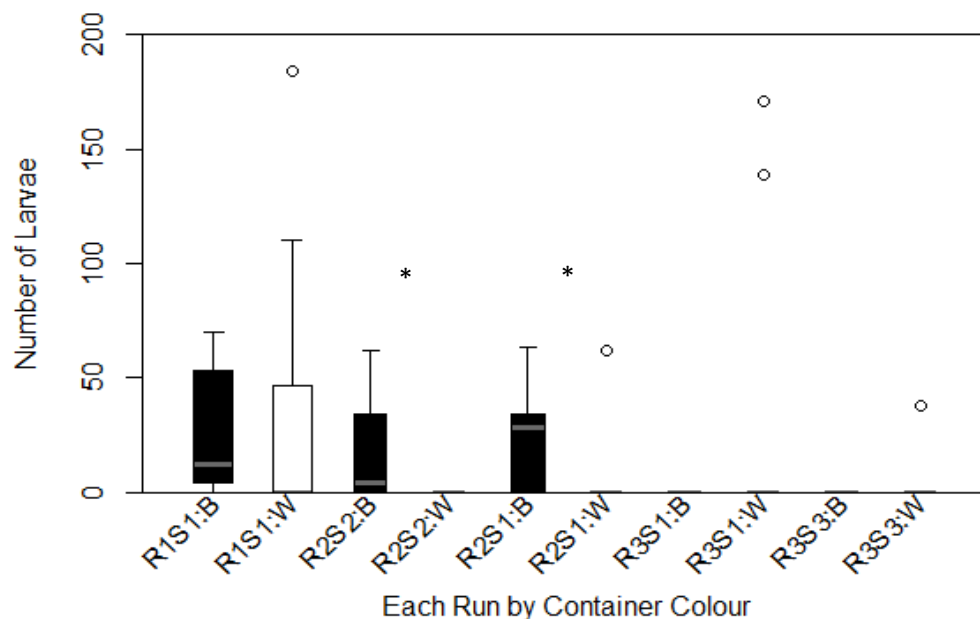
745 **Table 1:** A summary of the amount of rafts found for each trial and their significance
 746 using the Mann-Whitney U test. (Sig. code: **p=0.01-0.001;***p=0.001-0.0001;
 747 NS=p>0.05)

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

748

749 Larvae

750 The difference between larvae found in the black and white containers for the
 751 whole study was significant ($U=2279.5$, $n=110$, $df=1$, $p<0.001$) and there were
 752 more larvae found in the black containers than in the white containers (Figure 9).
 753 The first and third experimental run had higher counts of larvae in the white
 754 containers than in the black containers. It was only a few white containers that
 755 contributed to the large count. The larvae counts in the black container were more
 756 evenly distributed. The difference between these experimental runs (Run 1:
 757 $U=78.5$, $n=30$, $df=1$, $p=0.147$; Run 3: $U=152.5$, $n=40$, $df=1$, $p=0.350$) and trials
 758 (Run 3: Site1 – $U=54$, $n=20$, $df=1$, $p=0.670$; Site3 – $U=55$, $n=20$, $df=1$, $p=0.368$;
 759 Table 2) were not significant. The second experimental run had significantly more
 760 larvae in the black containers than in the white containers for both sites (Run 2:
 761 Site1 – $U=22.5$, $n=20$, $df=1$, $p=0.02124$; Site2 – $U=25$, $n=20$,
 762 At the end of the first experimental run a number of dead larvae were noted on the
 763 water surface. The dead larvae were only noticed in two containers and not taken
 764 into consideration for statistical analysis.



765

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

770

771 **Table 2:** The table gives a summary of the larvae found for each run and site of the
772 study. It further illustrates that the only trials with a significant difference in larvae
773 between the black and white containers was in the second run. (Sig. code: NS= $p > 0.05$;
774 $*p = 0.01 - 0.05$)

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

775

776 **4.1 WATER QUALITY AND CONTAINER COLOUR**

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

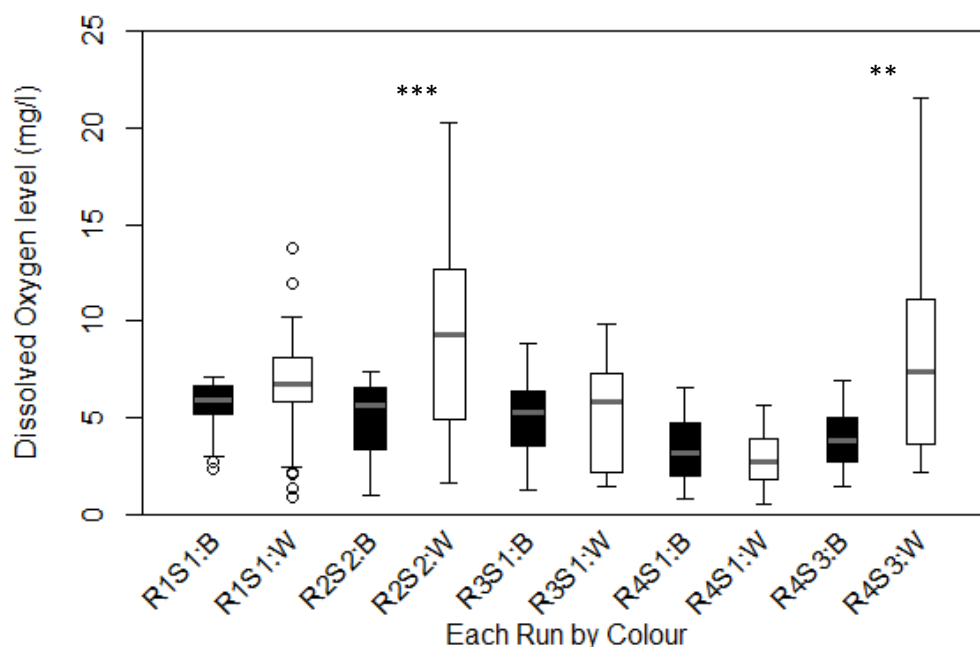
781 Dissolved oxygen (DO) levels were only significant in colour at site two ($U=100$,
782 $n=20$; $df=1$, $p=0.0002$) in the third trial and site three in the fifth trial ($U=96$, $n=20$,
783 $df=1$, $p=0.0006$; Figure 10). The measures were not significant for all the trials at
784 site one (Run1: Site1 – $U=159$, $n=30$, $df=1$, $p=0.056$; Run2: Site1 – $U=55$, $n=20$,
785 $df=1$, $p=0.734$; Run3: Site1 – $U=34$, $n=20$, $df=1$, $p=0.241$)

786 EC levels were also significantly influenced by colour with black containers having
787 higher EC readings than the white containers when looking at the first and second
788 experimental run (Run1: $U=38$, $n=30$, $df=1$, $p=0.0021$; Run2: $U=58.5$, $n=40$, $df=1$,
789 $p=0.0001365$; Figure 11). On further test on the two trials in the second run
790 separately indicated that only site one had a significant difference (Run2: Site1 -
791 $U=2$, $n=20$, $df=1$, $p=0.0003$; Site2 - $U=31$, $n=20$, $df=1$, $p=0.162$). There was no
792 significant difference in the EC levels between the container colours for the third
793 experimental run ($U=140.5$, $n=40$, $df=1$, $p=0.110$). There was neither a significant

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

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

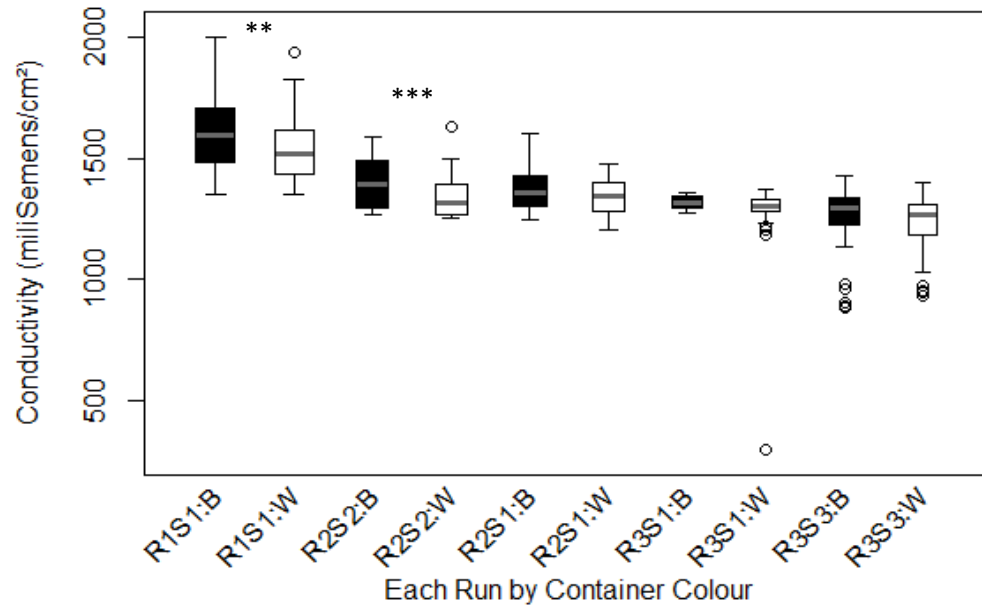
802 Last of the water quality measures that were taken was pH. Unfortunately, it could
 803 only be taken for the last experimental run of the study as the first pH probe broke
 804 just before the experiment started. The pH levels between the white and black
 805 containers were significantly different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



811

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

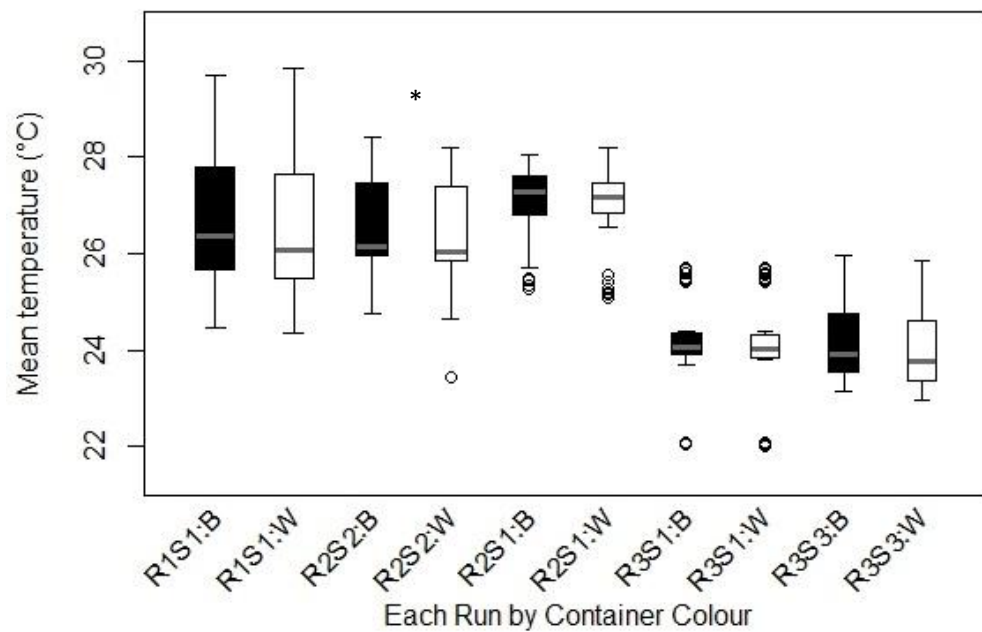
815



816

817 **Figure 11:** There was a decline in the mean EC over the periods of the experiment. (Code:
 818 R = Run e.g. R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code**
 819 $p=0.01-0.001$,*** $p<0.001$).

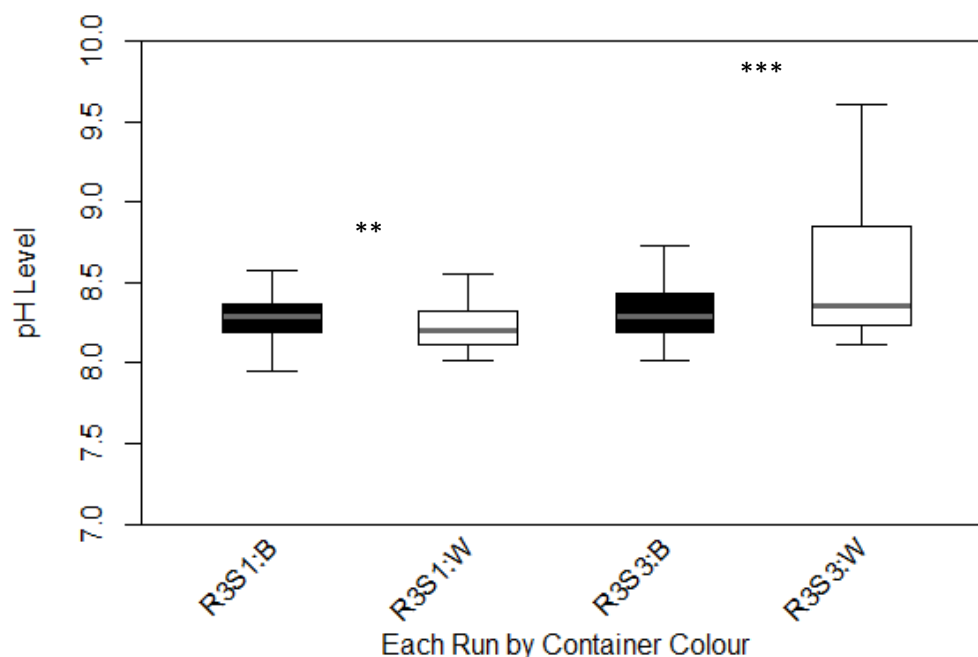
820



821

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

825



826

827 **Figure 13:** The difference in pH levels for the third run. The pH levels of the white
 828 containers at site three was higher than that of the black containers (Code: R = Run e.g.
 829 R1=Run 1; S= Site e.g. S1 = Site 1 and B/W indicates the colour; Sig. code: ** $p = 0.01$ -
 830 0.001 ; *** $p < 0.001$).

831 4.2 CHANGES THROUGH TIME

832 The mean EC, DO, and water temperature as well as the totals of raft and larvae
 833 counts of each container was used to compare the different periods using the
 834 Kruskal-Wallis test. The test was used to compare the changes in EC ($H=85.247$,
 835 $n=110$, $df=2$, $p < 0.0001$), DO ($H=28.691$, $p < 0.0001$, $n=110$, $df=2$), rafts numbers
 836 ($H=10.480$, $p=0.005$, $n=110$, $df=2$), larvae numbers ($H=14.519$, $p=0.001$, $n=110$,
 837 $df=2$), and water temperature ($H=76.039$, $p < 0.0001$, $n=110$, $df=2$) between the
 838 three time periods. The Kruskal-Wallis test was followed by a post hoc pairwise
 839 analysis with a Bonferoni adjustment to see how each of the variables related to
 840 each of the three experimental runs.

841 The test indicated that EC was significantly different between all three off the
 842 experimental runs (All experiments: $p < 0.0001$, $df=2$). As with EC, DO levels were
 843 significantly different between all three the experimental runs (All experiments:
 844 $p < 0.0001$, $df=2$). This outcome lead to analysing each of the trials on their own
 845 using a Mann-Whitney U test.

846 There was a significant difference in the amount of rafts found in each trial over the
847 experimental period ($H=16.114$, $p=0.001$, $df=2$) with the most rafts counted in the
848 first period of the study. The follow-up post hoc test indicated a significant
849 difference between the first and third ($H=18.458$, $p=0.029$, $df=2$), and the second
850 and third period ($H=19.363$, $p=0.01$, $df=2$) of the study. There was no significant
851 difference in raft counts between the first and second period of the study ($p=1$,
852 $df=2$).

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

861 For the mean water temperature the same adjusted analysis indicated a significant
862 difference between the first and the last or third period of the study ($H=52.4$,
863 $p<0.0001$, $df=2$) as well as between the second and third period ($H=56.950$,
864 $p<0.0001$, $df=2$). There was no significant relationship between the temperature of
865 the first and second period. There was a strong correlation between air and water
866 temperatures when temperatures were compared with the Pearsons Correlation
867 test in R ($t=4.8069$, $n=23$, $df=21$, $p=0.00009$, Figure 14). The decline in ambient
868 temperature will therefore have an impact on water temperature. The strong
869 relationship between temperature and larvae survival or production can clearly be
870 seen on Figure 1515 where the number of larvae fluctuated as the average
871 temperature fluctuated.

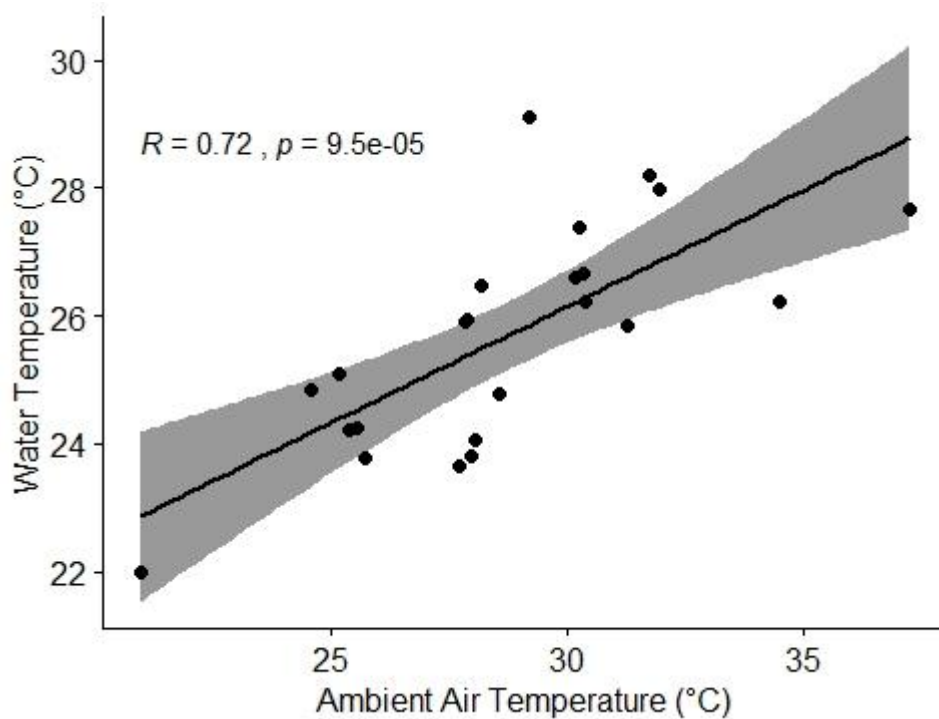


Figure 14: There was a significant correlation between water temperature in the containers and ambient temperature ($t=4.8069$, $n=23$, $df=21$, $p=0.00009$). The mean day temperature between 15h00 and 17h00 was used for ambient temperature and the daily mean water temperature of all containers were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

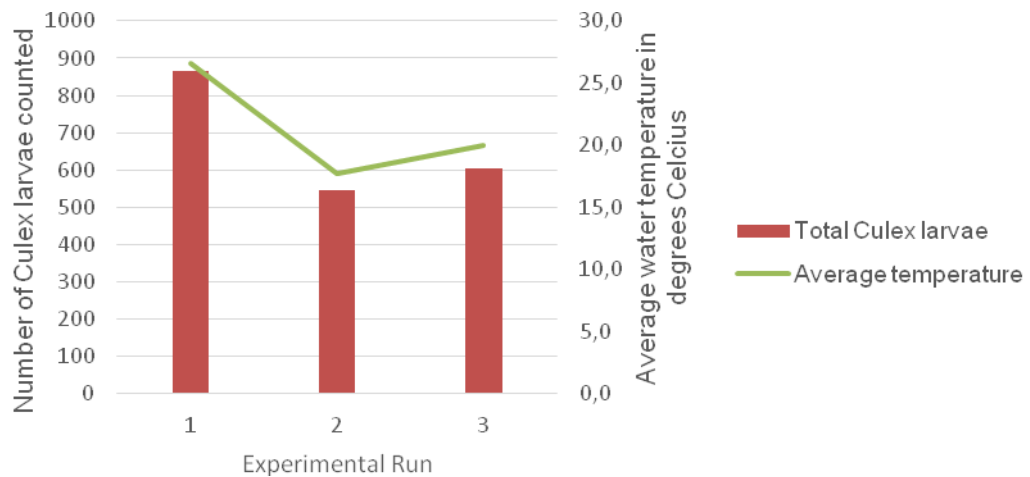


Figure 15: A comparison between *Culex pipiens* complex larvae survival and water temperature for the duration of the study.

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

897 5 DISCUSSION

898

899 ***Oviposition and Container colour***

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

906 ***Oviposition and experimental design***

907 In the study, identical one litre containers made from either black or white plastic
908 were used. This meant the inner surfaces of the containers only varied in colour,
909 and not in texture or surface area, or any other way, that may have unintended
910 consequences. *Culex* and *Aedes* mosquito larvae and chironomids were found
911 across all experimental runs and sites. As hypothesised, black containers had a
912 larger amount of *Culex* species egg rafts than white containers in at least some of
913 the experimental runs (Figure 8). This concurs with laboratory studies on *Aedes*
914 mosquitoes where darker colours attracted significantly more females than yellow
915 and white (Brett, 1938; Beehler *et al.*, 1992; Derraik and Slaney, 2005; Hoel *et al.*,
916 2011). These results also concur with a study done in Florida where gravid *Culex*
917 species mosquitoes chose green and black containers above the lighter containers
918 for oviposition (Allan and Kline, 2004).

919 ***Colour and larvae***

920 Colour did play a role in the amount of *Culex* larvae counted at the end of
921 experimental run. Like the egg rafts, the number of *Culex* spp. larvae found in black
922 containers was significantly more abundant than in the white containers. Although
923 gravid female mosquitoes take great care in selecting a site for oviposition (Day,
924 2016), and regards black containers more favourably than white ones, conditions
925 may change, influence the survival of larvae (Silver, 2008). The first experimental
926 run of the study had more rafts in general. This was most probably the result of
927 favourable weather conditions. During the second run there was a slight decline
928 and the third run a rapid decline in larvae numbers. The water temperature and

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

932

933 ***Water Quality***

934 The container colour not only had an influence on the amount of rafts and larvae
935 numbers, but also on water temperature, EC, and DO measurements. The EC
936 measures were lower in the white containers. EC is an indicator of the amount of
937 ions found in the water and can be used to calculate total dissolved solids. The
938 amount of dissolved solids is an indication of the pollution in the water (Miller *et al.*,
939 1988). Survival of mosquito larvae depends on various factors contributing to a
940 larvae's feeding and physiological niche such as bacteria, fungi (Pavlovich and
941 Rockett, 2000), organic matter, and maximum temperatures (Luhken *et al.*, 2015).
942 Changes in water quality would therefore have an influence on mosquito larvae.
943 The lower EC levels in the lighter containers could be a result of more sunlight in
944 the liquid which speeds up biochemical processes in water such as the formation
945 of hydrogen peroxide (Cooper and Zika, 1983; Bidin *et al.*, 2017), decreasing the
946 amount of ions found in the water. With the decrease in EC fewer processes use
947 oxygen, therefore higher oxygen levels were observed.

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

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

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

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

983 ***Climate, oviposition, and larvae***

984 For the period February, March, and April a decrease in average ambient
985 temperatures, and rainfall, was measured at the SAWC weather station. A
986 decreasing trend in rafts counted and larvae present was also noted between the
987 first, second, and third run of the study as the mean temperature changed. Earlier
988 studies also found a strong link between mosquito oviposition, larvae survival,
989 temperature (Madder *et al.*, 1983; Rueda *et al.*, 1990; Loetti *et al.*, 2011; Ruybal *et*
990 *al.*, 2016), and rainfall (Silver, 2008, p. 100). It is especially the first instar larvae
991 that are very sensitive to climatic changes (Collins *et al.*, 2019). This is in line with
992 the findings of Nguyen *et al.* (2013) where mosquito numbers and oviposition
993 responded to seasonal temperature change. Apart from influencing survival rates,
994 temperature also has a great effect on adult characteristics such as fecundity, body
995 size, longevity, and competence (Clements, 1992; Mottram *et al.*, 1994; Loetti *et*
996 *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

1032 smaller *Culex* individual. In another instance a fourth instar *Aedes* species larvae
1033 killed a second instar larvae. This suggested that cannibalism and predation, apart
1034 from overcrowding could have decreased the number of larvae found in the study.
1035 Koenraadt *et al.* (2004) studied *Anopheles gambiae* mosquitoes and found that
1036 limited space increases interactions between larvae which increases predation and
1037 cannibalism. Ruybal *et al.*, (2016) also found cannibalism in *Culex* species.
1038 However, predation and cannibalism may not be a result of food scarcity; Muturi *et*
1039 *al.* (2010) found that third instar *Anopheles gambiae* s.s. and *Culex*
1040 *quinquefasciatus* larvae predated on first instar larvae. In this study food was
1041 plentiful and not the reason for predation behaviour.

1042 The extent of larvae lost to predation and cannibalism is not known but it was
1043 possible as different instar sizes were found in one container. In this study
1044 observations were made that *Aedes* species develop much faster than *Culex*
1045 species. The reason for the observation is that *Aedes* species convert food to
1046 biomass at a faster rate than other species which then results in faster growth
1047 (Carrieri *et al.*, 2003). Their larger size could therefore make it possible to predate
1048 on the slower developing *Culex* species. In the first experimental run third and first
1049 instar larvae were found in the same container. Based on the difference in size,
1050 cannibalism could also have influenced the outcome of larvae counted on the last
1051 day of the experiment.

1052 The observations of evaporation, large number of larvae, and the influence of
1053 temperature is in line with other studies as the main cause of larval death (Subra,
1054 1981).

1055

1056 ***Limitations and future considerations***

1057 Site consideration and setup is important in any study. In this study the setup was
1058 changed from a randomized design to a checkerboard setup to take the influence
1059 of light on water quality into consideration. The change in setup further complicated
1060 the analysis process. The row of ten containers was used to mimic the SAWC on-
1061 site water treatment system. Since water quality and oviposition was considered,
1062 other setups such as a Latin square design could have been used to better
1063 understand the influence of light on the setup.

1064

1065 There was a limited amount of suitable sites with a protective wall and a roof within
1066 the SAWC campus. Moving sites also added variables to the study such as wind.
1067 The study took place under rooves to limit evaporation and keep conditions as
1068 natural as possible. This approach did work but for future studies it compensation
1069 for evaporation, by filling the containers on a daily base to keep water levels
1070 constant, should be considered. When there are concerns about open sky or trees
1071 dropping leaves into the containers influencing oviposition, sites can be set under
1072 a tarp to try keeping other variables constant.

1073

1074 Although *Culex* species are the only group that lay eggs in rafts, counting the rafts
1075 was harder than expected. The complexity was a result of the methodology to leave
1076 old rafts in the container to hatch. Differentiating between the old and new rafts
1077 was a bit more difficult than first anticipated. New rafts were a bit more shiny than
1078 older rafts. Some of the older rafts quickly disappeared while others stayed afloat
1079 for longer. Black containers did have higher levels of oviposition but in order to
1080 prevent over estimations rafts can be counted every third day. This will give time
1081 for eggs to hatch and can then be removed to prevent double counting (Blaustein
1082 *et al.*, 2005; Nguyen *et al.*, 2012). Some of the eggs may not yet have hatched and
1083 will then result in removal of possible larvae. In warm periods, eggs hatch within
1084 48 hours. The objective was to indicate which colour had the most larvae surviving
1085 relative to the other colour. Therefore it is argued that removing eggs from all
1086 containers on a three day cycle will not have a significant influence on the
1087 difference in survival between the containers.

1088

1089 Water quality also had some challenges as not all the equipment functioned as the
1090 HACH experts predicted. The DO probe had readings of higher than 22mg/l which
1091 is the upper limit of the probe and a DO saturation of more than 150%. The
1092 technology used to measure the presence of oxygen in the water is called
1093 Luminescent Dissolved Oxygen (LDO) technology. The device emits a blue light to
1094 an oxygen sensitive membrane which then reacts with the luminescent light and
1095 returns a red light. The time delay and intensity of the red light is measured and
1096 used to determine the dissolved oxygen levels in the water (HACH, 2019). It was
1097 therefore thought that the physical colour of the container absorbed or reflected
1098 the light emitted differently and therefore gave inaccurate DO readings. A 200 ml
1099 white plastic cup was inserted in the containers with the probe to minimize the

1100 effect. There was no need as Picot *et al.* (1992) and Richmond (1986) measured
1101 super saturations of DO in water with algae during periods with high light intensity.
1102 All the DO measures taken during this study could therefore be used. A future
1103 consideration is to use multiple DO probes or a probe that does not use light to
1104 confirm or deny the effect of container colour.

1105

1106 6 CONCLUSION

1107 It is important to human and animal health that mosquito populations are controlled
1108 as some of them are vectors of disease (Jupp, 2005). Both adults and larvae are
1109 targeted in population management strategies. All species need water for the
1110 larvae to be able to morph into adult mosquitoes (Day, 2016) therefore the water
1111 is a good starting point for mosquito control. Water scarcity is unfortunately not
1112 recognised by everyone. They waste and pollute the fresh water that is available
1113 (Lewin *et al.*, 2007). This could be a result of poor maintenance or a lack of
1114 infrastructure (Barlow and Clarke, 2002; Dos Santos *et al.*, 2017). The polluted
1115 water with high nutrient content results in waterbody eutrophication. This results in
1116 the loss of mosquito predators (Isife, 2012). It further creates favourable conditions
1117 for mosquito larvae development (Nguyen *et al.*, 2013).

1118

1119 Constructed wetlands are seen as a cheap alternative for secondary water
1120 treatment (Shutes, 2001). Their design can vary from large pond system down to
1121 small container system for cleaning wastewater (Vymazel, 1998) to use for crop
1122 irrigation (Emongor and Ramolemana, 2004; Kurniadie, 2011; Al-Gheethi *et al.*,
1123 2018). Direct sunlight, wind, and plants assist in treating waste water and killing
1124 bacteria (Webb, 2013). In the right balance these factors together with water quality
1125 also have an effect on mosquito breeding (Dale and Knight, 2008). Gravel can be
1126 used to prevent mosquitoes from reaching the water but is expensive and prone to
1127 clogging (Vymazal, 1996) which makes gravel less than ideal for rural communities.

1128

1129 Understanding mosquito biology and ecology, therefore, becomes important in the
1130 design process of a CW. In this study there were higher levels of oviposition in
1131 black containers. Although there were a few white containers with large numbers
1132 of larvae it was only black containers that had a significant impact on larvae
1133 numbers in some of the trials. The use of light colours could therefore be one of
1134 the design solutions to limit oviposition and larval development by making the
1135 habitat less favourable. Colour on its own may not be the ultimate solution for
1136 decreasing mosquito numbers as they also use chemical and tactile cues to select
1137 a breeding site (Bentley and Day, 1989).

1138

1139 The colour of the container can be changed by using paint (Collins and Blackwell,
1140 2000). Unfortunately paint does not attach easily to plastic and it reflects light rather

1141 than increasing light penetration through transparent walls. The paint may also
1142 influence mosquito oviposition as they use chemical cues for site selection. If
1143 colour can limit habitat selection in *Culex pipiens* complex species, it could be
1144 successful for many other species. The only research found that used light colours
1145 for attracting insects was to attract pollinator species (Clare *et al.*, 2000; Vrdoljak
1146 and Samways, 2012)

1147

1148 Water temperature and weather played a role in oviposition with raft and larvae
1149 numbers declining as the experimental run moved closer to winter. Should global
1150 temperatures increase, mosquito numbers may also increase and so also the
1151 spread of disease (Bhattacharya and Basu, 2016). On the other hand, constant
1152 high temperatures above 25 degrees Celsius (Ciota *et al.*, 2014) could also so the
1153 growth or kill off larvae and decrease population sizes.

1154

1155 Related to temperature, DO is affected by container colour with white containers
1156 having much higher DO levels than in the black containers. From these results it is
1157 difficult to directly link DO levels with larva survival or death. In the long term higher
1158 DO levels will result in more favourable conditions for mosquito predators. There
1159 was a study done that indicated that larva start dying at very low DO levels (Silver,
1160 2008). High temperatures result in lower DO levels which make it more difficult for
1161 mosquito predators to survive. Temperature and DO is highly correlated and their
1162 influence on mosquito larvae can therefore not be seen in isolation.

1163

1164 The conditions in the black containers were good for mosquito oviposition and
1165 larval growth. Warm water temperatures, low DO levels and sewage water did
1166 stimulate oviposition and assisted in larval growth. Colour also had a direct and
1167 indirect influence on the breeding success of the mosquitoes. However, there is a
1168 need to further investigate the interrelationship between container colour, water
1169 quality, temperatures and how they separately and as a whole influence mosquito
1170 reproduction. Further factors that can be considered is the role of direct sunlight,
1171 container aperture, and size has on the oviposition choice of various mosquito
1172 species (Derraik and Slaney, 2005).

1173

1174 There is a great need for treating wastewater using cost effective biological
1175 methods while minimizing the risk of breeding mosquitoes. There is still a lot of

1176 research needed to understand the physical qualities that attracts a female
1177 mosquito to lay her eggs (Reiskind and Zarrabi, 2012). This study highlights the
1178 complexity of mosquito breeding biology and how one variable such as colour can
1179 affect egg laying and water quality. There is potential to minimize mosquito
1180 population growth by changing the colour of the containers used to above ground
1181 CW's. Container colour will not only change mosquito behaviour but also the
1182 biochemical process in the wastewater. Minimizing mosquitoes in constructed
1183 wetlands while effectively treating the wastewater is more complex and will need
1184 to find a solution looking deeper into mosquito biology, water biochemistry and
1185 wetland design.
1186

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