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The effects of larval age composition and adult female body size on oviposition by *Anopheles gambiae* (Diptera: Culicidae).

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Period: October 2007 – July 2008
1st Examinator: Willem Takken
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Summary

The assessment of the suitability of an oviposition site by female mosquitoes increases their fitness and that of their offspring. Chemical ecology is an important strategy in oviposition behaviour of mosquitoes. Most studies on chemical cues have been performed on Culicinae, and only few could be found on anophelines. There are indications that *Anopheles gambiae* use semiochemicals to assess the quality of oviposition sites. Sources of chemical cues affecting oviposition behaviour by this species range from bacteria to potential breeding water and larvae. In this study I examined the role of chemical cues associated with larval development in female oviposition choice and the effect of body size on oviposition behaviour.

Gravid females are attracted towards water containing first instars and are deterred by 4th instars. When both stadia are abundant in one cup, mosquitoes do not make a clear choice anymore, although there seems to be a trend in which the mosquitoes lay more eggs in the cups without L4 larvae.

When larvae develop under high competition, smaller females will emerge. These smaller females have reduced fitness-related factors, like survival and number of eggs produced. The effects of adult body size on oviposition behaviour of *Anopheles gambiae* has never been tested. The results from the first experiment in which L1 larvae are attractive and L4 larvae are deterrent, was found to be valid only for medium-sized mosquitoes. Both small and large larvae do not respond in agreement with earlier results.

Different chemical compounds collected from headspace of water in which larvae are developing, have been found that might have an effect on oviposition behaviour of *Anopheles gambiae*. Nonane and 2,4-pentanedione were found in waters containing L1 larvae as well as L4 larvae and less in the control. Dimethyl disulfide and dimethyl trisulfide were found in significantly larger quantities in waters containing L4 larvae than in waters containing L1 larvae and the control. The biological effects of these chemicals on oviposition behaviour of *Anopheles gambiae* needs to be tested.

The results provide evidence that oviposition choice and behaviour of *An. gambiae* is mediated by chemical cues that are associated with developing larvae.

Preface

In this report the results of my study on the ovipositional behaviour of *Anopheles gambiae* will be shown. This study was done as a MSc thesis for the Master phase of Biology. I started this study in 2003 at the University of Nijmegen. Shortly after the start of my studies, I developed a great interest in insects and ecology. After doing some explanatory and experimental studies on insects in Nijmegen in my third and fourth year, there were no further opportunities on doing studies on insects there. Therefore, I had to come to Wageningen University.

I participated at the course 'Fundamental and Applied Aspects of the Biology of Insects' and wrote a proposal about mosquito control, of *Aedes albopictus* in Europe. My interest for mosquitoes was aroused, and it did not take long to decide what I wanted to do for my next internship, which would last for 6 months. But, because this was such an interesting study and the atmosphere at the Laboratory of Entomology is incredible, I expanded it with 3 months.

Because of these extra months, I was able to dig deeper into this interesting study, which resulted in some interesting results. Therefore I would like to thank Professor Takken, for this opportunity and help during this study. And of course, I would like to thank my other supervisor Jeroen Spitzen for is help and suggestions. Furthermore, I would like to thank the rearers, Leo Koopmans, Frans van Aggelen and André Giddink, for they provided me with biological material for the experiments. And of course Karel van Roey and Annet van Swaay, for our own rearing of mosquitoes could not be successful without them. At last, I would like to thank everyone who helped me in one way or the other, they are too much to name separately.

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1. Introduction

Mosquitoes (Diptera: Culicidae) are best known for their nuisance and their role in the spreading of diseases. *Anopheles gambiae* (Giles) and *Anopheles arabiensis* (Patton), for example, play a major role in the spreading and maintenance of malaria in Sub-Saharan Africa. These species are the vectors of *Plasmodium falciparum*, a parasite that causes a deadly form of malaria in humans, when infected (Koenraadt *et al*., 2004), which is responsible for over a million deaths each year, with about 90% of deaths occurring in Africa south of the Sahara (WHO, 2007).

An. gambiae is distributed almost universally in tropical Africa, with the exception of high altitude habitats and deserts, and is often strictly localized in areas with closed canopy forests. It is abundant from the extreme south of Mauritania across southern borders of the Sahara through the northern Sudan, to the Red Sea coast of Arabia near Jeddah. In the Southern part of Africa it is abundant in the central parts of South West Africa and in Botswana, but is absent from the central plateau. It is abundant in Malagay, Réunion, Mauritius, the Comores, Verde Islands, Principe, San Thomé and Fernando Poo, in the Indian Ocean (Gillies & De Meillon, 1968).

Other examples of disease transmitting mosquitoes are *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), both species can transmit dengue and yellow fever, and *Culex quinquefasciatus* (Say), which transmits filariasis (Navarro, 2003).

Understanding of the biology and ecology of these species is necessary for a proper control of these mosquitoes.

Culicidae have a life cycle consisting of two phases; an aquatic phase, in which early stage mosquitoes live, and a terrestrial phase, occupied by adult mosquitoes.

1.1 Biology of mosquitoes

1.1.1 Early stage mosquitoes

Mosquitoes are holometabolous insects, meaning that their life cycles undergo a complete metamorphosis, usually through a pupal stage. There are four different stages: eggs, larvae, pupae and adults. Adult females oviposit in aquatic habitats, where the insects complete the first three stages of their life-cycle.

A couple of days after oviposition the eggs hatch, where the hatching rate is dependent of the species.

The larval stage consists of four instar levels. During each instar level continuous growth occurs. Structurally, the larva is a combination of larval functional organs and slowly developing adult organs (Clements, 1992).

The growth rate of larvae is affected by various extrinsic factors, like temperature, nutrition and densities. Growth and development can only occur between a range of temperatures with a lower developmental threshold temperature and an upper lethal temperature. These ranges vary with species. Furthermore, there is a positive correlation between temperature and growth rate, within the temperature range.

Growth rates will diminish when the available food for larvae is below a certain amount (Clements, 1992). Although larvae are abundant in different aquatic habitats, the available food is generally the same and consists of micro-organisms, detritus and bio-film. The different types of food can be found in different locations of the habitat and different feeding modes have been developed by the larvae.

Collecting-filtering is the removal of particles, micro-organisms and detritus, suspended in the air-water interface (most *Anopheles* species), water column (*Culex* and some *Aedes* species) and stems and roots. Collecting-gathering is the resuspension and removal of micro-organisms and detritus on surfaces or substrata (many *Aedes* species). The removal and ingestion of bio-layers, adherent algae and protists from the surface of plants, stones and substrata is called scraping. Shredding is the biting-off of leaves and dead invertebrates in the water column and substratum. And finally, predation, the ingestion of living invertebrates in the water column (Clements, 1992).

Predation also occurs between mosquito larvae, and is observed between different species, as well as within the same species (cannibalism) (Koenraadt & Takken, 2003; Koenraadt *et al*., 2004). The degree of cannibalism is dependent on factors such as food availability, larval densities, size composition and age composition of the population. Individuals of the same age cannibalize each other rarely, whereas large differences in size between larvae may result in frequent cannibalization of the smaller larvae (Sherratt & Church, 1993).

Feeding behaviour consists of different steps. First, there is a movement towards the food source. Secondly, the food is captured. And finally, there is a coordination of the complex mouthpiece movement (Clements, 1999).

The food availability is closely related to the larval densities, higher densities usually result in less food available per larvae. This intraspecific competition results in a longer developmental time, reduced pupation success and a reduced pupal

weight. Some other factors affecting growth rate are salinity and water depth (Clements, 1992).

When fourth instar larvae moult they become pupae. This is the stage between the larvae (juvenile) and mosquitoes (adult), during which the larvae undergo a complete transformation within a hardened outer covering. Individuals in this stage do not feed.

The duration of the pupal stage is temperature-dependent and differs between species. Under optimal temperatures the pupal stage of *An. gambiae* takes 1 or 2 days. Emergence of adults of this species occurs early in the evening and is followed by a short flight to a suitable resting place, where the mosquitoes wait until their cuticle hardens (Gillies & De Meillon, 1968).

1.1.2 Adult mosquitoes

Compared to the earlier stages, adult mosquitoes have a higher mobility, and are less dependent of aquatic habitats. They are dependent of other resources than the larvae, namely: Sugar, blood, mates and resting sites. Additionally, females require oviposition sites (Clements, 1999).

Plant sugars are an important energy source during adult mosquito life of both sexes, and can be significant for survival. Plant sugars can primarily be found in floral nectar, but also in extra-floral nectaries, damaged fruits, vegetative tissues and honeydew. Plant sugars are the only food resource for male mosquitoes (Clements, 1992).

Females require a blood meal for the development of their eggs. Most species feed on blood from mammals and birds, while a few species are known to feed on reptiles, amphibians and fish. Female mosquitoes commonly ingest more blood (up to two to four times) than their own weight and are capable of separating nectar and blood in their digestive tract. Nectar is kept in the crop, while blood is transported to the midgut. By doing this, females make sure that their stomach is empty, even after a sugar meal, and can receive a blood meal at any time (Clements, 1992). It is the need of vertebrate blood for the production of the eggs, that accounts for the transfer of parasites (Takken & Costantini, 2006).

For mating to occur, males and females have to find individuals of the same species and the opposite sex. Clements (1999) formulated 3 manners in how male and female mosquitoes encounter each other: Swarming by males, assemblage at biological significant places and the approach of isolated resting females by males.

Males of *An. gambiae* swarm a short period during dusk (Clements, 1999; Gillies & De Meillon, 1968; Charlwood *et al*., 2002), mating with females they

encounter. Females that are receptive fly actively during the time the males are swarming (Clements, 1999), enhancing the chance of finding a mating-partner. Despite the short period in which swarming occurs, it is a very efficient mate-finding mechanism, at least for *Anopheles gambiae* (Charlwood *et al*., 2002).

Males of various *Aedes* species are known to respond to host odours when searching for females to mate with. Close-range species recognition is mediated by species-specific contact pheromones, at least for *Ae. aegypti* and *Ae. albopictus* (Takken & Knols, 1999).

In the first 30 – 60 hours of the adulthood of *An. gambiae*, females are non receptive to males. They try to avoid mating by kicking males off or by bending their terminalia away from the male genitalia. Males respond to female sexually 12 – 36 hours after emerging. At this time the antennae fibrilae are, or can be kept at least temporary, erect (Clements, 1999). Mating usually takes place once in the life of an *An. gambiae* female (Gillies & De Meillon, 1968).

The gonotrophic cycles consist of: host-finding and blood-feeding (mating might occur after these stages), inactivity during the ovarian development, pre-ovipositional behavior and oviposition. Eggs can be developed without insemination, oviposition, however will not take place then. Sugar-feeding may occur at each of these stages, but most *Anopheles* and *Aedes* species do not feed on plant sugars while blood fed or gravid. All of these stages are separated spatially and the females have developed resource-orientated locomotion behaviours (Clements, 1999).

Anautogenous mosquitoes need, in contrast to autogenous mosquitoes, at least one blood meal for the ovarian development. The specificity of anautogenous mosquitoes towards feeding-hosts differs between species and genera. *Anopheles* species feed on mammals above all, as do *Aedes* species, while *Culex* species feed on mammals, birds, or both and some species on amphibians and reptiles (Clements, 1999).

Many anautogenous mosquitoes use odour signals originating from the host, when searching for blood (Takken & Knols, 1999). The anthropophilic species *An. gambiae* mainly feeds indoors, which is connected with the late feeding behaviour. Most biting takes place after midnight. The biting will occur until shortly before dawn (Gillies & De Meillon, 1968), when the host defensive responses are low (Takken & Constantini, 2006), and usually no biting occurs during the day (Gillies & De Meillon, 1968). Other species, like *Ae. albopictus*, are diurnal feeders and feed on humans during daytime (Knudsen, 1995).

After a blood meal there is a period of inactivity, during which the ovarian development occurs (Clements, 1999). A substantial proportion of individuals of *An.*

gambiae rests indoors, while relatively few females leave the house after feeding (Gillies & De Meillon, 1968). The development of the habit of resting indoors provided mosquitoes of a relatively stable and favourable microclimate and the freedom from predators (Gillies & De Meillon, 1968; Takken & Constantini, 2006). At dusk there generally is a departure of large amounts of males and unfed and gravid females that have covered indoors for the day (Gillies & De Meillon, 1968).

The next step of the gonotrophic cycle is pre-ovipositional behaviour, which exists of the search for a favourable habitat for the eggs and larvae. Localization of these habitats occurs through behavioural responses to olfactory and optical cues (Clements, 1999). After the localization of a habitat, the female will determine the quality of the water using short distance olfactory, as well as gustatory cues (Clements, 1999). After assessing the water, the female reacts to the environmental circumstances of the habitat and searches for a better habitat or lays its eggs and starts a new life cycle.

1.1.4 Oviposition sites

Oviposition sites, which will form the larval habitats, differ between species. *An. gambiae* favors open sun-lit, temporary freshwater habitats, like shallow pools, car tracks, borrow-pits, drains and brick-pits filled with rainwater and hoof-prints (Koenraadt & Takken, 2003; Gillies & De Meillon, 1968; Clements, 1999), without emerging vegetation (Clements, 1999). It should be noted that the human influence in many of the *An. gambiae* habitats is implicit (Gillies & De Meillon, 1968).

Ae. aegypti is a container-dwelling species, meaning that females oviposit in small containers holding water. Mosquitoes of the domestic populations oviposit in storage vessels that contain clean water, and are sheltered from the rain (Clements, 1999), like pots, car tires and other discarded household refuse (Edman *et al*., 1998). Females of peridomestic populations oviposit in natural and artificial containers, like tree holes and barrels, while in the woods living populations oviposit in tree and rock holes (Clements, 1999).

Wyeomyia vanduzeei (Dyar & Knab) is very specific in its choice of an oviposition habitat, and has a very strong preference for *Tilandsia utriculata* (Frank & O'Meara, 1985), a tropical bromeliad which collects water in its pits.

Female mosquitoes are in some way attracted to a specific oviposition site, which will be the habitat for their offspring. Individuals assessing, and reacting to, the risks of that habitat enhance the survival of their offspring and are favoured by natural selection (Angelon & Petranka, 2001).

1.1.5 Cues for habitat selection

Most Culicid species are *r*-strategists, the larvae colonize short-lived biotopes, in which a rapid rate of population increase is favoured by selection (Clements, 1992).

Therefore, the location and selection of an oviposition site is an important part of the life history of Culicidae, and involves visual, olfactory, and tactile/gustatory responses (Bentley & Day, 1989; Reiskind & Wilson, 2004). The location of oviposition is especially important for mosquitoes, because the larvae are not capable of moving to another habitat when their habitat becomes unfavourable (Mokany & Shine, 2003).

Natural selection favours individuals that assess the risks of the habitat of oviposition, and maximize the survival of their offspring. When searching for a favourable habitat for oviposition, mosquitoes may encounter habitats of different qualities, in which components like the density of competitors, the risk of predation, the seasonal duration and the productivity of the habitat may influence the fitness of the offspring (Angelon & Petranka, 2001). It is suggested that, by choosing high-quality larval habitats for oviposition, females will enhance their own fitness (Reiskind & Wilson, 2004; Spencer *et al.*, 2002). Their choice may be influenced by factors that fit the female mosquito the best, like the availability of essential resources (Reiskind & Wilson, 2004).

Different cues are used when selecting an oviposition habitat, which can be divided in attractants and repellents; these cues will attract the mosquito towards the habitat of ovipositioning or will make the mosquitoes move away from the habitat, respectively. After reaching the habitat mosquitoes respond to chemotactile stimulants and deterrents, which will determine whether oviposition will occur or not (Bentley & Day, 1989).

Mosquitoes are known to choose among water bodies based on cues of environmental conditions, like temperature, light, shade, water depth, turbidity, salinity and pH (Bentley & Day, 1989; Navarro *et al*., 2003). Next to these environmental conditions, oviposition sites are also assessed on physical features like colour and texture of the surface, which are often visual cues. Females of *An. gambiae*, for example, laid more eggs in black Petri dishes, than in grey or white ones (Clements, 1999). Finally, ovipositional habitats are also assessed by chemical cues (see table 1).

Much research on oviposition sites has been done on *Aedes* and *Culex* species. Adult females are attracted, or repelled by conspecific larvae in the habitat, according to species and age of the larvae (Zahiri *et al.*, 1997a; Allan & Kline, 1998; Trexler *et al*., 2003a; Reiskind & Wilson, 2004; Sherratt & Church, 1993; Clements,

1999, Bentley *et al*., 1976). In some species the females are even attracted to habitats containing heterospecific species (Zahiri *et al*., 1997a; Zahiri *et al*., 1997b; Allan & Kline, 1998; Bentley *et al*., 1976).

Some species may also be attracted by conspecific and/or heterospecific eggs in the water (Allan & Kline, 1998; Trexler *et al*., 2003a; Clements, 1999). From *Culex quinquefasciatus* an attractive chemical compound has been identified as 6-acetoxy-5-decanolide, an aggregation pheromone (Clements, 1999). Although much research has been done on chemical cues used to assess water, a lot of the chemical compounds that affect the behaviour of female mosquitoes are unknown.

Culex restuans (Theobald) has been shown to be attracted to decomposing plant material, which can be nourishment for the larvae (Reiskind & Wilson, 2004). Other species are also attracted towards compounds released by decaying plant material, like hay, Bermuda grass and decayed wood. Some of these attractive compounds have been analyzed, like 3-methyl-indole (skatole), *p*-cresol and 4-methylcyclohexanol (Millar *et al*., 1992; Beehler *et al*., 1994; Bentley *et al*., 1982).

It has also been shown that females are attracted to chemical compounds released by bacteria. Most of the research on bacteria has been done with *Aedes* and *Culex* species (Trexler *et al*., 2003a). Bacteria are an important source of food for the larvae in nature (Navarro *et al*., 2003), this might explain the attractiveness towards bacteria by females.

By assessing the hazards of a habitat, females are able to enhance their fitness. In several species it has been shown that female mosquitoes are able to detect pathogens and predators (Spencer *et al*., 2002; Zahiri *et al*., 1997a; Mokany & Shine, 2003; Stav *et al*., 1999; Angelon and Petranka, 2002). It has been shown that females of *Culex* mosquitoes are capable of using solely chemosensory mechanisms to assess predator risks of the habitat. The responses to predators with chemical cues are often influenced by the predator's diet, like mosquito larvae (Angelon & Petranka, 2002). Furthermore, *Ae. aegypti* was able to recognize habitats in which starvation of larvae occurred and did not oviposit there (Zahiri *et al*., 1997b).

Table 1: Sources of chemical cues affecting mosquito oviposition, and their active compounds if known. In the third column the effect on the behaviour of the mosquitoes is given. Note that distinction is only made in attractants and repellents, stimulants and deterrents are included in these groups. This is because it is often not known if compounds act whether as attractants/repellents or stimulants/deterrents. The fourth and fifth column show the species in which the cues are found, and the authors publishing these results.
** in: Clements, 1999. Biology of mosquitoes, Vol. 2.*
† see Trexler et al., 2003 for a summation of mosquito species and bacteria they are attracted to.
‡ in: Navarro et al., 2003.
Ae.= Aedes; An.= Anopheles; Cx.= Culex; T.= Trichoprosopon; Tx.= Toxorhynchites.

Source of Chemical Cues	Compound	Attractant/ Repellant	Species	Authors
Larvae				
- Conspecific	Unknown	Attractant	*Ae. aegypti*	Zahiri *et al.*, 1997a
			Ae. atropalpus	
			Ae. albopictus	Allan and Kline, 1998
				Trexler *et al*., 2003a
			An. gambiae	Mwingira *et al.,* (in prep.)
			Ae. triseriatus	Bentley *et al.*, 1976
		Repellent	*Cx. restuans*	Ikeshoji, 1966*
				Reiskind and Wilson, 2004
			An. gambiae	McRae, 1984
- Older larvae	Unknown	Repellent	*T. digitatum*	Sherratt and Church, 1993
			An. gambiae	Mwingira *et al.,* (in prep.)
				Munga *et al.,* 2006
- Heterospecific	Unknown	Attractant	*Ae. aegypti* to *Ae. atropalpus* and	Zahiri *et al.*, 1997a
			Ae. albopictus	Allan and Kline, 1998
			Tx. splendens to *Ae. aegypti*	Benzon *et al.*, 1988
			Ae. triseriatus to *Ae. atropalpus*	Bentley *et al.*, 1976
- Starved	Unknown	Repellent	*Ae. aegypti*	Zahiri *et al*., 1997b
Eggs				
- Conspecific	Unknown	Attractant	*Ae. aegypti,*	Allan and Kline, 1998
			Ae. albopictus	
	6-acetoxy-5-decanolide		*Cx. quinquefasciatus*	Trexler *et al*., 2003a, Laurence and Picket, 1985*
- Heterospecific	Unknown	Attractant	*Ae. aegypti* to *Ae. albopictus*	Allan and Kline, 1998
	6-acetoxy-5-decanolide		*Cx. cinereus*	Mboera *et al.,* 1999
Bacteria				
- In general	Unknown	Attractant	*Aedes* species†	Trexler *et al*., 2003a
				Navarro *et al*., 2003
			Culex species†	Trexler *et al*., 2003a
			An. gambiae	Sumba *et al.,* 2004
- *P. aeruginosa*	7,11-dimethyl-octadecane	Attractant	*Ae. aegypti*	Ikeshoji *et al*., 1979‡

Table 1 (continued): Sources of chemical cues affecting mosquito oviposition, and their active compounds if known. In the third column the effect on the behaviour of the mosquitoes is given. Note that distinction is only made in attractants and repellents, stimulants and deterrents are included in these groups. This is because it is often not known if compounds act whether as attractants/repellents or stimulants/deterrents. The fourth and fifth column show the species in which the cues are found, and the authors publishing these results.
Ae.= Aedes; An.= Anopheles; C.= Culiseta; Cx.= Culex; O.= Ochlerotatus.
A.= Anax; G.= Gambusia; L.= Limnodynastes; N.= Notonecta; P.= Plagiorchis.

Source of Chemical Cues	**Compound**	**Attractant/ Repellant**	**Species**	**Authors**
Predators and pathogens				
- *N. maculata* (water bug)	Unknown	Repellent	*C. longiareolata*	Spencer *et al.*, 2002
- *P. elegans* (trematode)	Unknown	Repellent	*Ae. atropalpus*	Zahiri *et al*., 1997a
- *L. peronii* (anuran)	Unknown	Repellent	*O. australis*	Mokany & Shine, 2003
- *A. imperator* (dragonfly)	Unknown	Repellent	*C. longiareolata*	Stav *et al*., 1999
- *G. affinis* (fish)	Unknown	Repellent	*Cx. pipiens, Cx. quinquefasciatus*	Angelon *et al*., 2002
- *Notonecta* sp. (water bug)	Unknown	Repellent	*An. gambiae*	Munga *et al.,* 2006
Infusions				
- Bermuda grass/hay	3-methyl-indole (skatole)	Attractant	*Cx. quinquefasciatus, Cx. tarsalis, Cx. stigmatosoma*	Millar *et al*., 1992, Beehler *et al.*, 1994 Beehler *et al.*, 1994
- Decayed wood	*p*-cresol	Attractant	*Ae. triseriatus*	Bentley *et al.*, 1982
	4-methyl-cyclohexa-nol		*Ae. triseriatus*	Bentley *et al.*, 1982
- Decomposing plants	Unknown	Attractant	*Cx. restuans*	Reiskind and Wilson, 2004

In contrast to the Culicines, only few published records of semiochemicals in the oviposition behaviour of Anophelines could be found.

There are indications that *An. gambiae* females also uses chemical cues to assess the larval habitat. Blackwell and Johnson (2000) gave the first indication that chemoattractants may have an important role in the oviposition behaviour of females, based on electroantennogram response (EAG) studies. Furthermore, Sumba *et al.* (2004) showed that the oviposition behaviour is influenced by the abundance, or absence, of living microorganisms in the water or soil.

McRae (1984) showed that mosquitoes were repelled by larvae, but did not mention the larval stage that was tested. This repellent effect was also shown by Munga *et al* (2006). More eggs were laid in cups containing untreated rain water or rain water treated with few L2 larvae, than in cups containing rain water treated with 50 second instars. Mwingira *et al*. (in prep.), also showed that that gravid females are repelled by fourth instars, but they also proved that gravid females of *An. gambiae* are attracted towards water containing first instars.

Cannibalism occurs between larvae when there is a great difference in age between the larvae (Koenraadt & Takken, 2003; Koenraadt *et al*., 2004). Females seem to be able to assess the age of the larvae in a particular larval habitat, and react to it by ovipositing in habitats with none or young larvae, enhancing the survival of their offspring and the females fitness.

So, the fitness of adult mosquitoes is influenced by the conditions of the habitat of the larval stage. A very high larval density, for example, leads to a high intraspecific competition leading to a longer development time, reduced pupation success and reduced pupal weight (Clements, 1999), resulting in smaller females.

It is known that small females have a reduced fitness, because they can only contribute to the next generation after they have had a blood meal, which they need to build up reserves for survival. So, small females need at least two blood meals to complete ovarian development. Large females can use most of the blood meal for the development of the eggs, because they have emerged with sufficient reserves to survive the first days after emergence (Takken *et al.*, 1998).

The survival of small females is lower than that of large females when kept with access to water only. Next to a lower survival, small females show a reduced host-seeking response, compared to large females (Takken *et al.*, 1998). The effects of adult female body size on responses to ovipositional cues are yet not known, but may be important for the ability to assess the quality of the oviposition habitat and the survival of the larvae. Based on the preliminary evidence of semiochemicals affecting oviposition of *An. gambiae* a detailed study of these effects has been designed and is described below.

2. Research questions and Hypotheses

2.1 Research questions

1. Does age structure of the larval population affect oviposition of adult females of *Anopheles gambiae*?
2. What is the effect of body size on oviposition behaviour of *Anopheles gambiae* females?

2.2 Hypotheses

1. Females are attracted to L1 larvae and deterred by L4 larvae. When both larval stages are present in one cup, females will choose the most suitable cup for the survival of the larvae. They will avoid ovipositioning in cups containing L4 larvae. When both cups contain L4 larvae, and one of them also has L1 larvae present, mosquitoes will oviposit in the latter cup.
2. Smaller females have a lower survival rate and lower fecundity, therefore they cannot be as selective as larger females in their oviposition site and will respond less specific to the chemical cues they receive. Meaning that they will oviposit in less favourable habitats more often than larger females.

3. Materials & Methods

3.1 Age structure

Previous research has shown that gravid females are attracted to cups with tap water containing 100 L1 larvae en are repelled by cups with 50 L4 larvae. But the effects of a mixed age structure on oviposition are unknown.

Oviposition behaviour was investigated using 7 treatments:

1. Control (tap water) vs. Control
2. 100 L1 vs. Control
3. 50 L4 vs. Control

3b. 100 L4 vs. Control

4. 100 L1 + 50 L4 vs. Control
5. 100 L1 + 50 L4 vs. 100 L1
6. 100 L1 + 50 L4 vs. 50 L4

Treatments 1, 2 and 3(b) were designed as control treatments to determine whether females had a preference to oviposit in plain tap water, or water containing L1 or L4 larvae, and whether the addition of larvae to the cups containing water rendered these oviposition sites attractive or repellent.

Treatments 4, 5 and 6 were designed to determine the effects of a mixed age structure of larvae in an oviposition site.

The treatments were run simultaneously, as much as possible.

9-days old females were fed blood on a human arm for ten minutes, two days before the start of the experiment and were kept on a 6% glucose solution.

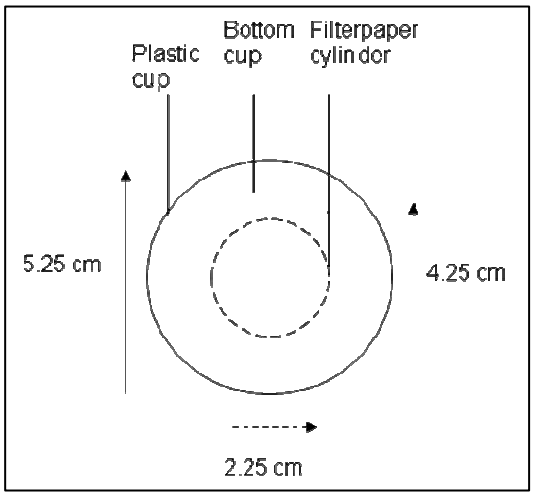

Figure 1: Top view of a oviposition cup, with a filter paper cylinder.

One gravid mosquito was held in a Bugdorm® cage of 30x30x30 cm, for 48 hours. The cages were kept in a climate cell with a temperature of 28°C and a 80% relative humidity, under a 12-h-light:12-h-dark photo cycle.

A small bottle with a 6% glucose solution, containing a long piece of filter paper sticking out, was placed in the cage, as a food source.

Each cage was provided with a pair of oviposition cups (5.25 cm diameter x 3 cm height), containing 30 ml of tap water with or without larvae. On top of the cup wet filter paper was placed, which acted as an oviposition site for the mosquitoes. To prevent drying out of the oviposition papers, a cylinder, made of filter paper, was placed in the cups (Figs. 1 and 2). This cylinder ensured that when the water level in the cup decreased, the oviposition paper remained wet, because water elevated through this cylinder to the oviposition paper.

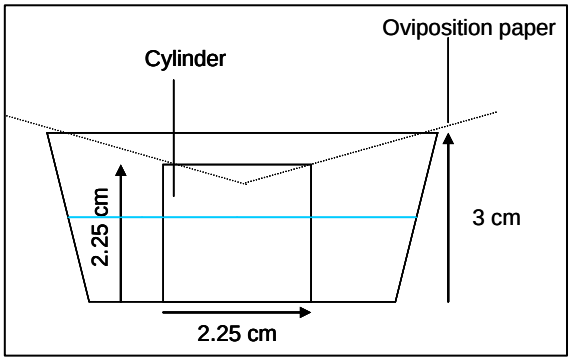

Figure 2: Side view of oviposition cup, containing a filter paper cylinder and oviposition paper.

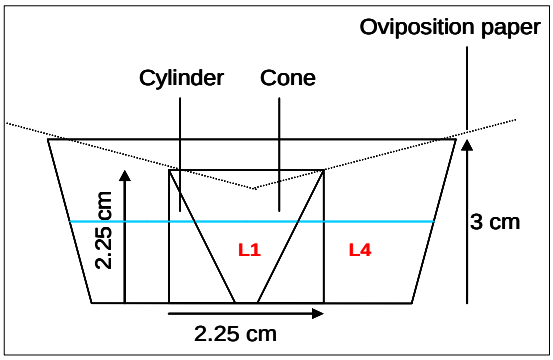

Figure 3: Side view of oviposition cup, containing an oviposition paper, filter paper cylinder and a filter paper cone, used for mixed populations to prevent cannibalism.

To prevent cannibalism between the different larval stages, it was necessary to separate these larvae within the same water body. The filter paper cylinder was not sufficient to keep the larvae from the different stages separated, the small L1 larvae were able to swim underneath the cylinder, and a filter paper cone was placed inside it. This cone prevented the L1 larvae to escape to the surrounding water, and was kept in place by the cylinder (Fig. 3).

Larvae from the different treatments from the same stage were kept in the same part of the cup, within the cylinder or outside.

Each cup was placed diagonally in the opposite side of the cage, with a distance as far as possible from the other cup (30 cm). The position of the cups was altered at each replicate (Fig. 4).

After 24 and 48 hours the eggs were counted, as well as larvae that pupated. When after 24 hours larvae did pupate, the pupae were removed and replaced by new L4 larvae.

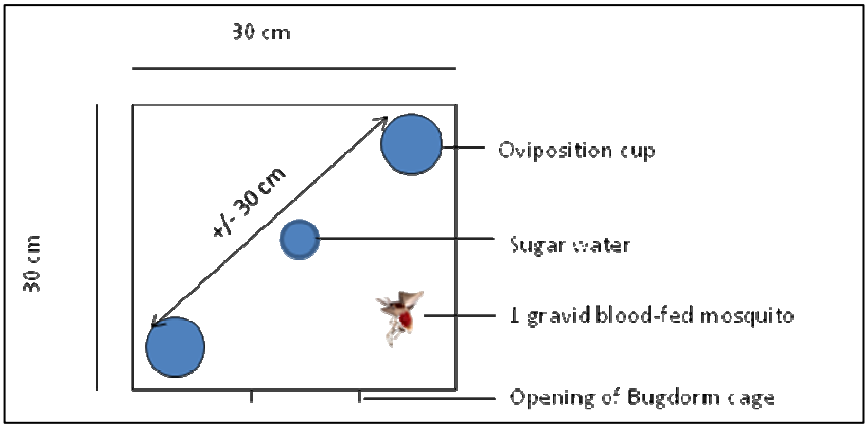

Figure 4: Top view of experimental set up. Inside a Bugdorm cage of 30x30x30 cm, 2 ovipostion cups were placed at a distance of approximately 30 cm. In the centre of the cage a bottle filled with a 6% glucose solution was placed. One gravid female was kept in the cage for 48 hours.

Directly after the experiments, females were immobilized by putting them in the freezer for 45 – 60 minutes. The right wings of the females were removed and measured, for there is a strong correlation between wing length and body size (Lyimo et Koella, 1992). The wings were placed between two glass plates, on top of a drop of nail polish. Wing lengths were measured from the distal end of the alula to the tip of the wing, excluding the outer scales. The various stages of development of the ovarian follicles of the females (Christophers' stages I-V) were recorded, as well as the number of retained eggs (stage V). Therefore, the ovaries were extracted according to the classical method described in 'Manual on Practical Entomology in Malaria' (WHO, 1975).

9-days old female mosquitoes were taken from the research population. Eggs were provided, or obtained after feeding the mosquitoes by providing them blood from an arm. Larvae were reared after hatching of the eggs.

3.2 Body size

Adults of different body sizes were obtained by rearing larvae with different food densities. To obtain large females 200 first instars were kept in standard rearing trays containing 1 litre of tap water. The larvae were fed 0.05 mg of Tetramin® (Tetra Werke, Germany) per larva per day, for the first two days. Subsequently, the larvae were fed 0.1 mg of Tetramin® per larva per day, for two days. After this period larvae were fed 0.3mg of Tetramin® per larva per day during the remaining period of

development. Small females were obtained by keeping 200 first instars in 1 litre of water in standard rearing trays while feeding them 0.05 mg of Tetramin® per larva per day, for four days and 0.1 mg Tetramin® per larva per day during the remaining period of development. Based on Takken *et al*. (1998), a short pilot has been conducted to determine the quantity of food given to the larvae.

Pupae were placed in cages assigned for 'large' and 'small' mosquitoes. Pupae from the first three days were used for the large females. While for the small females, pupae that pupated 6 to 7 days after the first pupae emerged, were used. This was done to ascertain that the larvae actually did have a food shortage during the development, compared to larvae provided with sufficient food.

After emergence, mosquitoes were kept in a 30*30*30 Bugdorm® cage. 7-9 days old mosquitoes were blood fed on a human arm for ten minutes. Large mosquitoes were only blood fed once, while small females were fed for a second time 48 h later, to ascertain the development of eggs.

One gravid mosquito was held in a Bugdorm® cage of 30x30x30 cm, for 48 hours. The cages were kept under the same conditions as in the age structure experiments. In this experiment only two treatments were tested. Gravid females were given the choice between a cup containing 100 L1 larvae and a control cup (Treatment 2) or between a cup containing 100 L4 larvae and a control cup (Treatment 3b).

Each cup was placed diagonally in the opposite side of the cage, with a distance as far as possible from its control, that is 30 cm. After 24 and 48 hours the eggs were counted.

After the experiments, females were immobilized by putting them in the freezer for 45 – 60 minutes. The right wings of all females were removed and measured and the development of the ovarian follicles has been recorded, as described above.

3.3 Chemical analysis

The chemical compounds released by water, water containing L1 larvae and water containing fourth instars were collected and analysed. Cups filled with tap water and water with larvae were placed in a separate air-tight cuvette.

Volatiles were entrained using the "purge and trap" approach on an adsorbing polymer, Tenax-TA 20/35 (Alltech, USA). To reduce background volatiles, air was sucked into the cuvette through a carbon filter and a cartridge containing 100 mg

Tenax-TA. Headspace volatiles were entrained at a flow rate of 100 ml/min for 24 hours on a second cartridge containing 100 mg Tenax TA connected to the outlet of the cuvette. Samples were analyzed using thermodesorption followed by gas chromatography - mass spectrometry (GC-MS), on a Markes International Ltd/Thermo Scientific system consisting of a thermal desorption autosampler (Ultra 50:50 TD, Markes, USA), electrically cooled cold trap for focusing (Unity, Markes), flow controller (air server, Markes) for thermal desorption injection into a Trace GC Ultra (Thermo Scientific) coupled to a quadrupole mass detector (Trace DSQ, Thermo Scientific).

The program for thermal desorption consisted of a 3 min He dry purge (residual moist removal), and 1 min He prepurge (residual oxygen removal) both at 30℃. Tube desorption was performed at 250℃ for 3 minutes and the volatiles were focused on a cold trap (general purpose hydrophobic, Markes) at 0℃. Injection onto the analytical column was achieved by heating of the cold trap at the maximum speed (>60℃/s) to 250℃ and splitting of the carrier gas (He) resulting in an injection of 1/6 of the total amount. The transfer line between the cold trap and the GC was kept at 160℃.

A 30 m x 0.25 mm ID x 1.0 µm capillary GC column (Rtx-5 MS, Restek, USA) with He (5.0 grade) as carrier gas at a flow rate of 1.0 mL/min was used. The GC temperature was programmed as follows: 3 minutes at 45℃ followed by a ramp of 8℃/min to 280℃ and a 2 minute hold at 280℃, the transfer line between the GC and MS was kept at 275℃.

MS-spectra were recorded by ionization of the column effluent by electron impact (EI) ionization at 70 eV, scanning in positive mode from 35-300 *m/z* with a scan speed of 5 scans/s and an ion source temperature of 250℃. The filament was switched off from 13.6-13.8 min because of a high background peak.

Peak identification was performed using Xcalibur software (Thermo Electron Corporation, San Jose, USA). Comparison of the obtained spectra was performed with the NIST library (version 2.0 d), using calculated and reported retention indices and injection of authentic synthetic reference compounds.

Tenax-TA cartridges were conditioned with the TC-20 Tube Conditioner (Markes, UK) by heating for 1 hour at 320℃ under a flow of He at 60 mL/min.

A semi-quantitative analysis was performed. Peak surface estimates performed using the software Xcalibur (Thermo Electron Corporation, San Jose, USA). This analysis was done on 16 compounds, of which selection was based on the graphs showing the relative abundance of these chemical compounds. Chemical

compounds that seemed to show differences in peak surface between the three different cups were selected (see Figs. 5a and 5b for some examples).

It was not possible to add a standard chemical to the cups, because a chemical that could be added to the water and was gradually emitted from the water to the headspace for 24 hours was not available. To comply with the provision of a standard, 4-methylheptane was found to be abundant in equal amounts in all three cups, and was therefore used as an internal standard. Data was corrected for aberrations, using this internal standard. If, after this correction, series were still aberrant, they were left out of the analysis. The treatments have been performed for six times.

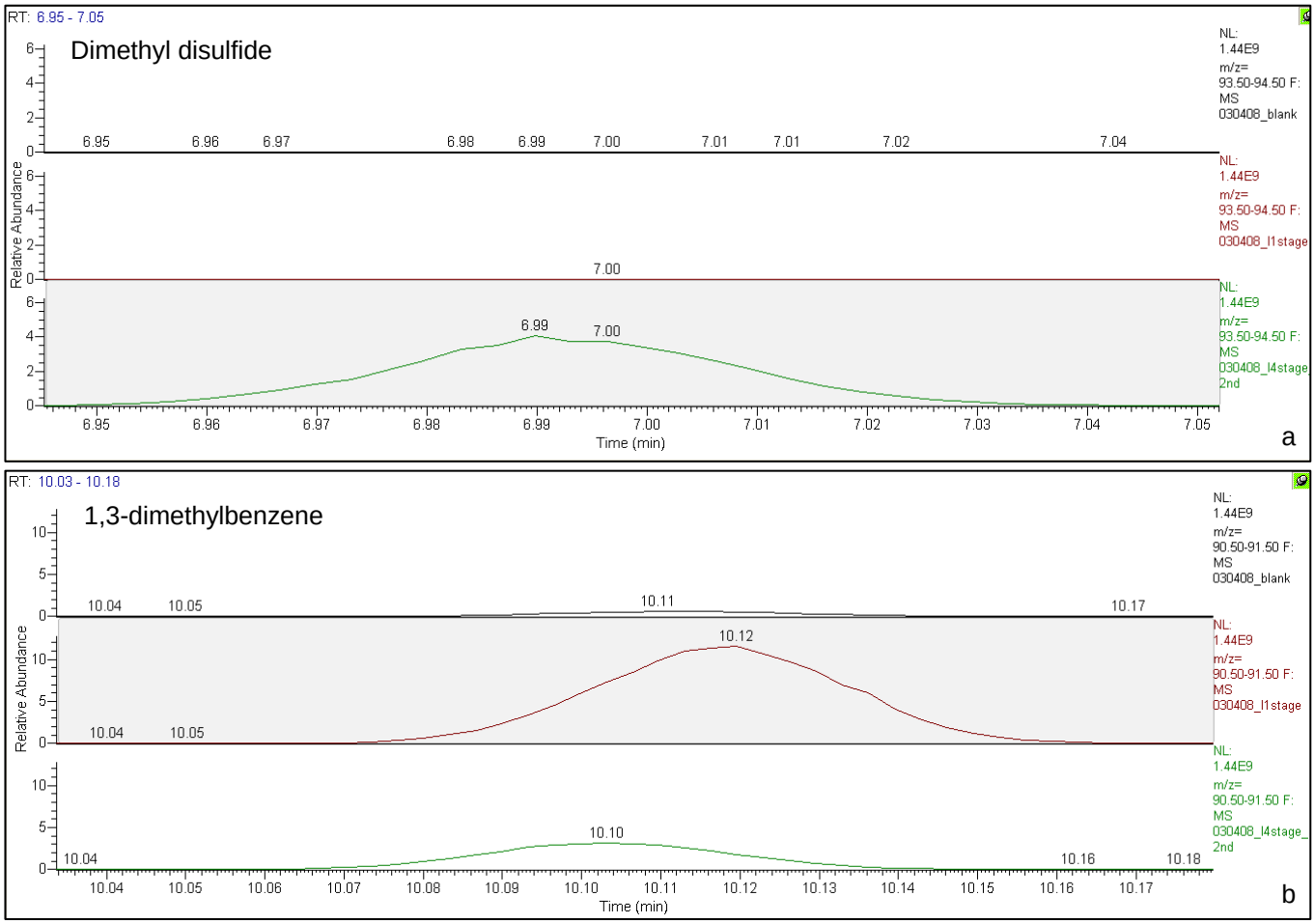

Figure 5a - 5b: Examples of GC-MS of a) dimethyl disulfide and b) 1,3-dimethylbenzene. Selection of the 16 chemicals is based on differences in peak surfaces. The upper black graph shows the relative abundance of the specific chemical in cups containing water, the middle red graph and the lower green graph show the relative abundance of the specific chemical in water containing L1 larvae and water containing L4 larvae, respectively.
In this treatment dimethyl disulfide was abundant more in cups containing water and L4 larvae than in the other cups. 1,3-dimethylbenzene was abundant more in cups containing water and L1 larvae, than in the other cups.

3.4 Statistical analysis

For the statistical analysis the following tests have been performed: the Wilcoxon signed rank test for the differences in oviposition preference of the mosquitoes. A linear regression, for the relation between wing length and number of eggs. A Mann Whitney U test was used to test the differences in wing length of three different size groups and the wing lengths of mosquitoes spreading their eggs, and to test the differences between chemical compounds. All tests were performed using SPSS 15.0 for Windows.

4. Results

4.1 Age structure

When the different larval stages in the mixed age experiments were not separated, the rate of cannibalism was very high. After 24h, between 90 and 100% of the L1 larvae were eaten by the larger larvae. Another 24 h later, there were no L1 larvae left. Therefore, the larvae were separated within the water body, using filtration paper (as described in the Materials and Methods section). It was assumed that chemicals could freely pass the paper membrane. In this arrangement almost all larvae survived.

Not all mosquitoes oviposited. In treatment 1 nine of the fifteen mosquitoes laid eggs, for treatment 2 this proportion was ten out of eighteen. In the remaining treatments the amounts of egg-laying mosquitoes were: twelve out of eighteen, eleven out of fourteen, twelve out of fifteen and eleven out of eleven for treatments 3, 4, 5 and 6, respectively.

Mosquitoes did not always deposit all developed eggs, often mature eggs were found in the ovaries of mosquitoes that had laid eggs (see Appendix for further details). Furthermore, they did not always lay developed eggs at all, probably because they were not inseminated. Unfertilized eggs do not turn black. Not all mosquitoes were able to finish the development of the eggs, resulting in a gonotrophic stage described as the second stage of Christopher's stages of egg development (WHO, 1975). These mosquitoes suffered a blood shortage, limiting the development of the eggs.

Figures 6a – 6g show the boxplots of the number of eggs laid in the different cups. Mosquitoes oviposited randomly when two control cups were provided with an average of 14.1 to 17.9 eggs per cup (Treatment 1, P= 0.767, Wilcoxon signed rank test). In this treatment, spreading of the eggs over the two cups was rare and almost all eggs were placed in one of the two cups.

The mosquitoes were attracted towards L1 larvae (Treatment 2); significantly more eggs were laid in the cups containing 100 L1 larvae than in the control cup (P= 0.013, Wilcoxon signed rank test). Although more eggs were deposited in the control cups, compared to cups containing 50 L4 larvae (Treatment 3), this difference was not significant (P= 0.505, Wilcoxon signed rank test). When the number of L4 larvae was doubled, the mosquitoes placed significantly more eggs in the control cup (P= 0.017, Wilcoxon signed rank test).

The differences in the choice of oviposition site in the remaining treatments were not significant (Treatment 4: P= 0.423, Treatment 5: P= 0.530, Treatment 6: P= 0.374, Wilcoxon signed rank test). However, there seemed to be a trend in which mosquitoes were deterred by a mixed population containing L1 and L4 larvae, when the other cups contained no L4 larvae (Figs. 6e, 6f and 6g).

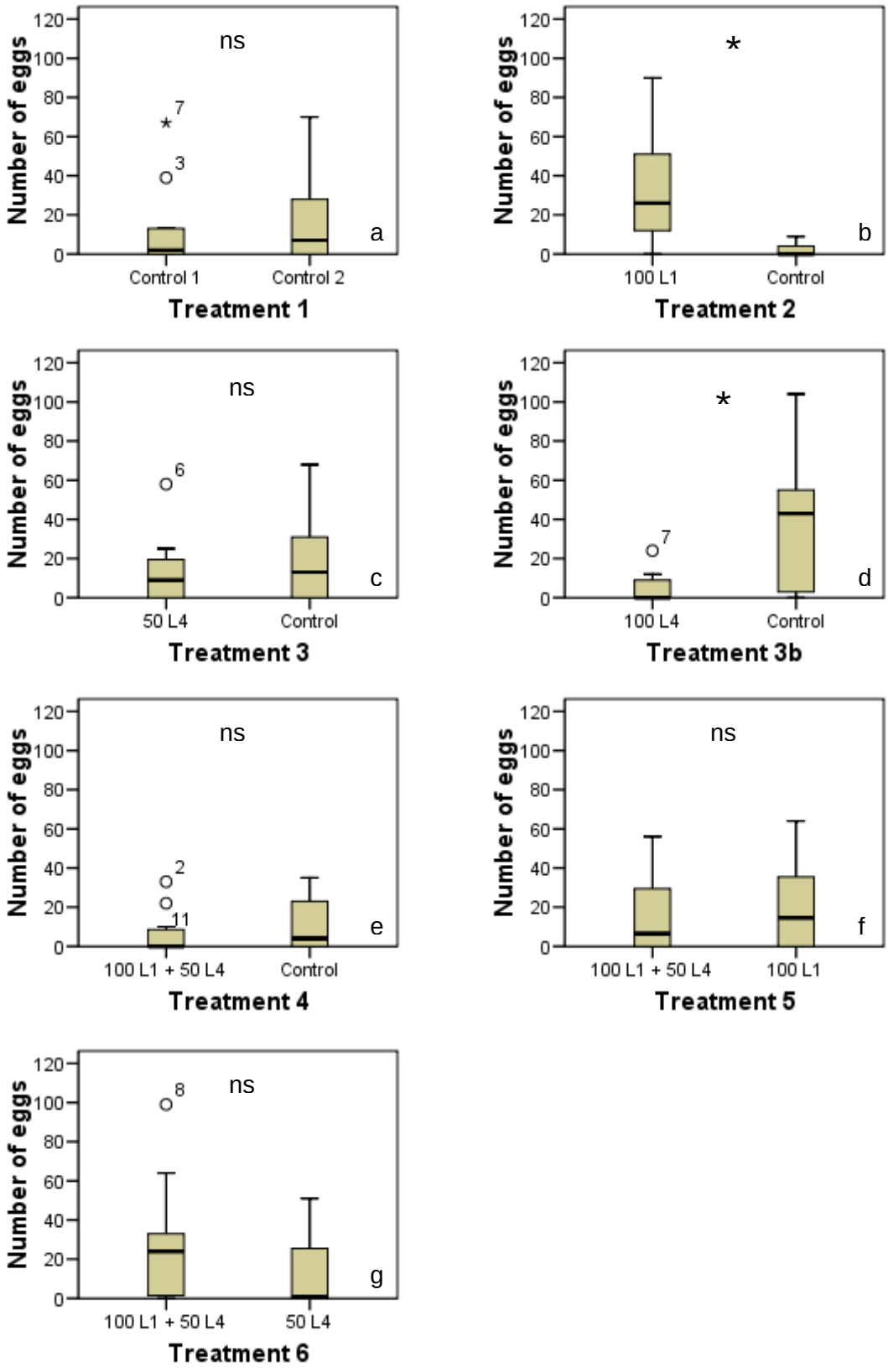

Figure 6a - 6g: Boxplots of number of eggs laid in the cups, showing the median (fat line), first and third quartiles and the maximum and minimum of the amount of eggs laid.
Treatment 1: containing only tap water (control) (N=9); Treatment 2: a cup containing 100 L1 and a control (N=10); Treatment 3: cup containing 50 L4 and a control (N=12); Treatment 3b: 100 L4 larvae versus control cup (N=14); Treatment 4: one cup with 100 L1 and 50 L4 and a control cup (N=11); Treatment 5: 100 L1 and 50 L4 versus 100 L1 (N=12); Treatment 6: 100 L1 and 50 L4 versus 50 L4 larvae (N=11).
** indicates a significant difference, with α= 0.05. Ns means a significant difference was not found.*

The wing length of the mosquitoes was taken as a fitness-related factor, and was related to aspects concerning oviposition.

The variation in wing length between the different individuals was large. There was a clear correlation between the amount of eggs that were produced and the wing length (R^2= 0.42, *P*< 0.001, linear regression) (Fig. 7). Smaller mosquitoes produced fewer eggs than did the larger ones.

When only eggs were counted that were laid by the female mosquitoes, there still was a strong correlation between wing length and amount of eggs (R^2= 0.31, *P*< 0.001, linear regression).

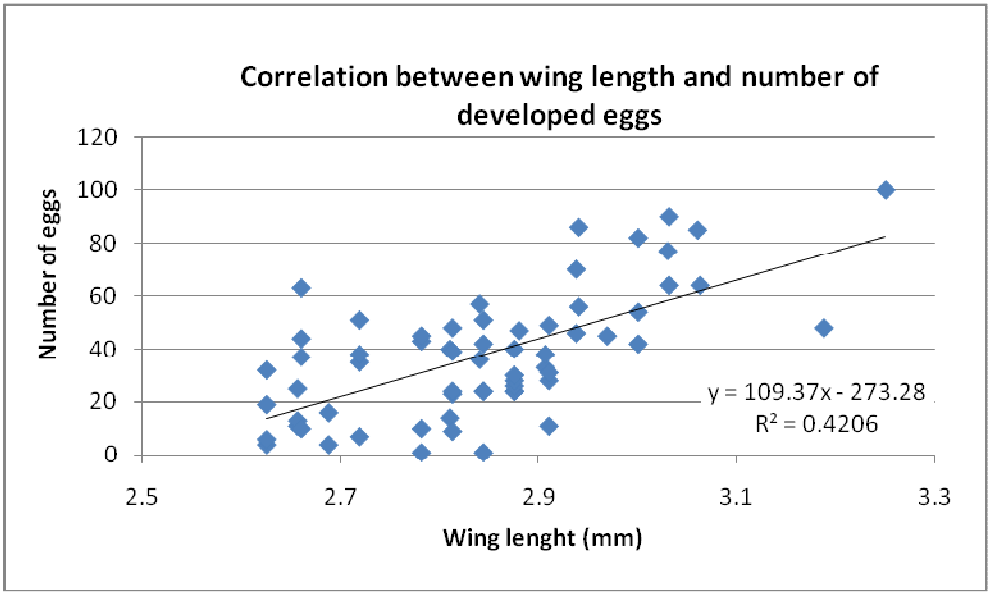

Figure 7: Correlation between wing length and number of developed eggs of the individual females. N=63.

Based on these, and the results of the research of Mwingira *et al*. (in prep.), it is concluded that mosquitoes prefer to lay their eggs in a cup containing 100 L1 larvae, when they have to choose between this cup and a control cup (Treatment 2). When both a control cup and a cup containing 100 L4 larvae is provided, mosquitoes mostly lay their eggs in the control cup and are deterred by the L4 larvae (Treatment 3b). Although this difference was significant in this study, there were mosquitoes that did not respond in agreement to the hypothesis that gravid female mosquitoes are attracted towards oviposition sites containing L1 larvae and are deterred by sites containing fourth instars.

When mosquitoes were selected based on their response regarding this hypothesis, mosquitoes could be assigned to three different groups, i.e. a group of mosquitoes that did not respond in agreement with the hypothesis, a group that did

respond in agreement with this hypothesis and a group that could not be placed in the former groups, because they laid their eggs in both cups.

Mosquitoes that did not lay their eggs in the expected cup, were significantly smaller than the mosquitoes dividing the eggs (*P*= 0.034, Mann Whitney U), and the mosquitoes laying in the expected cups (*P*= 0.025, Mann Whitney U) (Fig. 8). The average wing lengths of the mosquitoes laying their eggs not in agreement with the hypothesis were 2.74 ± 0.04 mm. The average wing lengths of mosquitoes that spread their eggs over both cups and of the mosquitoes that laid their eggs in agreement with the hypothesis were 2.90 ± 0.03 mm and 2.91 ± 0.03 mm, respectively.

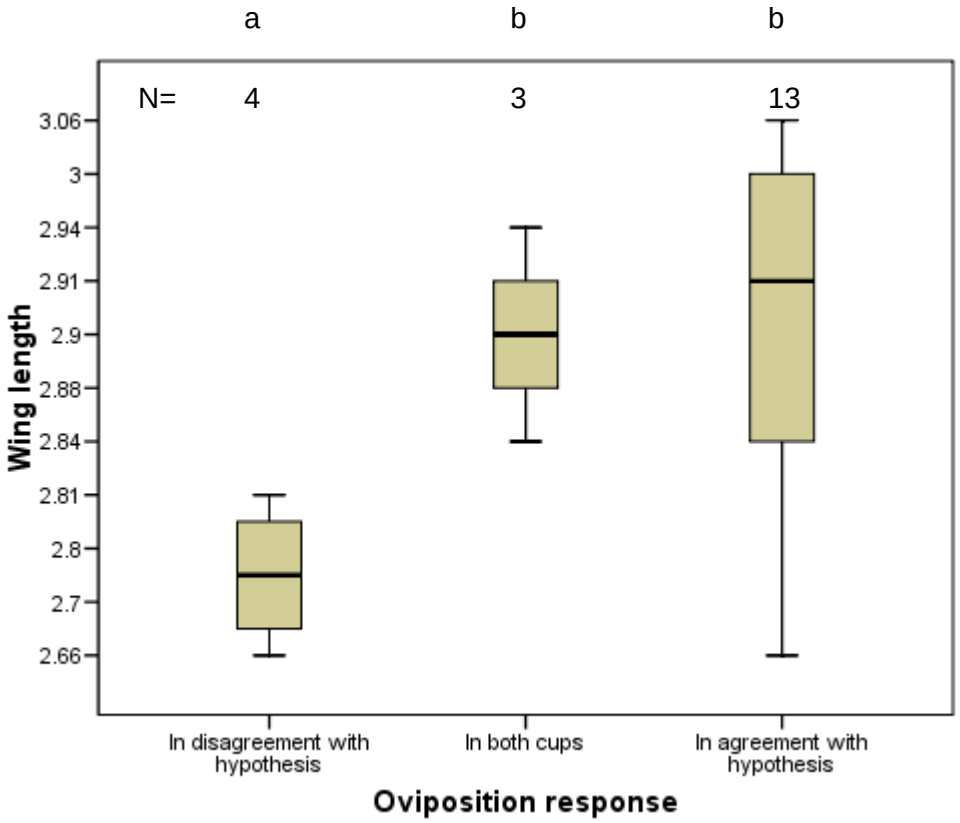

Figure 8: Boxplot of 3 groups of mosquitoes, showing the median (fat line), first and third quartiles and the maximum and minimum of the wing lengths of the females. Mosquitoes were grouped, based on their ovipositional response: In disagreement with the hypothesis, oviposition in both cups and in agreement with the hypothesis. The hypothesis was that gravid females were attracted towards cups containing L1 larvae and deterred by cups containing L4 larvae.
This analysis was done with the mosquitoes used in treatments 2 and 3b (N=20). Letters above boxes indicate significance of differences, different letters mean a significant difference. Numbers represent the number of females present in the specific group.

4.2 Body size

The mosquitoes that have had a malnutrition were significantly smaller than both the mosquitoes that received much food (P< 0.001) and non-selected mosquitoes that were used in the age-structure experiments (P< 0.001). Furthermore, the mosquitoes used in the age-structure experiments were significantly smaller than the mosquitoes that have had sufficient food to grow well (P< 0.001).

Because of these differences, the mosquitoes with different nutritional backgrounds could be classified as being ‘Small’, ‘Large’ and ‘Medium sized’ (Fig. 9). The average wing lengths ± SD of the groups were 2.63 ± 0.12 mm for the small mosquitoes, 3.10 ± 0.17 mm for the large mosquitoes and 2.88 ± 0.13 mm for the medium sized mosquitoes, ranging wider than the mosquitoes reared by Suwanchaichinda and Paskewitz (1998), whose wing lengths of small, medium-sized and large mosquitoes were 2.68 ± 0.08, 2.83 ± 0.12 and 3.00 ± 0.10 mm, respectively.

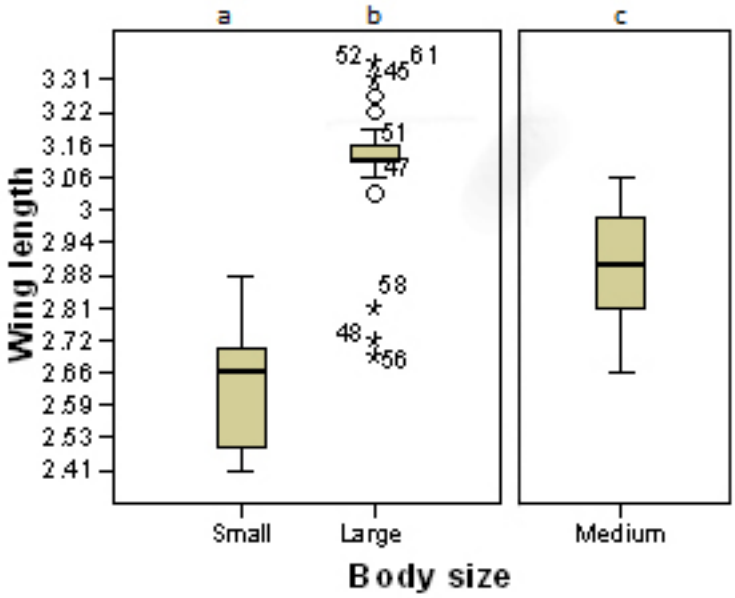

Figure 9: Wing lengths of mosquitoes, differing in larval food availability. Boxplots show the median (fat line), first and third quartiles and the maximum and minimum of the wing lengths of the mosquitoes. Small mosquitoes (N=20) have had a malnutrition, large mosquitoes (N=21) have had sufficient food, medium-sized mosquitoes (N=20) were the non-selected mosquitoes used in the age-structure experiments.
Different letters above graph indicate significant difference (Mann Whitney U test).

There was no difference in the amount of eggs laid in the cups when small mosquitoes had a choice between a control and a cup with 100 L1 larvae (*P*= 0.612) and between a control and a cup with 100 L4 larvae (*P*= 0.338).

Large mosquitoes showed a similar response. There was no difference in the number of eggs between a control cup and a cup containing 100 first instars (*P*= 0.612) or a control cup and a cup containing 100 L4 larvae (*P*= 0.583) (See Figs. 10a - 10f). The mosquitoes of medium size did show significant differences, as has been shown in the age-structure experiments.

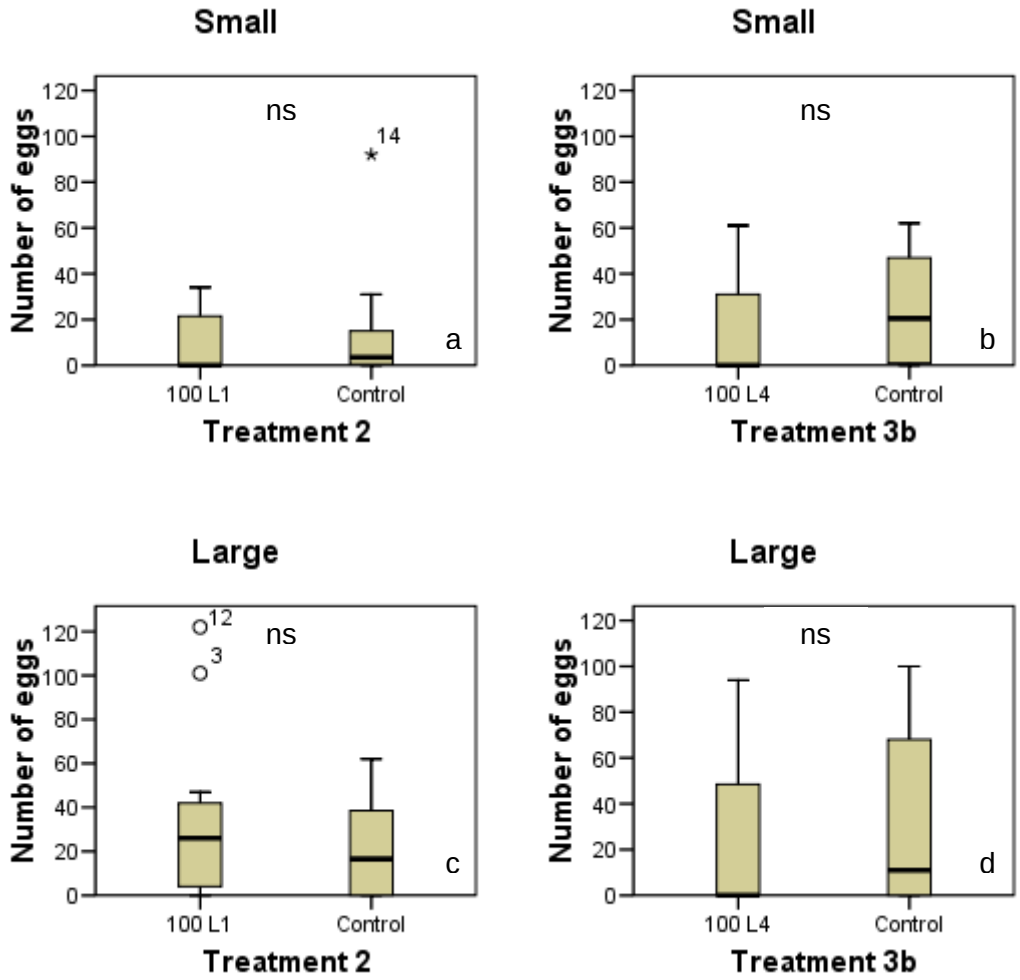

Figure 10a - 10d: The median (fat line), first and third quartiles and the maximum and minimum of the number of eggs laid in the cups containing larvae of different larval stages, for small (a; N=12 and b; N=12) and large mosquitoes (c; N=12 and d; N=12). Ns indicates there was no difference in the amount of eggs laid in the cups (Wilcoxon signed rank test).

Based on the results from Mwingira *et al*. and from the age-structure experiment, it was hypothesized that mosquitoes were attracted towards 100 first instar larvae, and repelled by 100 fourth instar larvae. The response of mosquitoes that laid their eggs in the cup that was expected by this hypothesis was regarded as consistent with this hypothesis. The response of mosquitoes that did not oviposit in the expected cup, was regarded as inconsistent with the hypothesis. The response of mosquitoes that spread their eggs over both cups was regarded as division of their eggs.

When the 'Small', 'Medium' and 'Large' mosquitoes were divided into wing length classes of 0.1 mm, and the proportions of the mosquitoes showing a specific response were plotted, there was a distribution in which there was an optimum in wing length, regarding the mosquitoes that oviposited in consistency with the hypothesis. This optimum length was found in the wing length class of 2.9 – 2.99 mm, close to the average wing length of the medium sized mosquitoes. The proportion of mosquitoes that did not oviposit in consistency with the hypothesis was highest in the smallest wing length classes (Fig. 11).

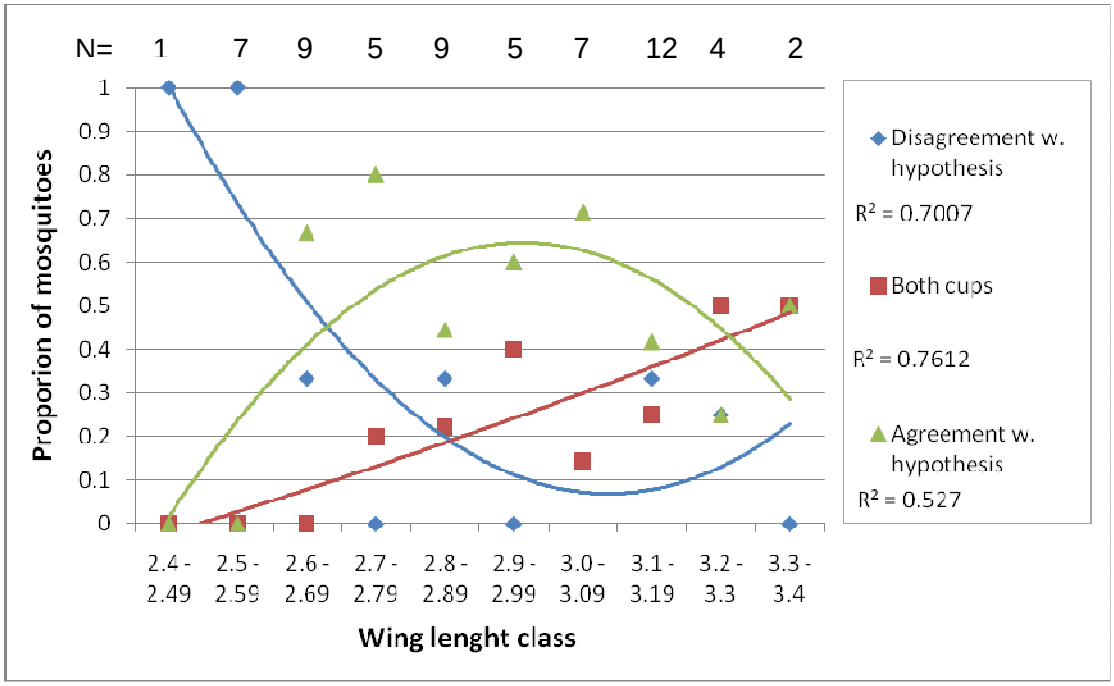

Figure 11: Proportion of mosquitoes in a wing length class that show an ovipositional response in disagreement or agreement with the hypothesis, or spread their eggs over both cups. The hypothesis was that gravid females were attracted towards cups containing L1 larvae and deterred by cups containing L4 larvae. Numbers above graph show the number of mosquitoes in a wing length class. Curve estimation regression with a quadratic model has been performed to obtain significances of curves.

The spreading of the eggs occurred by mosquitoes with a wing length of at least 2.7 mm. There was a positive correlation between wing length class and the proportion of mosquitoes spreading their eggs. The significances of the curve estimations were 0.015 for the curve of mosquitoes in disagreement with the hypothesis, 0.007 for the curve of mosquitoes that oviposited in both cups and 0.073 for the curve of mosquitoes that showed a response in agreement with the hypothesis.

Females that spread their eggs over both cups were larger than females that laid their eggs in one cup only (*P*= 0.010, Mann Whitney U test) (Fig. 12).

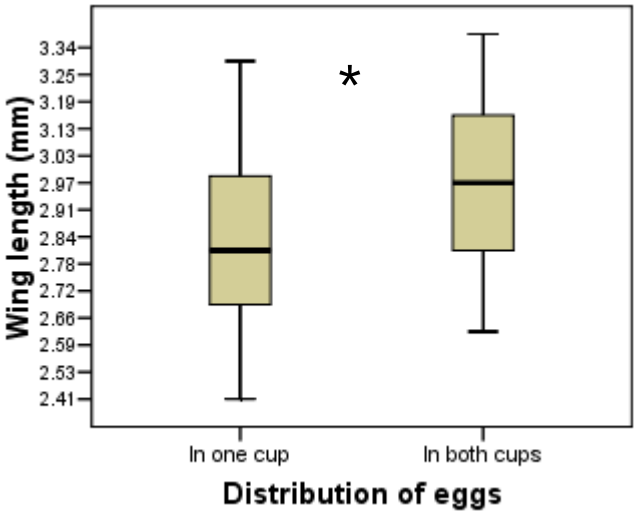

*Figure 12: Distribution of eggs laid in one (N=84) or in both cups (N=22) versus wing length. Females of both the age structure experiments and body size experiments are included in analysis. * indicates a significant difference, with α= 0.05, Mann Whitney U test.*

4.3 Chemical analysis

From the chemical analysis of the headspace collections for developing larvae, 16 compounds appeared of interest, as they were relatively more abundant in treated water compared to water controls (Fig. 13).

Of the 16 compounds tested, 4 were of interest (Table 2). Dimethyl disulfide and dimethyl trisulfide (Figs. 14a and 14b) were found significantly more in cups containing L4 larvae than in both the control and cups containing L1 larvae. Both nonane and 2,4-pentanedione were found more in cups containing larvae, irrespective of larval stage, although these differences were not significant for nonane (Figs. 14c and 14d).

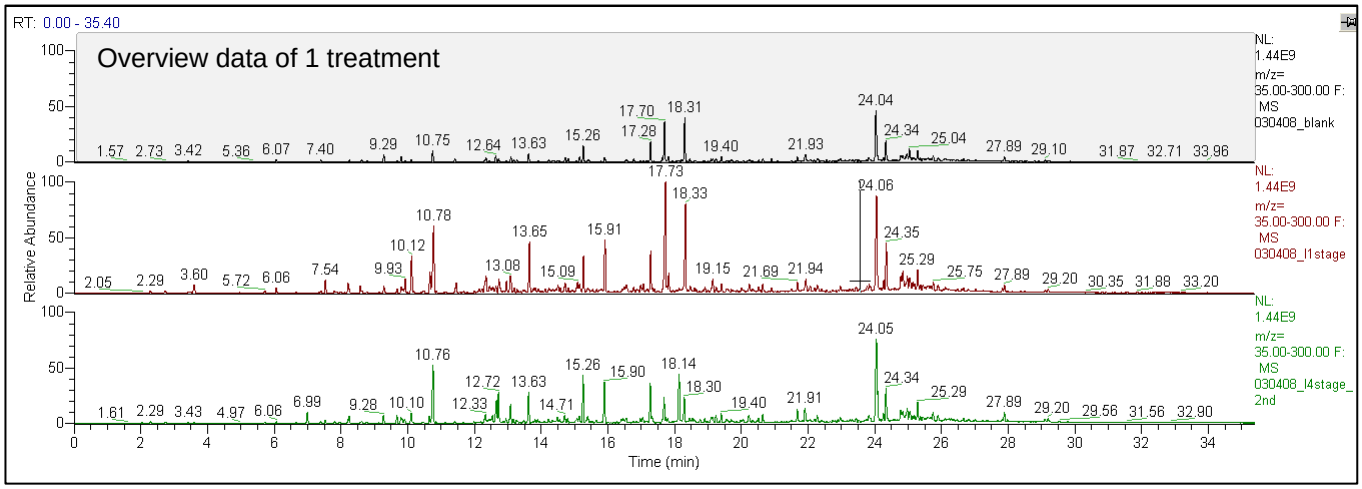

Figure 13: GC-MS profiles of an overview of the profiles from water (upper black graph), water with L1 larvae (middle red graph) and water with 4th instar larvae (lower green graph), from one replicate (see Appendix for more details). Notice the differences in peaks between the profiles of cups containing water only and the profiles of cups containing water and larvae.

*Table 2: Significances of semi-quantitative analysis of chemicals emitted by a control (tap water), L1 larvae and L4 larvae. Chemicals of interest for further study are marked with *.*

	Control - L1	Control - L4	L1 - L4
Hexane	0.602	0.917	0.754
2-methyl-2-propenoic acid methyl ester	0.465	0.754	0.465
Dimethyl disulfide *	**0.175**	**0.016**	**0.047**
Toluene	0.754	0.917	0.917
Acetic acid buthylester	0.754	0.754	0.754
Ethylbenzene	0.754	0.917	0.917
1,3-dimethylbenzene	0.917	0.917	0.754
Nonane *	**0.113**	**0.245**	**0.834**
2,2-dimethyl-1,3-propanediol	0.465	0.917	0.465
Dimehyl trisulfide *	**0.175**	**0.009**	**0.009**
2-ethyl-1-hexanol	0.917	0.917	0.917
Itaconic acid diethylester	0.347	0.917	0.465
Diethylethylidene malonate	0.465	0.917	0.215
Benzothiazole	0.251	0.602	0.465
Ethylcitrate	0.465	0.602	0.745
2,4-pentanedione *	**0.076**	**0.047**	**0.917**

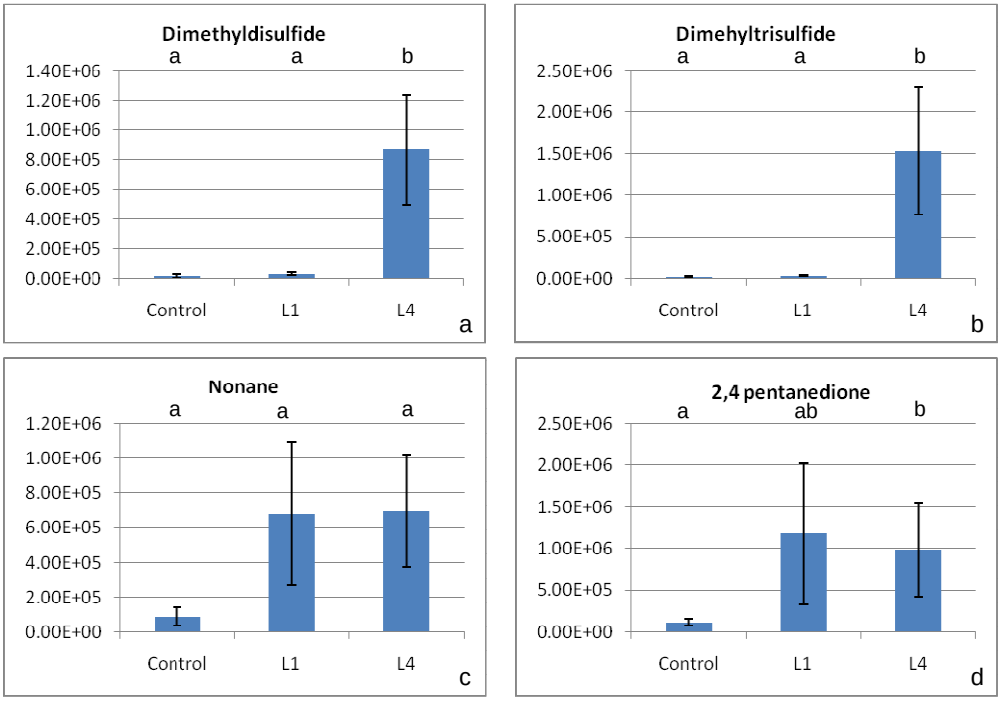

Figure 14a - 14d: Graphs of averages ± S.E. of relative abundance of chemical compounds interesting for further study; a) dimethyl disulfide, b) dimethyl trisulfide, c) nonane, d) 2,4-pentanedione. Different letters above bars indicate significant difference in relative abundance was found (Mann Whitney U test). Note that graphs can only be used to compare differences of chemical compounds between treatments, not between chemicals.

5. Discussion

Gravid female mosquitoes are capable of discriminating between oviposition sites with or without larvae. Oviposition cups containing young larvae are more attractive than cups containing no larvae and cups containing old larvae are deterrent. This result is in consistency with the results shown by Mwingira *et al*., but is in contradiction to the statement of McCall *et al*. (1995), who state that anophelines do not show ovipositional aggregation behaviour. This research provides strong support for the hypothesis that the oviposition behaviour must be chemically mediated as the females could not see the larvae, which were hidden from view by filter paper.

A high density of young larvae may indicate a suitable site, that has been assessed by other females before. Furthermore, it might be a strategy to have large number of larvae in case the habitat becomes less suitable, for example when predators enter the site. The chance of a larva of a specific female being eaten is smaller when there is a high amount of larvae from other mosquitoes. It might also be a strategy to induce mass adult emergence, which could assist mate selection in species where males form swarms (McCall *et al.*, 1995), like *Anopheles gambiae.*

Older larvae are known to cannibalize the smaller individuals (Koenraadt & Takken, 2003; Koenraadt *et al*., 2004). Furthermore, the competition with the L4 larvae might be too difficult for the first instars to survive. So, the chance of survival for the offspring of the ovipositing mosquitoes is larger in waters where fourth instars are absent. The results from Munga *et al.* (2006) suggest that conspecific larvae are deterrent as soon as they reach their second larval stage, indicating that larvae of only a couple of days older render the oviposition site unfavourable.

Even though mosquitoes did not discriminate between waters containing 50 L4 larvae and a control, the addition of 50 L4 larvae to waters containing 100 L1 larvae altered the behaviour of the mosquitoes. They were not attracted towards water containing L1 larvae, which they were when only L1 larvae were present.

Furthermore, the chemical compounds released by the L1 larvae seemed to overrule the compounds released by the older larvae. While mosquitoes preferred to lay eggs in cups containing L1 larvae when a control cup was also provided, the mosquitoes did not show this behaviour for the cup containing 100 L1 larvae when in the other cup 50 L4 larvae were put together with 100 L1 larvae.

When gravid females were given the choice between a cup containing both larval stages and a cup with L4 larvae, most eggs were laid in the cup containing L1

as well as L4 larvae, suggesting that the mosquitoes choose for the less unsuitable cup, although this difference was not significant.

These results suggest that females seem to have trouble in assessing the suitability of the habitat when contradicting odours are emitted from the oviposition site. When in this experiment 100 L4 larvae are used, the trend in which mosquitoes avoided the L4 larvae will most likely push on.

The results from 50 fourth instars and a control cup were not in consistency with the results of Mwingira *et al*. This difference was probably caused by the oviposition paper that has been used in this experiment, which might have blocked part of the emitted odours.

In the age structure experiments, there is a difference in the degree in which mosquitoes translate the perception of ovipositional cues into behaviour. Whereas the larger mosquitoes in these medium-sized mosquitoes (average wing length of 2.90 mm) seem to make a deliberate choice regarding the oviposition site, smaller mosquitoes in this range (average wing length of 2.74 mm) seem to respond to the signals transmitted by the ovipositional source less frequently. This is in line with observed differences in host-seeking response, where smaller females respond less well to chemical cues than larger ones (Takken *et al*., 1998).

When next to these mosquitoes small and large mosquitoes are tested, the pattern in which small mosquitoes do not seem to respond to the chemical cues emitted by the larvae and larger mosquitoes seem to make a deliberate choice, is no longer visible. Neither small, nor large mosquitoes showed a difference in the number of eggs laid in the different cups. It was expected that there was a positive correlation between the size of the mosquitoes and their response, like it was shown in the age structure experiment. Instead of a linear relation, there is a parabolic relation between wing length and response, with an optimum response at 2.90 mm. Close to the average wing size of medium sized mosquitoes (2.88 mm). The range (2.41 – 3.38) and average ± SD (2.89 ± 0.24) of wing lengths of mosquitoes reared in this study where comparable to the range (2.41 – 3.52) and average ± SD (2.92 ± 0.17) of a natural population of *An. gambiae* (Lyimo and Koella, 1992).

Small mosquitoes have a reduced survival, as compared to large mosquitoes (Takken *et al*., 1998; Fernandez & Briegel, 2004), and do not react towards host odours as well as large individuals (Takken *et al*., 1998). Small mosquitoes probably do not have enough energy reserves to be very selective in their oviposition choice. Furthermore, their survival is lower than of larger mosquitoes. So, it might be more advantageous to oviposit in any given site, instead of searching for a suitable site

with an increased chance of dying without ovipositing at all, when compared to larger mosquitoes. Another explanation for the diverged behaviour of small mosquitoes might be that because of the malnutrition their behavioural threshold towards odours is altered, as has been shown in bumblebees (Spaethe, 2007).

It is known that body size is a good parameter for several survival and fitness related factors (Takken *et al.*, 1998; Charlwood, 2003), which is also shown with the positive correlation between wing length and the number of eggs that have been produced and laid. Large mosquitoes develop more eggs than do the smaller individuals. Because of the previously shown relations between body size and factors related to fitness (Honek, 1993; Takken *et al.*, 1998; Charlwood, 2003), it was expected that the large mosquitoes would show a response that was in agreement with the hypothesis (that L1 larvae are attracting and L4 larvae deterring) the most, but this was not the case. An explanation of this behaviour might be that, because of the increased number of eggs in larger individuals, other factors play an important role in oviposition of large mosquitoes. When many eggs are produced, the spreading of eggs over more oviposition sites can be an advantage. If, for example, external factors alter the circumstances in one oviposition site, and the environment is no longer suitable for the abundant larvae. Females may still have surviving offspring, when they are present in another site, which initially might be less suitable than the former site.

Because small mosquitoes do not produce as many eggs as larger ones do, and because their survival and energy reserves are lower, the first blood meal is used for maternal energy supplementation (Lyimo and Takken, 1993), spreading their eggs may not be possible for them.

Possible chemicals affecting the oviposition behaviour of *Anopheles gambiae* were found to be dimethyl disulfide, dimethyl trisulfide, nonane and 2,4-pentadione. Because both dimethyl disulfide and dimethyl trisulfide were emitted significantly more by waters containing L4 larvae than by the younger larvae and the control, and L4 larvae have a deterrent effect on oviposition, these compounds are most likely deterrents. These compounds have also been described elsewhere as oviposition mediating cues. Trexler *et al.* (2003b) showed that, in field trials, *Aedes albopictus* laid fewer eggs in waters containing dimethyl disulfide than in control water. This compound can be found in hog lagoon odours, suggesting that *Ae. albopictus* prefers to oviposit in habitats that are not highly organic or polluted. Dimethyl disulfide shows insecticidal toxicity, for which isoptera and hymenoptera are more sensitive than diptera (Auger *et al*., 2004). A blend of L-lactic acid, acetone and dimethyl disulfide

approached the attraction potency of some humans for *Aedes aegypti* (Bernier *et* al., 2007). Dimethyl trisulfide was found to be attractive to oviposit by *Culex tarsalis,* at one concentration (1*µ*g/L). Dimethyl trisulfide is derived from infusions of Bermuda grass (Du and Millar, 1999). *An. gambiae* favors open sun-lit, temporary freshwater habitats (Koenraadt & Takken, 2003), without emerging vegetation (Clements, 1999). The organic content of these sites will most likely be very low. The abundance of dimethyl trisulfide might indicate a highly organic site. This might explain the deterrent effect of L4 larvae to oviposition by gravid females.

Nonane and 2,4-pentanedione are emitted by both L1 and L4 larvae more than by the control. These chemicals could be potential attractants. Concerning L4 larvae, it is very well possible that the attractiveness of these chemicals is masked by dimethyl disulfide and dimethyl trisulfide. This is also hypothesised with host odours. Logan *et al*. (2008) found that the attractiveness of hosts might be decreased because of masking effects of odorous compounds. In the study of Logan *et al.* it could not be determined whether this was the case, or that mosquitoes were repelled by these compounds.

Clear differences in which a compound was emitted by L1 larvae and not by the control or L4 larvae were not found, although 2,2-dimethyl-1,3-propanediol, itaconic acid diethylester and benzothiazole seem to show such patterns, these differences were not significant.

Behavioural experiments have to be conducted to test the biological function of these chemicals, regarding oviposition. Application of these chemicals could be found in oviposition traps, as, for example, is also discussed for *Culex pipiens* (Michaelakis, *et al.*, 2005). When chemical compounds emitted by larvae can be used in traps, it is expected that medium sized mosquitoes will be caught most. They respond best to the odours released by the larvae. It has been shown by Lyimo and Koella (1992), that the mosquitoes surviving long enough for *Plasmodium* to develop sporozoites are medium sized mosquitoes (2.84 mm). Furthermore, medium sized females of *Anopheles arabiensis* in Ethiopia were considered epidemiological more important than large mosquitoes, because of their high proportion of medium-sized mosquitoes (50.8%) compared to the large mosquitoes (17.8%) (Ameneshewa, 1996).

Infection with *Plasmodium* can reduce egg development of the mosquito (Hogg *et al*. 1997a; Hogg *et al*. 1997b). But, it is not yet known whether an *Plasmodium* infection alters the behaviour of these mosquitoes towards the odours released by larvae. If oviposition behaviour is not altered by *Plasmodium* infections,

these traps will catch the most risky group of mosquitoes. This should be tested in future research.

When the oviposition trap would prove effective, it can be used best in combination with other odour traps, like host odour traps. When mosquitoes, despite the host odour trap, bite a human being, the oviposition trap may still catch the mosquito before it lays its eggs and starts searching for another blood meal. In this way, mosquitoes can be caught in a critical moment of both the mosquito life cycle, namely just before it releases its offspring to the environment, and the *Plasmodium* life cycle, before the sporozoites are transmitted to the human host.

But, it should be noted that with these experiments, it is assumed that gravid mosquitoes were *attracted* towards water containing L1 larvae. But this could not be proven with this setting. It is very well possible that mosquitoes were *stimulated* to oviposit in the sites containing L1 larvae, and were not attracted, as was shown by *Culex quinquefasciatus,* using sticky screen bioassays and egg raft bioassays of 10 chemicals in odours from Bermuda grass infusions. Whereas sticky screens caught three times more mosquitoes from water treated with these infusion, the number of egg batches laid in this water, without sticky screens and mosquitoes having full access to the oviposition site, was ten times higher as the control. Suggesting that these volatile chemicals play a lesser role on the attraction of mosquitoes, than in the stimulation to oviposit (Du and Millar, 1999). Whether the effects of chemicals released by L1 larvae are attractants, or stimulants should be tested in further studies.

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7. Appendix

7.1 Age-structure experiments

Table 3: Number of eggs laid by individual females in a specific cup, the gonotrophic stage of the ovarian follicles, the number of retained eggs of the dissected females and the wing lengths of the right wings. Data in bold font are used in the analysis.

Treatment 1	Control 1	Control 2	Stage	# Retained eggs	Wing length
14-11 - 16-11	0	0	*	*	*
21-11 - 23-11	**13**	**0**	*****	*****	**2.66**
28-11 - 30-11	**0**	**21**	*****	*****	*****
5-12 - 7-12	0	0	*	*	2.63
19-12 - 21-12	**39**	**0**	**1**	**0**	**2.81**
19-12 - 21-12	0	0	5	30	2.88
23-01 - 25-01	**0**	**70**	**1**	**0**	**3.00**
30-01 - 1-02	0	0	5	27	2.88
30-01 - 1-02	0	0	2+5	3	*
05-03 - 07-03	**2**	**35**	**1**	**0**	**2.66**
05-03 - 07-03	**8**	**0**	**5**	**34**	**3.00**
05-03 - 07-03	0	0	5	10	2.88
05-03 - 07-03	**67**	**0**	**5**	**3**	*****
05-03 - 07-03	**0**	**7**	*****	*****	*****
05-03 - 07-03	**0**	**28**	**5**	**1**	**2.91**
Average	**14.3**	**17.9**			**2.84**

Treatment 2	100 L1	Control	Stage	# Retained eggs	Wing length
14-11 - 16-11	**90**	**0**	*****	*****	**3.03**
21-11 - 23-11	**0**	**9**	*****	*****	**2.81**
28-11 - 30-11	**0**	**4**	*****	*****	**2.69**
5-12 - 7-12	0	0	2	0	*
5-12 - 7-12	**12**	**0**	**5**	**2**	*****
19-12 - 21-12	0	0	5	45	2.78
19-12 - 21-12	0	0	5	43	2.78
30-01 - 1-02	**22**	**9**	*****	*****	**2.91**
30-01 - 1-02	0	0	1	0	
6-2 - 8-2	0	0	5	46	2.94
6-2 - 8-2	**30**	**0**	**5**	**1**	*****
6-2 - 8-2	**64**	**0**	**1**	**0**	**3.03**
6-2 - 8-2	**43**	**0**	**5**	**2**	**2.97**
13-2 - 15-2	**14**	**0**	**5**	**10**	**2.88**
13-2 - 15-2	**51**	**0**	**1**	**0**	**2.72**
13-2 - 15-2	0	0	2	0	2.72
13-2 - 15-2	0	0	5	40	2.88
13-2 - 15-2	0	0	5	30	*
Average	**32.6**	**2.2**			**2.88**

Treatment 3	50 L4	Control	Stage	# Retained eggs	Wing length
14-11 - 16-11	**0**	**61**	*	*	*
21-11 - 23-11	**19**	**13**	*	*	**2.63**
5-12 - 7-12	**0**	**29**	**1**	**1**	**2.81**
19-12 - 21-12	**20**	**0**	**5**	**6**	*
19-12 - 21-12	**16**	**0**	**1**	**0**	**2.69**
30-01 - 1-02	0	0	2	0	*
6-2 - 8-2	**58**	**0**	**1**	**0**	*
6-2 - 8-2	0	0	5	48	3.19
6-2 - 8-2	0	0	5	54	*
6-2 - 8-2	**0**	**68**	**5**	**2**	**2.94**
6-2 - 8-2	**1**	**33**	**1**	**0**	*
6-2 - 8-2	0	0	2	0	*
13-2 - 15-2	0	0	2 + 5	1	2.84
13-2 - 15-2	**7**	**0**	**2**	**0**	**2.72**
13-2 - 15-2	0	0	2	0	2.88
13-2 - 15-2	**0**	**25**	**5**	**1**	**2.88**
13-2 - 15-2	**25**	**0**	**1**	**0**	**2.66**
13-2 - 15-2	**11**	**13**	**1**	**0**	**2.81**
Average	**13.1**	**20.2**			**2.77**

Treatment 3b	100 L4	Control	Stage	# Retained eggs	Wing length
26-3 - 28-3	0	0	2	*	3.00
26-3 - 28-3	**0**	**104**	**5+1**	**2**	**2.94**
26-3 - 28-3	**12**	**0**	*	*	**2.81**
26-3 - 28-3	0	0	5	86	3.19
26-3 - 28-3	**0**	**9**	**1**	**0**	**3.00**
26-3 - 28-3	**0**	**3**	**1**	**0**	*
26-3 - 28-3	**0**	**42**	**5+1**	**4**	**2.88**
26-3 - 28-3	**0**	**2**	**5+1**	**1**	*
16-4 - 18-4	**24**	**11**	**5+1**	**1**	**2.84**
16-4 - 18-4	**9**	**0**	**5+1**	**1**	**2.66**
16-4 - 18-4	**0**	**85**	**1**	**0**	**3.06**
16-4 - 18-4	**0**	**53**	**1**	**1**	**3.00**
16-4 - 18-4	**12**	**73**	**5+1**	**1**	**2.94**
16-4 - 18-4	**0**	**55**	**5+1**	**2**	**2.84**
16-4 - 18-4	**0**	**48**	**5+1**	**1**	**2.91**
16-4 - 18-4	**0**	**44**	**1**	**0**	**2.66**
Average	**4.1**	**37.8**			**2.88**

Treatment 4	100 L1 + 50 L4	Control	Stage	# Retained eggs	Wing length
14-11 - 16-11	**0**	**23**	*	*	**2.81**
21-11 - 23-11	**33**	**0**	*	*	**2.91**
26-11 - 28-11	**6**	**0**	*	*	**2.63**
5-12 - 7-12	0	0	1	1	2.84
5-12 - 7-12	**0**	**35**	**2**	**1**	**2.72**
19-12 - 21-12	0	0	5	19	2.63
19-12 - 21-12	**7**	**15**	**5 + 1**	**2**	*
20-2 - 22-2	**0**	**4**	**5 + 2**	**1**	**2.75**
20-2 - 22-2	**0**	**1**	**2**	**0**	**2.78**
20-2 - 22-2	**0**	**28**	**1**	**0**	**2.88**
20-2 - 22-2	**10**	**0**	**1**	**0**	**2.78**
27-2 - 29-2	**0**	**23**	**5 + 1**	**1**	*
27-2 - 29-2	**22**	**0**	**5 + 1**	**1**	*
27-2 - 29-2	0	0	5 + 2	2	2.88
Average	**7.1**	**11.7**			**2.78**

Treatment 5	100 L1 + 50 L4	100 L1	Stage	# Retained eggs	Wing length
14-11 - 16-11	**11**	**0**	*	*	**2.66**
21-11 - 23-11	**48**	**0**	*	*	**2.81**
26-11 - 28-11	**0**	**4**	*	*	**2.63**
5-12 - 7-12	**0**	**38**	**1**	**0**	**2.91**
5-12 - 7-12	**0**	**33**	**2**	**0**	*
19-12 - 21-12	**0**	**20**	**5**	**4**	**2.88**
19-12 - 21-12	**0**	**27**	**1**	**0**	*
20-2 - 22-2	**2**	**9**	**5 + 1**	**3**	**2.81**
20-2 - 22-2	0	0	2	0	*
27-2 - 29-2	**43**	**0**	**5 + 1**	**4**	**2.88**
27-2 - 29-2	0	0	5	40	2.81
27-2 - 29-2	0	0	5 + 2	26	*
05-03 - 07-03	**16**	**64**	**5 + 1**	**2**	**3.00**
05-03 - 07-03	**56**	**0**	**1**	**0**	**2.94**
05-03 - 07-03	**13**	**64**	**1**	**0**	**3.03**
Average	**15.8**	**21.6**			**2.85**

Treatment 6	100L1 + 50 L4	50 L4	Stage	# Retained eggs	Wing length
14-11 - 16-11	**42**	**0**	*	*	**2.84**
21-11 - 23-11	**64**	**0**	*	*	**3.06**
5-12 - 7-12	**24**	**0**	**1**	**0**	**2.84**
5-12 - 7-12	**0**	**51**	**1**	**0**	**2.84**
19-12 - 21-12	**24**	**0**	**1**	**0**	**2.84**
19-12 - 21-12	**0**	**38**	**1**	**0**	**2.72**
20-2 - 22-2	**3**	**1**	**5 + 2**	**7**	**3.13**
20-2 - 22-2	**99**	**0**	**5 + 1**	**1**	**3.25**
20-2 - 22-2	**0**	**48**	**1**	**0**	*
27-2 - 29-2	**24**	**13**	**1**	**0**	**2.66**
27-2 - 29-2	**8**	**3**	**1**	**0**	**2.91**
Average	**28.0**	**14.0**			**2.91**

7.2 Body size experiments

7.2.1 Large mosquitoes

Table 4: Number of eggs laid by large females in a specific cup, the gonotrophic stage of the ovarian follicles, the number of retained eggs of the dissected females and the wing lengths of the right wings. The ovipositional response is given a value of 0, 0.5 or 1, corresponding to a response in disagreement with the hypothesis, spreading of eggs or a response in agreement with the hypothesis, respectively. When eggs are spread, this is indicated with a value of 1. Data in bold font are used in the analysis.

Treatment 2	100 L1	Control	Stage	# Retained eggs	Wing length	Response	Divided?
14-3 - 16-3	0	0	*	*	3.09		
14-3 - 16-3	**0**	**51**	**5**	**7**	**3.19**	0	0
14-3 - 16-3	0	0	5	50	3.13		
14-3 - 16-3	**17**	**62**	**1**	**0**	**3.16**	0.5	1
14-3 - 16-3	**101**	**0**	**5**	**5**	**3.16**	1	0
14-3 - 16-3	**35**	**59**	**1**	**0**	**3.13**	0.5	1
2-4 - 4-4	0	0	5	77	3.16		
2-4 - 4-4	0	0	5	90	3.13		
2-4 - 4-4	0	0	5	58	3.06		
2-4 - 4-4	0	0	5	62	3.00		
2-4 - 4-4	0	0	5	3	3.13		
2-4 - 4-4	0	0	5	74	3.13		
23-4 - 25-4	**37**	**12**	**1**	**0**	**3.25**	0.5	1
23-4 - 25-4	**47**	**6**	**5**	**10**	**3.16**	0.5	1
23-4 - 25-4	**37**	**21**	**5**	**4**	**3.03**	0.5	1
23-4 - 25-4	**12**	**0**	**5**	**1**	**2.69**	1	0
23-4 - 25-4	**8**	**0**	**5+1**	**4**	**3.13**	1	0
23-4 - 25-4	**0**	**22**	**5**	**0**	**3.06**	0	0
30-4 - 2-5	0	0	5	56	2.75		
30-4 - 2-5	34	0	5	1	3.09		
30-4 - 2-5	0	0	5	86	2.97		
30-4 - 2-5	0	0	5	78	2.88		
30-4 - 2-5	44	0	5	5	2.81		
30-4 - 2-5	0	0	5	70	2.91		
15-5 - 17-5	**0**	**26**	*****	*****	**3.22**	0	0
15-5 - 17-5	**122**	**0**	*****	*****	**3.31**	1	0
Average	**38**	**18.2308**					

Treatment 3b	100 L4	Control	Stage	# Retained eggs	Wing length	Response	Divided?
2-4 - 4-4	**0**	**43**	**1**	**0**	**3.13**	1	0
2-4 - 4-4	**55**	**0**	**1**	**1**	**3.16**	0	0
2-4 - 4-4	0	0	5	94	3.00		
23-4 - 25-4	**83**	**0**	**5**	**1**	**3.13**	1	0
23-4 - 25-4	**0**	**68**	**1**	**0**	*****	0	0
23-4 - 25-4	0	0	5	1	3.09		
23-4 - 25-4	**0**	**8**	**1**	**0**	**2.72**	0	0
23-4 - 25-4	**7**	**0**	**5**	**10**	**3.13**	1	0
23-4 - 25-4	0	0	5	130	*		
30-4 - 2-5	80	0	1	0	3.09		
30-4 - 2-5	37	46	5	5	3.03		

30-4 - 2-5	21	0	1	0	2.88		
30-4 - 2-5	0	0	*	*	*		
30-4 - 2-5	0	4	1	0	3.00		
30-4 - 2-5	92	0	5	1	2.88		
15-5 - 17-5	**94**	**0**	*****	*****	**2.81**	1	0
15-5 - 17-5	0	0	*****	*****	3.22		
15-5 - 17-5	0	0	*****	*****	3.28		
15-5 - 17-5	**0**	**100**	*****	*****	**3.13**	0	0
15-5 - 17-5	**0**	**90**	*****	*****	**3.13**	0	0
28-5 - 30-5	**42**	**14**	**1**	**0**	*****	0.5	1
28-5 - 30-5	**0**	**5**	**5**	**9**	**3.34**	1	0
Average	**25.5455**	**29.8182**					

7.2.2 Small mosquitoes

Table 4: Number of eggs laid by small females in a specific cup, the gonotrophic stage of the ovarian follicles, the number of retained eggs of the dissected females and the wing lengths of the right wings. The ovipositional response is given a value of 0, 0.5 or 1, corresponding to a response in disagreement with the hypothesis, spreading of eggs or a response in agreement with the hypothesis, respectively. When eggs are spread, this is indicated with a value of 1. Data in bold font are used in the analysis.

Treatment 2	100 L1	Control	Stage	# Retained eggs	Wing length	Response	Divided?
17-3 - 19-3	**0**	**5**	**5+1**	**5**	**2.56**	0	0
17-3 - 19-3	0	0	5	32	2.56		
17-3 - 19-3	**0**	**92**	**1**	**0**	**2.41**	0	0
17-3 - 19-3	**0**	**10**	**5**	**34**	**2.59**	0	0
17-3 - 19-3	0	0	5	62	*		
17-3 - 19-3	0	0	5+1	25	2.56		
9-4 - 11-4	**0**	**1**	**5**	**31**	*****	0	0
9-4 - 11-4	0	0	5	48	2.88		
9-4 - 11-4	**34**	**0**	**5**	**1**	**2.72**	1	0
9-4 - 11-4	**1**	**0**	**5**	**11**	**2.53**	1	0
9-4 - 11-4	0	0	5	78	*		
9-4 - 11-4	**0**	**31**	**1**	**0**	**2.63**	0	0
8-5 - 10-5	**17**	**2**	*****	*****	**2.78**	0.5	1
8-5 - 10-5	0	0	*	*	2.78		
8-5 - 10-5	0	0	*	*	2.97		
8-5 - 10-5	**5**	**0**	*****	*****	*****	1	0
21-5 - 23-5	**26**	**0**	**5+1**	**30**	**2.66**	1	0
21-5 - 23-5	0	0	5	82	2.66		
21-5 - 23-5	0	0	5	40	2.53		
21-5 - 23-5	**0**	**6**	**1**	**0**	**2.50**	0	0
21-5 - 23-5	**34**	**1**	**5**	**24**	*****	0.5	1
21-5 - 23-5	**0**	**20**	**1**	**0**	**2.50**	0	0
Average	**9**	**12.9231**					

Treatment 3b	100 L4	Control	Stage	# Retained eggs	Wing length	Response	Divided?
9-4 - 11-4	0	0	5	50	2.69		
9-4 - 11-4	**0**	**31**	**5**	**2**	**2.69**	1	0
9-4 - 11-4	0	0	5	72	2.69		
9-4 - 11-4	0	0	5	12	2.72		
9-4 - 11-4	0	0	5	52	2.59		

9-4 - 11-4	0	0	5	40	*		
8-5 - 10-5	0	0	*	*	2.56		
8-5 - 10-5	**0**	**11**	*****	*****	**2.66**	1	
8-5 - 10-5	0	0	*	*	2.75		
8-5 - 10-5	**22**	**2**	*****	*****	**2.81**	0.5	1
8-5 - 10-5	**0**	**55**	*****	*****	**2.88**	1	0
8-5 - 10-5	**0**	**44**	*****	*****	*****	1	0
8-5 - 10-5	0	0	*	*	2.63		
8-5 - 10-5	**0**	**50**	*****	*****	**2.69**	1	0
21-5 - 23-5	0	0	**5**	**29**	**2.50**		
21-5 - 23-5	**45**	**0**	**1**	**0**	**2.66**	**0**	**0**
21-5 - 23-5	**0**	**62**	*****	*****	**2.72**	**1**	**0**
28-5 - 30-5	**40**	**0**	**1**	**0**	**2.50**	**0**	**0**
28-5 - 30-5	**1**	**4**	**5**	**4**	*****	**0.5**	**1**
28-5 - 30-5	**61**	**0**	**1**	**0**	**2.66**	**0**	**0**
28-5 - 30-5	**0**	**30**	**5**	**2**	**2.50**	**1**	**0**
Average	**14.0833**	**24.0833**					

7.2.3 Medium sized mosquitoes (age-structure experiment)

Table 5: Number of eggs laid by small females in a specific cup, the gonotrophic stage of the ovarian follicles, the number of retained eggs of the dissected females and the wing lengths of the right wings. The ovipositional response is given a value of 0, 0.5 or 1, corresponding to a response in disagreement with the hypothesis, spreading of eggs or a response in agreement with the hypothesis, respectively. When eggs are spread, this is indicated with a value of 1. Data in bold font are used in the analysis.

Treatment 2	100 L1	Control	Stage	# Retained eggs	Wing length	Response	Divided?
14-11 - 16-11	**90**	**0**	*****	*****	**3.03**	1	0
21-11 - 23-11	**0**	**9**	*****	*****	**2.81**	0	0
28-11 - 30-11	**0**	**4**	*****	*****	**2.69**	0	0
5-12 - 7-12	0	0	2	0	*****		
5-12 - 7-12	**12**	**0**	**5**	**2**	*****	1	0
19-12 - 21-12	0	0	5	45	2.78		
19-12 - 21-12	0	0	5	43	2.78		
30-01 - 1-02	**22**	**9**	*****	*****	**2.91**	0.5	1
30-01 - 1-02	0	0	1	0	*****		
6-2 - 8-2	0	0	5	46	2.94		
6-2 - 8-2	**30**	**0**	**5**	**1**	*****	1	0
6-2 - 8-2	**64**	**0**	**1**	**0**	**3.03**	1	0
6-2 - 8-2	**43**	**0**	**5**	**2**	**2.97**	1	0
13-2 - 15-2	**14**	**0**	**5**	**10**	**2.88**	1	0
13-2 - 15-2	**51**	**0**	**1**	**0**	**2.72**	1	0
13-2 - 15-2	0	0	2	0	2.72		
13-2 - 15-2	0	0	5	40	2.88		
13-2 - 15-2	0	0	5	30	*****		
Average	**32.6**	**2.2**					

Treatment 3b	100 L4	Control	Stage	# retained eggs	wing length	Response	Divided?
26-3 - 28-3	0	0	2	*****	3.00		
26-3 - 28-3	**0**	**104**	**5+1**	**2**	**2.94**	1	0
26-3 - 28-3	**12**	**0**	*****	*****	**2.81**	0	0

26-3 - 28-3	0	0	5	86	3.19		
26-3 - 28-3	**0**	**9**	**1**	**0**	**3.00**	1	0
26-3 - 28-3	**0**	**3**	**1**	**0**	*	1	0
26-3 - 28-3	**0**	**42**	**5+1**	**4**	**2.88**	1	0
26-3 - 28-3	**0**	**2**	**5+1**	**1**	*	1	0
16-4 - 18-4	**24**	**11**	**5+1**	**1**	**2.84**	0.5	1
16-4 - 18-4	**9**	**0**	**5+1**	**1**	**2.66**	1	0
16-4 - 18-4	**0**	**85**	**1**	**0**	**3.06**	1	0
16-4 - 18-4	**0**	**53**	**1**	**1**	**3.00**	1	0
16-4 - 18-4	**12**	**73**	**5+1**	**1**	**2.94**	0.5	1
16-4 - 18-4	**0**	**55**	**5+1**	**2**	**2.84**	1	0
16-4 - 18-4	**0**	**48**	**5+1**	**1**	**2.91**	1	0
16-4 - 18-4	**0**	**44**	**1**	**0**	**2.66**	1	0
Average	**4.07143**	**37.7857**					

7.3 Pilot studies

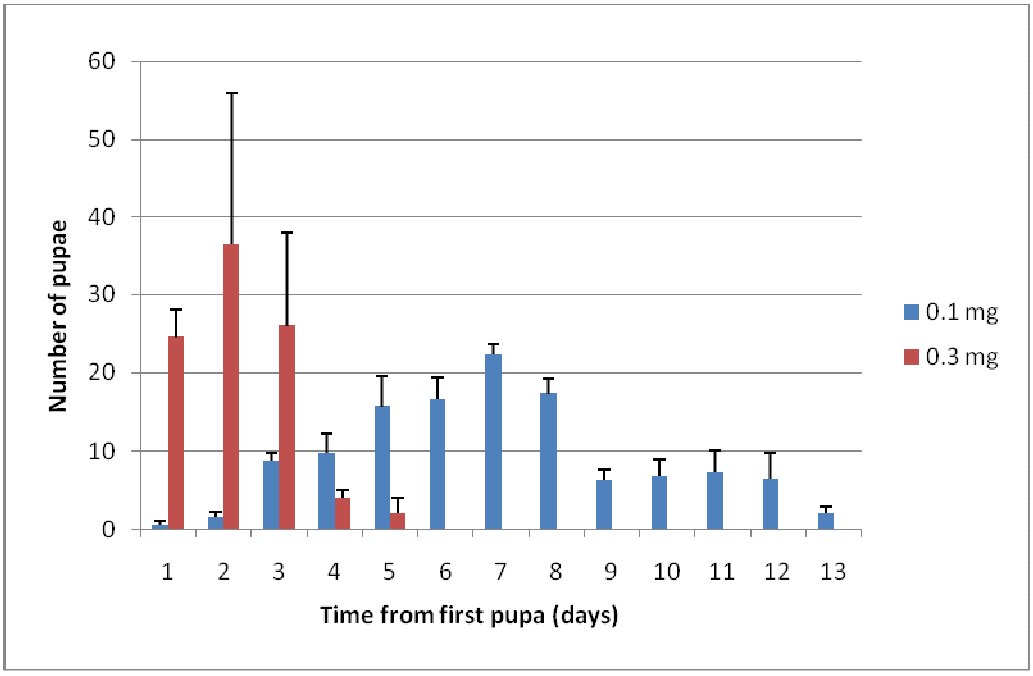


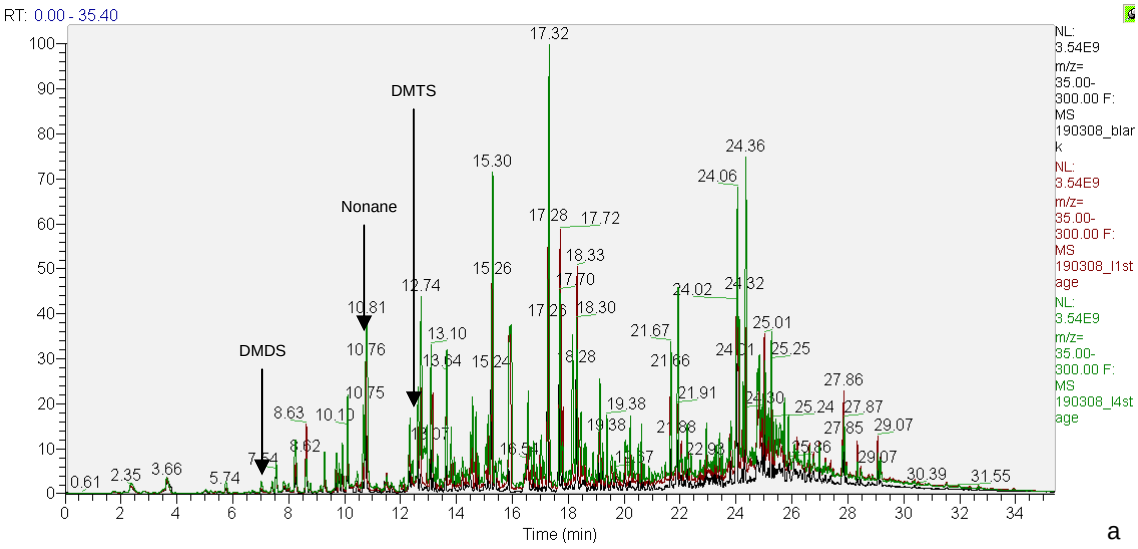
Figure 15: Number of pupae versus time, for 200 larvae that were fed 0.1 mg Tetramin/larvae/day during the entire larval stage (N=5) and 200 larvae that were fed 0.1 mg for the first two days and 0.3 for the rest of their larval stage (N=2)

7.4 Chemical analysis

Table 6: Peak surfaces of 16 chemical compounds derived from a control, L1 larvae and L4 larvae. Empty cells are from data that have been excluded from the analysis, because of a very low (replications 4 and 5) or high (replication 3) output.

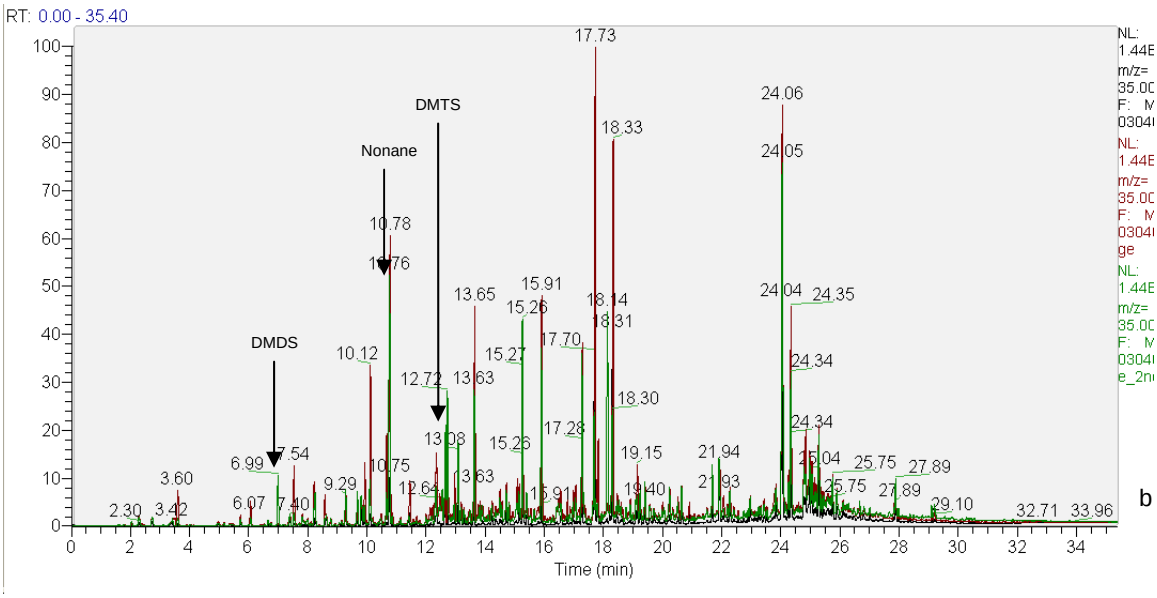
Chemical compound	Treatment	1	2	3	4	5	6	Average	SE
Hexane	Control	5.59E+04	1.17E+06		2.93E+05	5.00E+05	1.22E+05	4.27E+05	2.58E+05
	L1	1.52E+06	6.56E+05	5.62E+04		2.83E+05	4.14E+05	5.85E+05	2.52E+05
	L4	3.47E+05	8.54E+05	5.52E+04	5.00E+05		3.14E+05	4.14E+05	1.31E+05
2-methyl-2-propenoic	Control	4.06E+05	2.59E+04		3.04E+04	6.69E+04	0.00E+00	1.06E+05	7.57E+04
acid methyl ester	L1	9.69E+05	2.34E+04	4.40E+05		4.25E+04	2.69E+04	3.00E+05	1.85E+05
	L4	3.06E+05	1.90E+04	4.81E+05	3.60E+04		2.16E+04	1.73E+05	9.42E+04
Dimethyldisulfide	Control	3.26E+03	6.85E+04		1.71E+04	1.02E+03	3.10E+03	1.86E+04	1.28E+04
	L1	1.83E+04	6.65E+04	4.45E+04		1.26E+04	3.24E+04	3.49E+04	9.67E+03
	L4	2.14E+06	9.08E+05	1.04E+06	2.37E+05		2.48E+04	8.69E+05	3.71E+05
Toluene	Control	6.84E+04	3.19E+06		2.66E+06	4.29E+06	1.10E+05	2.06E+06	8.48E+05
	L1	3.53E+06	2.99E+06	6.54E+04		2.46E+06	6.21E+05	1.93E+06	6.77E+05
	L4	6.85E+05	2.96E+06	9.90E+04	4.52E+06		1.37E+06	1.93E+06	8.06E+05
Acetic acid buthylester	Control	1.68E+04	1.40E+05		6.83E+05	1.81E+06	1.04E+05	5.51E+05	3.36E+05
	L1	4.86E+05	2.67E+05	5.65E+04		1.02E+06	2.55E+05	4.18E+05	1.66E+05
	L4	4.53E+05	3.76E+05	5.39E+04	1.06E+06		4.44E+05	4.78E+05	1.64E+05
Ethylbenzene	Control	2.20E+05	2.06E+06		1.67E+06	3.90E+06	1.13E+05	1.59E+06	6.94E+05
	L1	3.07E+06	2.80E+06	7.73E+04		2.20E+06	5.80E+05	1.75E+06	6.01E+05
	L4	7.56E+05	2.44E+06	9.42E+04	3.03E+06		1.16E+06	1.50E+06	5.42E+05
1,3-dimethylbenzene	Control	4.24E+05	5.41E+06		3.01E+06	7.77E+06	2.03E+05	3.36E+06	1.45E+06
	L1	7.37E+06	6.43E+06	7.23E+04		4.29E+06	9.23E+05	3.82E+06	1.45E+06
	L4	1.88E+06	5.55E+06	9.34E+04	5.60E+06		1.87E+06	3.00E+06	1.10E+06
Nonane	Control	8.68E+04	2.92E+05		0.00E+00	0.00E+00	6.66E+04	8.90E+04	5.36E+04
	L1	2.28E+06	5.37E+05	1.00E+05		0.00E+00	4.92E+05	6.82E+05	4.13E+05
	L4	6.94E+05	1.74E+06	5.71E+04	0.00E+00		1.00E+06	6.99E+05	3.23E+05
2,2-dimethyl-	Control	4.45E+05	6.09E+05		3.34E+05	6.13E+05	3.86E+05	4.78E+05	5.72E+04
1,3-propanediol	L1	1.33E+06	1.20E+06	5.36E+05		3.19E+05	4.53E+05	7.68E+05	2.07E+05
	L4	2.37E+05	7.50E+05	2.99E+05	9.38E+05		5.66E+05	5.58E+05	1.33E+05
Dimehyltrisulfide	Control	1.14E+04	1.42E+04		2.51E+04	5.18E+04	8.07E+03	2.21E+04	7.95E+03
	L1	2.13E+04	4.29E+04	3.44E+04		2.51E+04	6.31E+04	3.74E+04	7.44E+03
	L4	4.23E+06	1.04E+06	2.17E+06	1.76E+05		7.13E+04	1.54E+06	7.72E+05
2-ethyl-1-hexanol	Control	1.22E+07	1.95E+06		4.11E+06	1.05E+07	1.10E+06	5.97E+06	2.26E+06
	L1	7.13E+06	6.33E+06	6.43E+05		6.14E+06	7.33E+06	5.51E+06	1.24E+06
	L4	3.92E+06	6.36E+06	6.07E+05	6.50E+06		1.24E+07	5.96E+06	1.94E+06
Itaconic acid diethylester	Control	4.51E+06	4.07E+06		5.47E+06	1.08E+07	1.07E+07	7.12E+06	1.51E+06
	L1	1.10E+07	8.88E+06	4.28E+06		6.47E+06	1.65E+07	9.42E+06	2.10E+06
	L4	2.23E+06	4.57E+06	3.99E+06	1.46E+07		1.46E+07	8.01E+06	2.73E+06
Diethylethylidene	Control	1.25E+06	1.09E+06		1.52E+06	7.89E+06	3.14E+06	2.98E+06	1.28E+06
malonate	L1	3.76E+06	3.92E+06	1.19E+06		1.78E+06	4.74E+06	3.08E+06	6.78E+05
	L4	6.51E+05	1.54E+06	1.16E+06	3.80E+06		3.82E+06	2.19E+06	6.74E+05
Benzothiazole	Control	4.02E+05	1.23E+06		2.22E+07	1.84E+05	2.54E+06	5.31E+06	4.24E+06
	L1	9.36E+05	1.63E+06	1.49E+06		2.39E+07	6.29E+07	1.82E+07	1.20E+07
	L4	1.26E+07	6.90E+06	5.42E+05	6.65E+05		1.82E+06	4.50E+06	2.33E+06
Ethylcitrate	Control	7.03E+05	7.47E+05		8.04E+05	3.35E+07	1.60E+06	7.46E+06	6.50E+06
	L1	2.79E+06	4.69E+06	3.12E+05		3.46E+06	5.59E+06	3.37E+06	9.05E+05
	L4	9.25E+05	3.54E+06	2.40E+05	1.38E+07		2.96E+07	9.62E+06	5.55E+06
2,4-pentanedione	Control	9.26E+04	2.37E+05		1.86E+05	2.43E+04	5.30E+04	1.18E+05	4.02E+04
	L1	7.31E+05	3.38E+05	9.03E+04		2.13E+05	4.54E+06	1.18E+06	8.46E+05
	L4	5.45E+05	5.24E+05	5.71E+04	3.21E+06		6.13E+05	9.90E+05	5.64E+05

190308



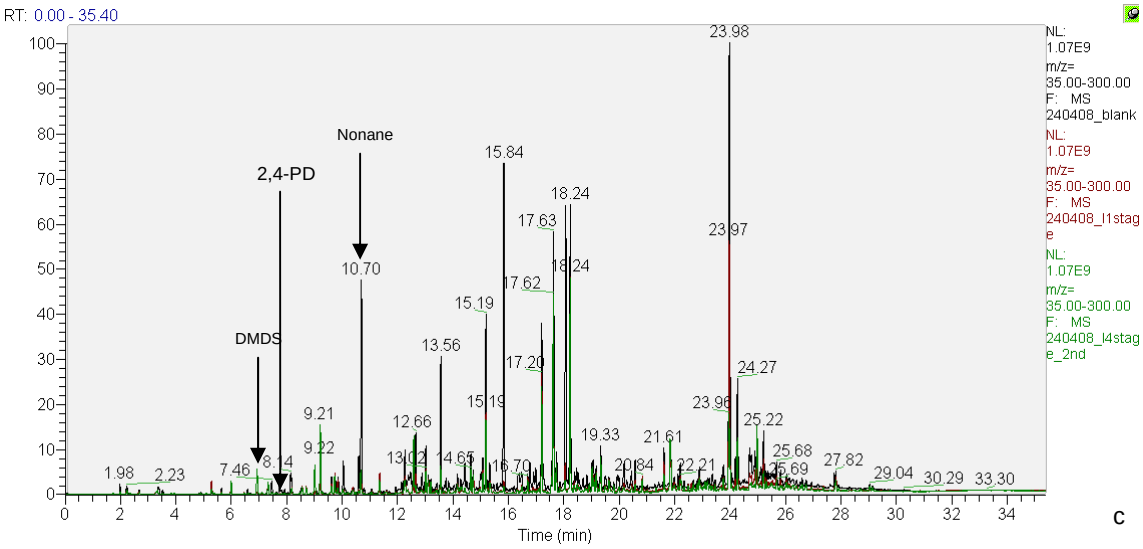
a

030408



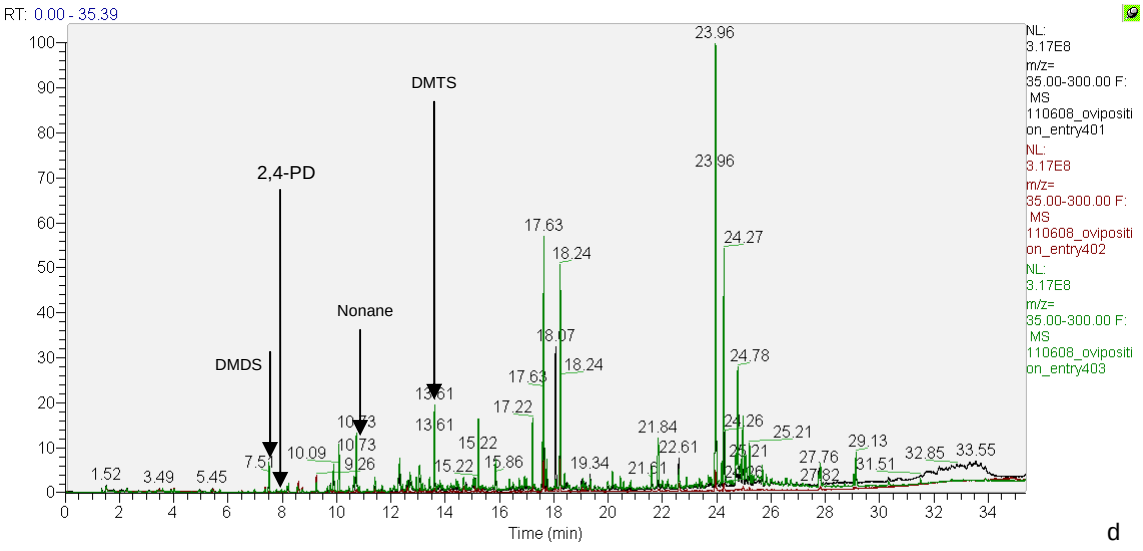
b

240408 (Control high output)



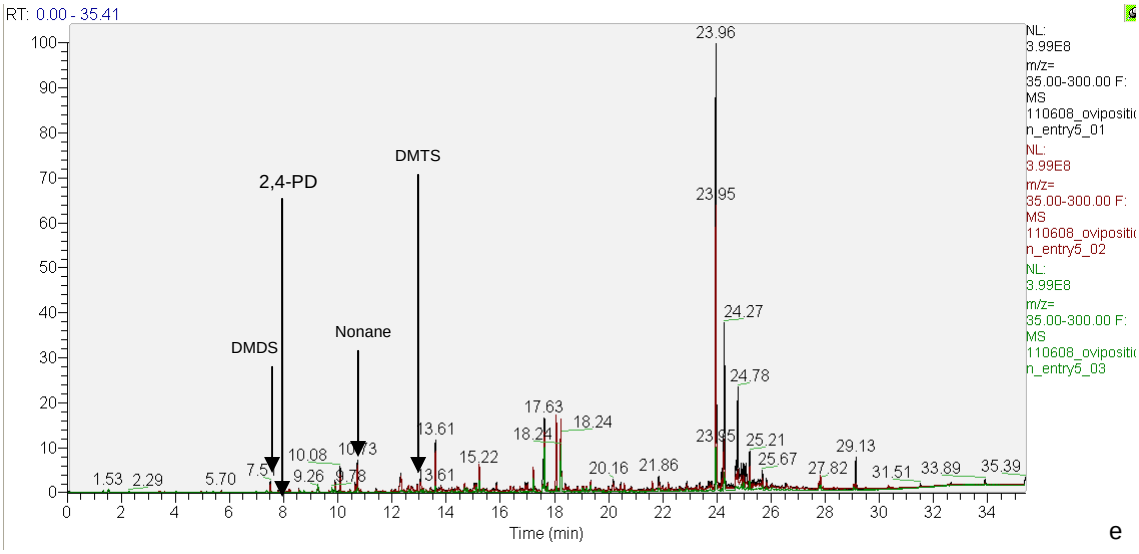
c

110608-04 (L1 low ouput)



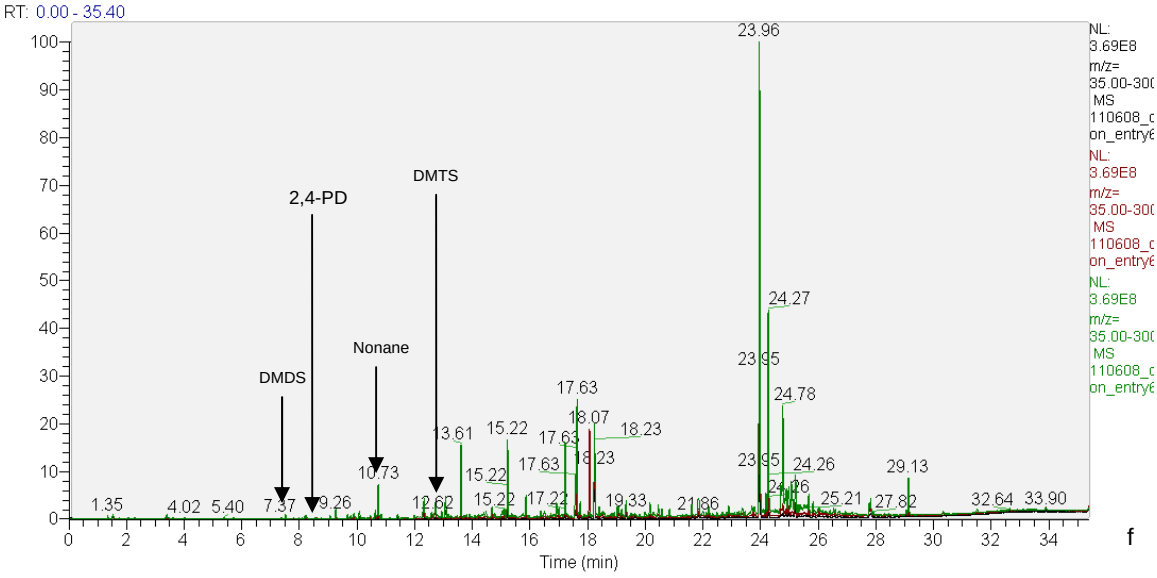
d

110608-05 (L4 low output)



e

110608-06



f

Figure 16a - 16f: Chromatograms of the chemical analyses performed using GC-MS. Black lines indicate compounds released by the control. Red and green lines show compounds released by L1 and L4 larvae, respectively.
DMDS= Dimethyl disulfide; 2,4-PD= 2,4-pentadione; DMTS= Dimethyl trisulfide.

Data from the control of Figure 16c, from L1 larvae of Figure 16d and from L4 larvae of Figure 16e were excluded from analysis, because of a high (Fig. 16c) or low output (Figs. 16d and 16e), even after the correction with the internal standard.