Impacts of sex, age, and desiccation on cuticular hydrocarbon profiles of Anopheles gambiae pupae

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Impacts of sex, age, and desiccation on cuticular hydrocarbon profiles of *Anopheles gambiae* pupae

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Abbreviations

*CHC – Cuticular Hydrocarbon

GC- MS – Gas Chromatography Mass Spectrometry

RT – Retention time

PCA – Principal Component Analysis

RH – Relative humidity

*Please note: CHC, hydrocarbon and compound will be used interchangeably throughout

1. Introduction

1.1. Project motivation

Malaria kills on average 445,000 people a year (WHO Global, 2019). Almost 90% of the world's malaria deaths occur in Africa (Health Organization, 2016). *Anopheles gambiae* complex of at least 7 species of mosquitoes is the most important vector of malaria in sub-Saharan Africa (Zoh et al., 2020).
An. gambiae carry one of the most dangerous malaria parasites, *Plasmodium falciparum*, that are responsible for 50% of all human malaria cases and more than 400,000 deaths per year (Rich et al., 2009). Mosquitos are such an effective and resilient transmitter of malaria in part because they can withstand a wide variety of temperatures and humidity levels (Chaves et al., 2008). Most vector species, such as *Aedes Albopictus*, can survive a broad temperature range between 8-40 °C (**Figure 1**) (Tyagi., 2004; Shocket et al., 2018). Although the range is of course species dependent for example *Culex pipiens* can survive very low temperatures of -10 °C (Reiter, 2001). On the other side of the spectrum is Sudanese *An. gambiae* that can survive the outdoor summer temperatures exceeding 55 °C (Reiter, 2001). *An. gambiae* mosquitoes are also shown to thrive in high humidity conditions as well as temperatures. According to Tyagi, *An. gambiae* mosquitoes tend to have lower mortality rates in environments with higher humidity levels (2004).

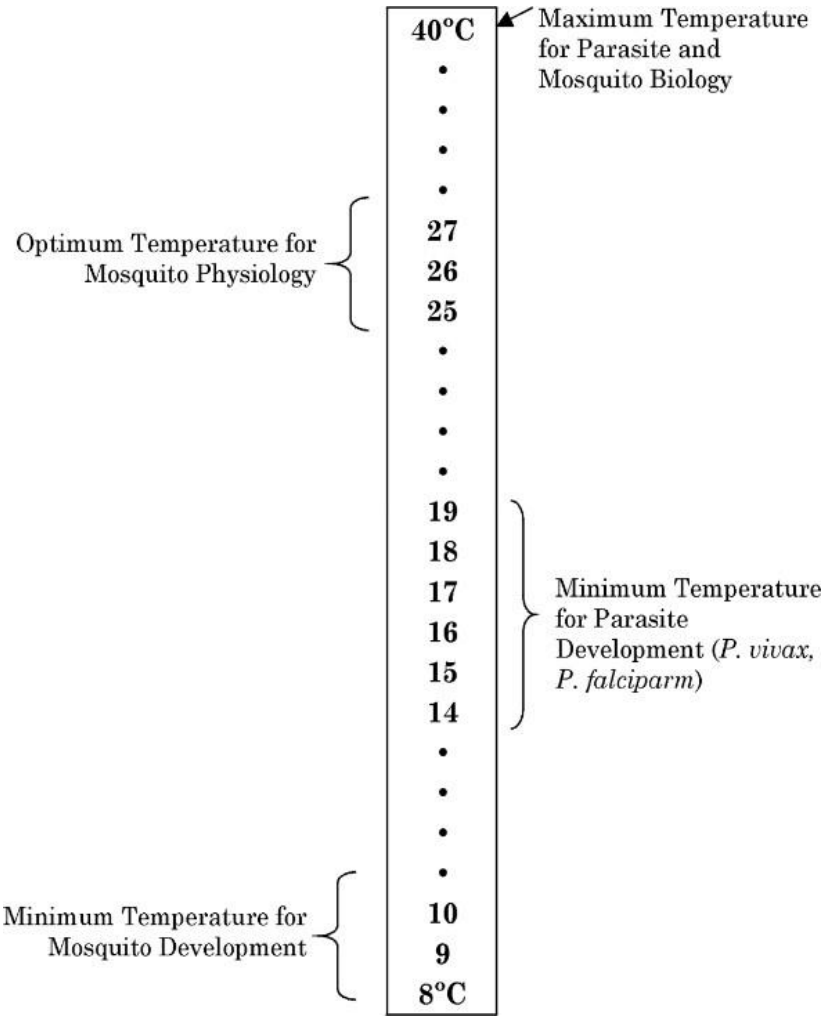


Figure 1. Effect of temperature ranges for the development of *An. gambiae* mosquito and the parasite in the body of the vector. (Image from Tyagi., 2004).

High temperature and humidity tolerance allows these mosquitoes to live in a range of different geographical locations from cooler, humid forests to hotter, arid areas, increasing the scale of disease transmission (Arcaz et al., 2016). An apt example of increasing temperatures creating conditions for the Anopheles genus to thrive is the unusual increase in outbreaks of malaria in 2012, in Djibouti, a country in East Africa (M. E. Sinka et al., 2020). Investigations revealed the cause of this increase was the new presence of an Asian mosquito, Anopheles stephensi in urban areas. Some have suggested that An. stephensi's spread is a result of both urbanisation with the urban heat island effect increasing temperatures, inducing a situation where this species can thrive. This phenomenon is of course exacerbated by climate change (Abbasi et al., 2020). This is due to An. Stephensi's impressive thermotolerance (Patil et al., 1996). However, major gaps remain in our understanding of how *An. gambiae* can withstand such a variety of temperatures and humidity at every stage of their lifecycle. The information about the survival of the pupal life stage of *An. gambiae* is particularly lacking. This is relevant as survival in the pupal stage is equally important in reaching adulthood as the larval or egg stages in all insects and *An. gambiae* is no exception (Radchuk et al., 2013). This study focuses on understanding how *An. gambiae* pupae are well adapted to a variety of environmental conditions, especially the extreme environmental conditions which induce desiccation. I will examine a range of physiological and chemical changes of pupae under extreme conditions, with specific emphasis given to changes in the composition of cuticular hydrocarbons (CHCs). CHCs coat the outside of nearly all insects, acting as a desiccation barrier

(Hadley, 1989). The CHC composition can also reflect variations in age and reproductive physiology (Hadley, 1989). Furthermore, this study seeks to investigate how pupal age, pupal sex, water salinity, air humidity and desiccation impact CHC profiles and mortality of *An. gambiae* during their pupal stage. This will develop a better understanding of the robust resistance of *An. gambiae* pupae to environmental stresses and how their level of resistance is related to their CHC profiles. This could outline possible areas for vector control efforts, by identifying possible "weak links" in the life cycle of *An. gambiae*.

1.2. Background

Life cycle

An. gambiae go through 4 main life stages (**Figure 2**). They begin their life cycle as eggs, which are between 0.47 to 0.48 mm long (Gillies, 1968), laying their eggs singly and directly on water. However, *An. gambiae's* eggs are not drought resistant (CDC, 2015). *An. gambiae* go through holometabolism and hence experience 4 distinct life stages: egg, larva, pupa and imago (adult) (Rolff et al., 2019). The larvae lack the respiratory siphons used for breathing, and hence lie parallel to the water surface to breathe. Mosquito larvae develop through 4 stages of larval size (instars) before pupating (Foster and Walker, 2002). The biggest instar can result in larvae reaching between 5 to 6 mm (Gillies and de Meillon 1968). The pupae phase of the mosquito, depending on the species, can last between 1.5 to 4 days. During this stage, they do not eat. However, they are mobile and can use their abdomen to propel themselves through the water.

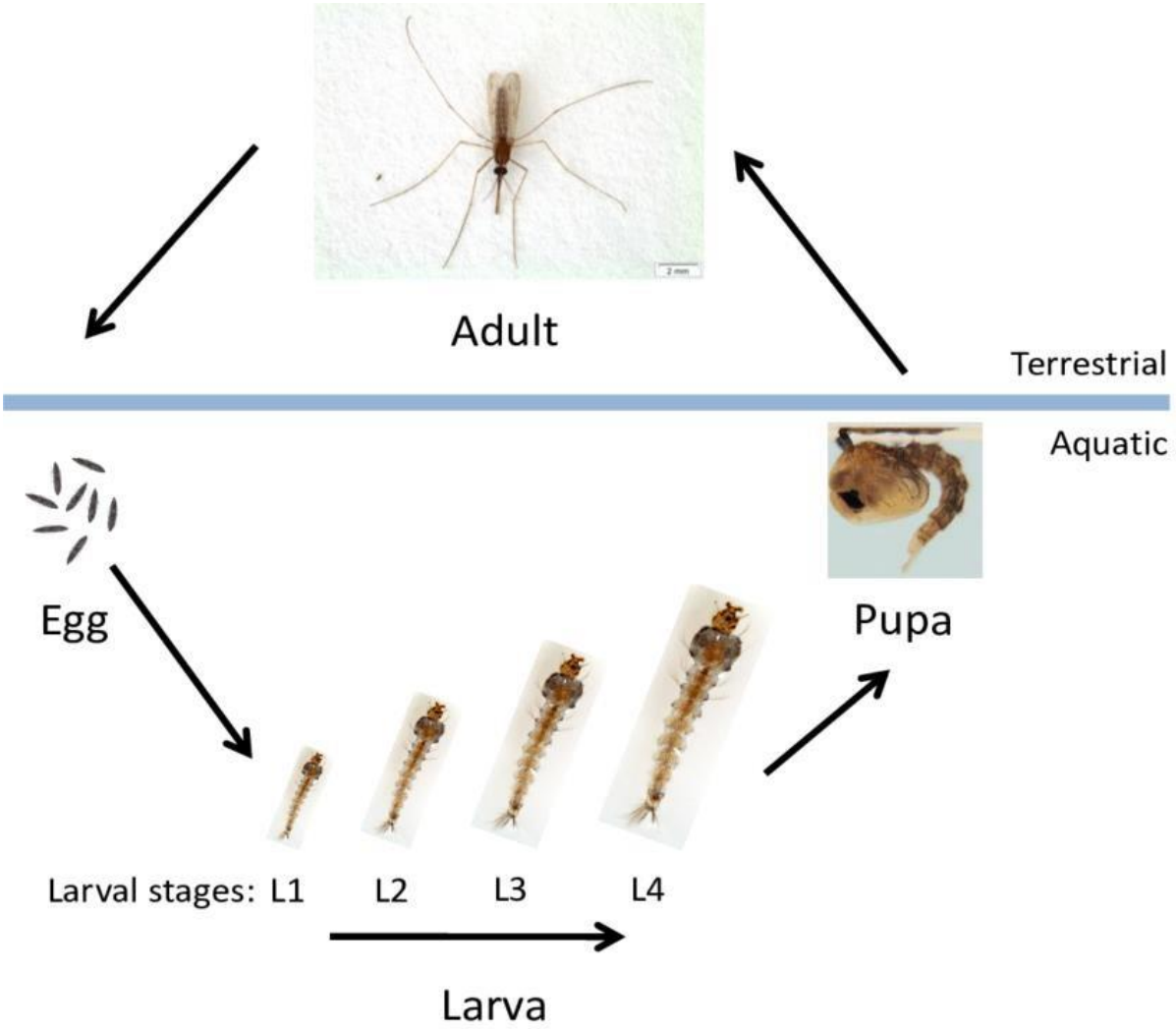


Figure 2. Stages of the Anopheles mosquito life cycle (Williams & Pinto, 2012).

Mosquito distribution with regards to climate and temperature

Anopheles is a transcontinental genus of mosquito able to survive in a broad variety of geographical zones and climates (**Figure 3**). *An. gambiae* is the most abundant species in Sub-Saharan Africa, where there is the highest incidence of malaria cases globally (Marianne E. Sinka et al., 2012). Malaria transmission is highly seasonal (Cairns et al., 2012). This is thought to be due to the availability of suitable larval sites (Craig et al., 1999). Understanding the ecological processes could present us with information about possible weaknesses in the life cycle of *An. gambiae*. Efforts to try and target mosquitos in their larval or pupal stages is also seen as potentially a more cost-effective way to reduce transmission of malaria (Sogoba et al., 2007). *An. gambiae* eggs are known to survive up to 12 days in dry field conditions (Beier et al., 1990). In dry seasons *An. gambiae* does not necessarily suffer die back. This is due to the ability of the eggs to remain dormant, thus resisting desiccation (Urbanski et al., 2010). *An. gambiae* larvae can survive up to 113 hours without water (Koenraadt et al., 2003). They are also able to seek water up to 10 cm away (Koenraadt et al., 2003). It is also shown that adult mosquitos can go through a process of aestivation, a dormant state, associated with physiological changes leading to suppressionof reproduction as well as extended

longevity of adult females in the dry season (Lehmann et al., 2010). Different species of mosquitos were also found to exhibit different traits, depending on the geographic zone they are adapted for (Barnard & Mulla, 1977). Three primary features *in Culex pipiens*, were found to contribute to suppression of water loss: large body size, reduced metabolic rate and increased quantity of cuticular hydrocarbons (Benoit & Denlinger, 2007). A reduction in sizeof spiracles in warmer climates was also noted in *An. stephensi* (Nagpal et al., 2003). It was shown that in *An. gambiae*, on average, females reared under dry seasonal conditions had 28% more CHCs than females reared under wet-seasonal conditions (Xu et al., 2018). Contrary to predictions, the mean CHC length was surprisingly shorter in mosquitoes reared under simulated dry-seasonal conditions in comparison to those that were reared under simulated wet-seasonal conditions (Xu et al., 2018). It is hypothesised that the production of shorter CHCs allows for an increase in total CHC quantity and that total quantity may be more important than hydrocarbon length in terms of reducing the loss of water through the trans-cuticular layer (Xu et al., 2018). It is highly possible that smaller n-alkanes compact better, and therefore are better at sealing the cuticle against water loss (Wagoner et al., 2014a). Wagoner et al also highlighted the effect of photoperiod on morphological changes that possibly shape the initial composition of CHCs (2014). A combination of photoperiod and relative humidity affects the total CHC amount and possibly its future composition (Wagoner et al., 2014a). Most importantly, morphological characteristics of adult *An. gambiae* seem to be predetermined from the larval and/or pupal stages (Wagoner et al., 2014a). However, the weighting that each one of these stages has on the morphological characteristics of the adult is unknown.

Photoperiod also can impact the CHC composition of mosquitoes. Mosquitoes reared under long photoperiods and high humidity levels had significantly higher mean n-alkane retention times then those under low humidity (Wagoner et al., 2014a). A faster CHC build-up was also noted in mosquitos undergoing photoperiod induce diapause (Benoit & Denlinger, 2007).

Two major African malaria vectors *An. gambiae* and *Anopheles arabiensis* occupy different climatic niches but their larvae are often found in similar geographic locations. Kirby and Lindsay investigated the survival and development of aquatic larval at different stages in their development (2009). Survival rate to adulthood was highest in both species when reared at 25 ^{o}C and declined with increasing temperature. Furthermore, whether the larvae were reared independently or with their sister species, depending on the temperature, had an impact on survivability. The mean age at eclosion and the size of the adult both decreased when reared in waters at higher temperature. Overall *An. arabiensis*larval seems to be more adapted to survival in slightly hotter climate condition according to this study.

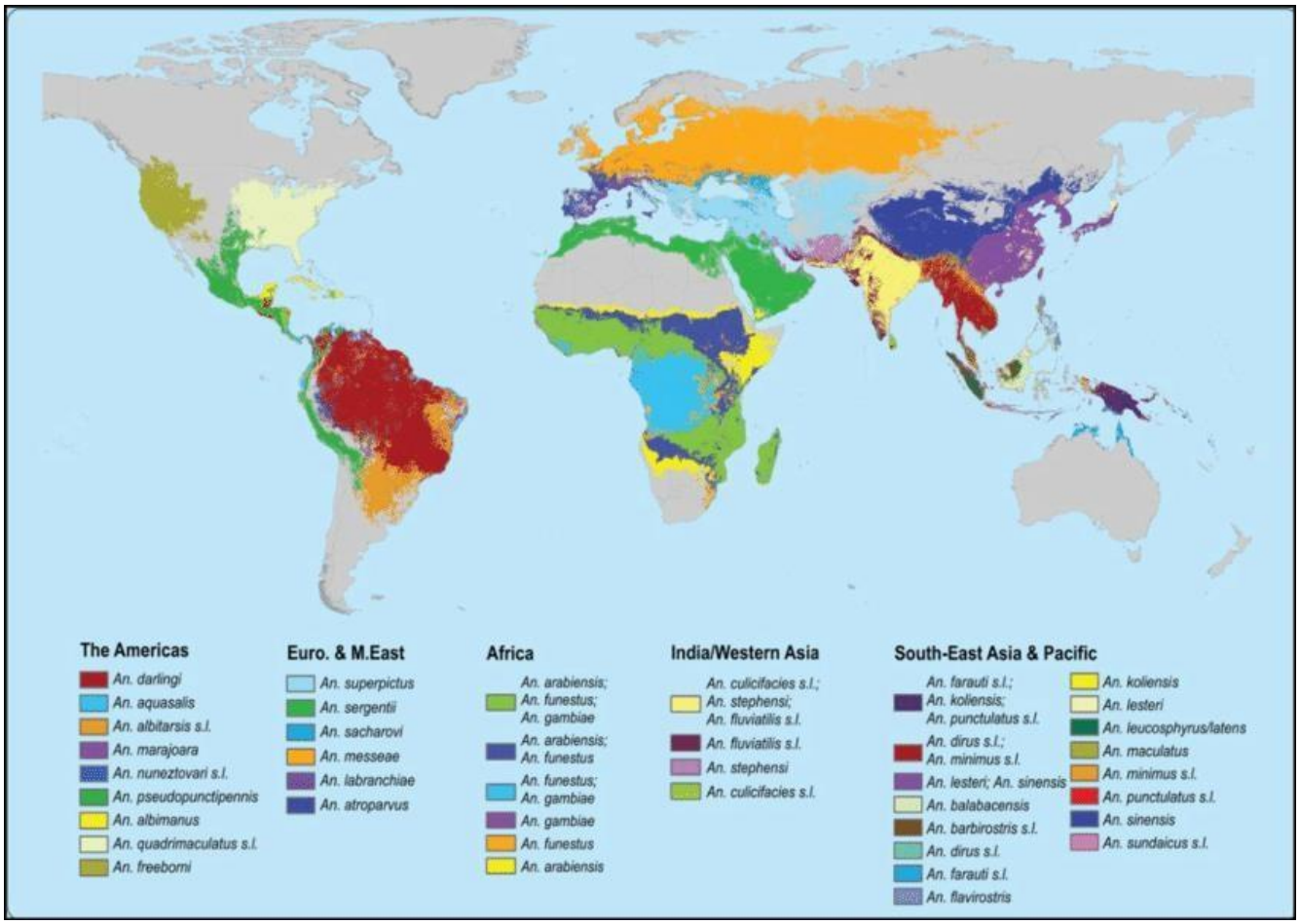


Figure 3. A global map of dominant or potentially important malaria vector species. The Anopheles mosquito is the global vector of the malaria parasite. *An. gambiae* is the predominant species especially in Sub- Saharan Africa. (Adapted and modified from Sinka et al., 2012).

Introduction to CHCs

CHCs or cuticular hydrocarbons are found on nearly all insects. This waxy layer consists of a complex mixture of lipids (Hadley, 1989). The layer helps to reduce the rate of transpiration (Wigglesworth, 1945). The hydrocarbon layer typically consists of a complex mixture of N-alkanes, methyl-substituted alkanes (the terms methyl-substituted alkanes, methyl, methylalkanes and monomethyl alkanes are used interchangeably in this study) dimethylated alkanes and unsaturated hydrocarbons (Stanley-Samuelson et al., 1988; Blomquist & Bagnères, 2010). The CHC composition is largely genetically determined (van Zweden et al., 2009).

CHC production is biosynthesised by the insect specific CYP4Gs, in specialized cells known as oenocytes. These cells are located in the abdominal walls (Qiu et al., 2012). After biosynthesis, the CHCs are bound to lipophores in the haemolymph, from where they are distributed to different body tissues, as well as deposited on the insect's surface. (Fan et al., 2002; Schal et al., 2001). However, the exact mechanism of uptake and deposition through the epidermal cells remains unknown.

The CHCs act as a desiccation barrier. There are separate types in each species. Even in closely-related species it was shown that CHC profiles hardly exhibited any of the same hydrocarbons in common (Morrison & Witte, 2011). They usually range from C23 to C37 and can also have differing positions of the double bond or methyl branch. CHCs can reflect variations in age. One study found that the CHC combination and quantity of the tropical blowfly*, Chrysomya putoria*, vary with age (Braga et al., 2016). Females of *C. putoria* were shown to have higher amounts of n-C27, n-C29 and n-C31 with increasing age, whereas males had increases in n-C29 and n-C31 and n-C33 (Braga et al., 2016). This indicates that within *C. putoria* CHCs can be a useful forensic entomology tool to identify the age of the fly. A more recent study by Moore *et al* Investigated the impact of age on empty pupa cases of *Calliphora vicina* (2017). They found a statistically significant change in CHC composition between younger and older pupa cases. The team also noted that n-C31 was most abundant in the younger 5-week pupa cases and 2-methlyhexacosane increased until month 5 where it peaked.
Whereas trimethyl-pentacosane demonstrated a U-shaped curve, where it was found in higher abundance at months 1-3 and then again at months 7-9. In both papers the GC-MS analysis is used more as a forensic device than of an indication of desiccation tolerance. This study was further supported by Beani *et al* who investigated European paper wasps, *Polistes dominula* (2019). They found that the relative quantities of CHCs were the same between sexes; however, females and males tended to have different CHC profiles. They also found that parasitic *strepsipteran* infection also influenced the CHC profiles, particularly in females. Showing that pressure from parasites can be a driver in CHC diversity in *P. dominula,* demonstrating that CHC profiles are an indicator of health and fitness. Hence, CHC profiles, along with visual signals and territorial displays come together to signal the health of the individual. Cuticular hydrocarbons are also very useful in identifying potential mates (Würf et al., 2020). The Würf study showed parasitoid wasps use CHCs to recognise females, showing that the composition of CHC profiles is typically species and sex specific. However, interestingly, the study found that artificially synthesised 7-MeC31 and 7-MeC33 (which were identified as being responsible for the sexual attraction) were not sufficient to make the males attractive to other males. Indicating that CHCs is only one aspect of sexual attraction in invertebrates.

The mechanisms behind how CHCs act as such an effective desiccation barrier is very well researched. A general outline of the impact of chain length and CHC structure on its properties is shown (**Figure 4**).

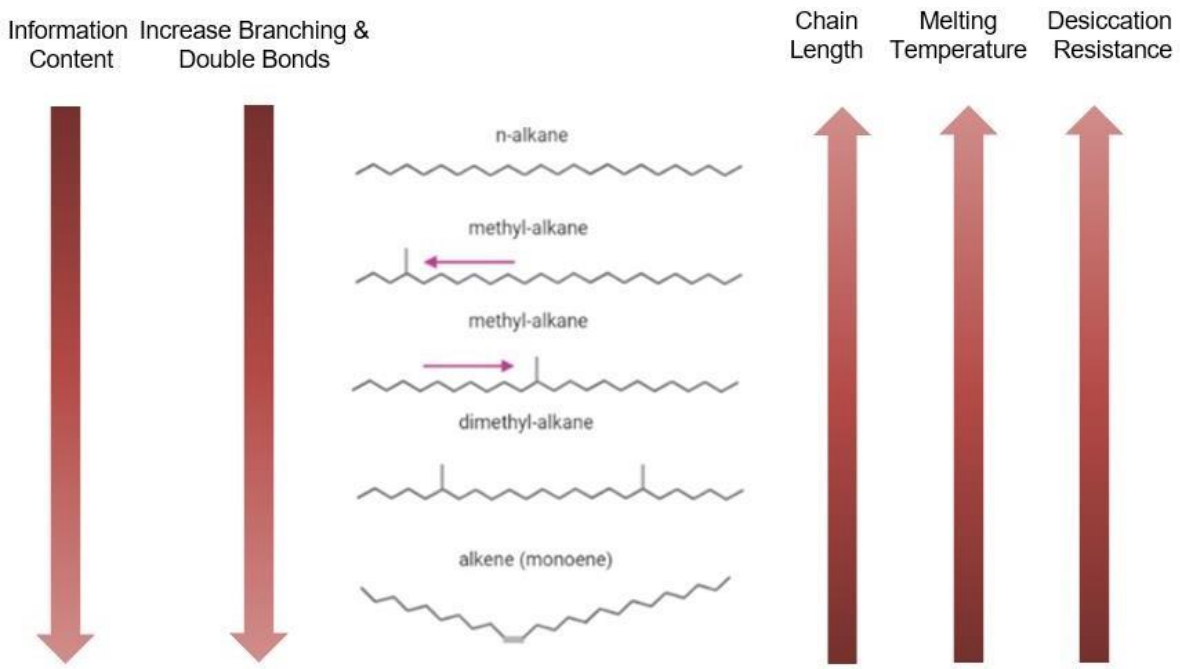


Figure 4. The general correlation between molecular structure and properties. The melting temperature increases with the chain length, independently of the structural class of the compound. Methyl-alkanes, with methyl groups located towards the end of the chain, have a higher melting point than those with methyl-groups closer to the centre (pink arrows).

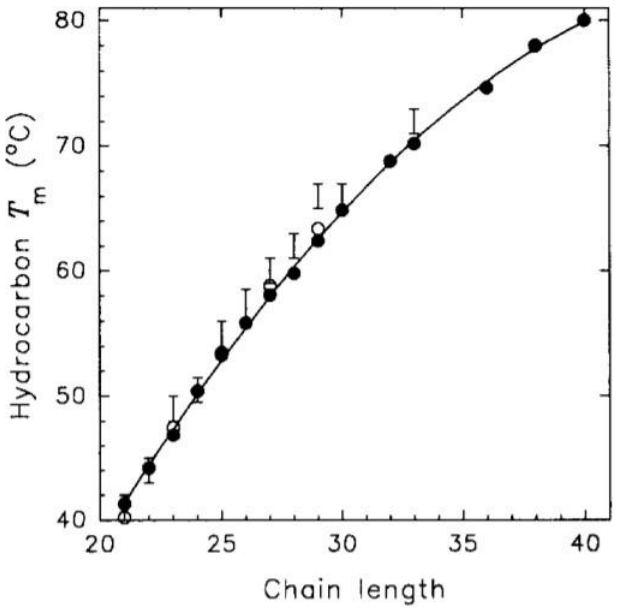


Figure 5. Increases in chain length of n-alkanes increases the melting point of the hydrocarbon, indicating stronger Van der Waals forces. The same is true for methylalkanes, dimethylalkanes and alkenes. Filled symbols indicate FTIR (Fourier Transform InfraRed spectroscopy) determinations. The vertical bars indicate Tm ranges. The open symbols are Tm values determined using differential scanning calorimetry (Figure reproduced from A. Gibbs & Pomonis, 1995).

The types and proportions of hydrocarbons within the cuticle have an important impact on the individual. Pure n-alkanes of insects normally have a melting point between 40 °C and 60 °C (Lide 2007). The melting point of n-alkanes is relatively high because alkane molecules aggregate better

than. methyl branched or unsaturated hydrocarbons due to an increase in Van der Waals forces (Brooks et al., 2015). Monomethyl alkanes have lower melting points, because the branched methyl groups prevent them packing as closely (Brooks et al., 2015). Hydrocarbons with methyl groups near the centre of the molecule (e.g., 12-MeC28) melt earlier than, for example, 2- MeC27 (Gibbs and Pomonis, 1995). A greater CHC chain length increases the melting point, as shown in **Figure 5**. However, most importantly for this study, the effect of branching is a more important factor than chain length in determining melting point and hence desiccation and insecticide resistance (**Figure 4**) (Gibbs & Rajpurohit, 2010). If the melting point is higher, it is an indication that the intermolecular forces between the compounds are stronger. This means they will be more tightly packed, reducing the number of water molecules that can leave via the cuticular layer, increasing desiccation resistance (Wigglesworth, 1945).

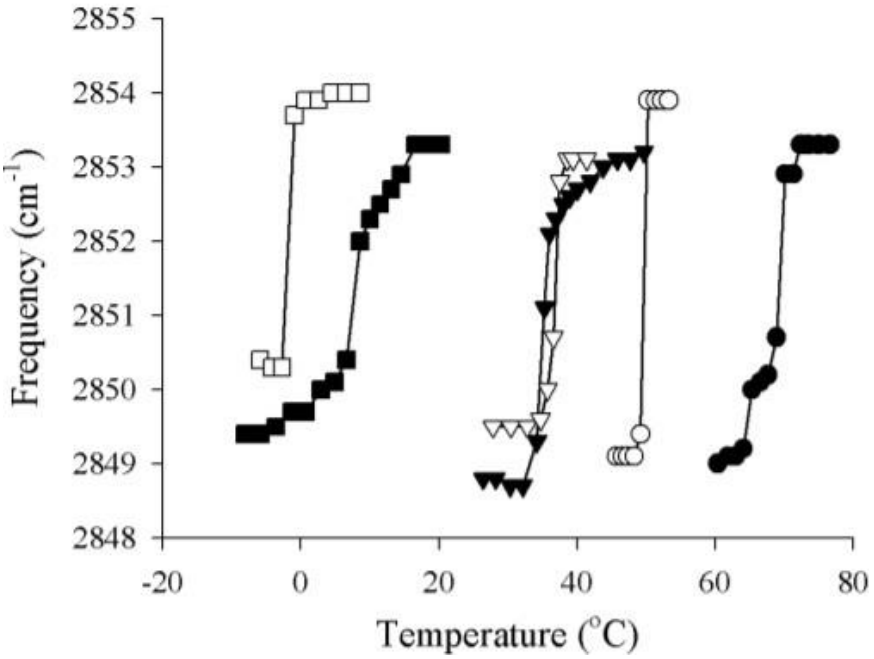


Figure 6: Effects of hydrocarbon chain modifications on melting points of similar-sized cuticular lipids.When lipids melt, the absorption frequency of C-H symmetric stretching vibrations increases. Each line represents a given CHC. This shows how the melting point changes depending on the compound, from -10 °C to 78 °C (Gibbs and Pomonis, 1995).

Figure 6 illustrates how structural differences greatly outweigh the effects of molecular size (ie chain length) on melting point. The lowest molecular weight compound shown, n -dotriacontane (MW = 450), has the highest melting point of around 69 °C while the highest molecular weight compound, oleyl acid oleic ester (MW=532), has a melting point below 0 °C. This shows that methyl branching is more closely correlated to melting point than chain length, even though every additional carbon atom in the chain increases the melting point by around 2 °C (**Figure 6**) (Gibbs et al., 1991; Gibbs and Mousseau, 1994). Dimethylalkanes tend to melt at ba lower temperature than monomethyl alkanes (A. G. Gibbs, 2002). Lastly, alkenes have an even lower melting point and are usually liquid atroom temperature (Blomquist, 2010). Their low melting point is due to the kinks in both E- and Z-alkenes, which prevents tight packing (Menzel et al., 2019; Gibbs and Pomonis, 1995).
Hence, in summary, hydrocarbons can be ranked in terms of melting points as such:

n-alkanes > methylalkanes > dimethylalkanes > n-alkenes

The more branched chain compounds and alkenes are in the CHC profile, the less resistant the pupae are to increases in temperatures and insecticides, which is shown in other species such as *Rhyzopertha dominica* (Alnajim et al., 2020). This is also confirmed in field studies, with ants from humid climates having more alkenes than ones from drier climates (Menzel et al., 2017a).

Aging and the impact it has on CHCs is important in the sexual selection process. As the CHC profile can reflect the individual's fitness and hence reproductive potential (Kuo et al., 2012). The study conducted by Kuo *et al* investigated *Drosophila melanogaster* (2012). It was found that changes in age had a direct impact on the CHCs produced, using analysis via both GC-MS analysis and desorption/ionization mass spectrometry, showing how age and sex have an impact on the CHC combination. Age has an impact on individual CHCs, overall hydrocarbon profiles shifting to favour compounds with longer chain lengths (Braga et al., 2016). Hence the older the fly, the longer the hydrocarbons. They also observed that an increase in age correlated to a decrease in sexual attractiveness. These studies demonstrate that CHC production is a reliable indicator of animal health and fertility. CHCs can also be used as a good indicator of mating status (Everaerts *et al*, 2010). Sex specific variations in CHCs were noted with age, as well as alterations in the profiles depending on whether the male or female was a virgin.

Also, CHCs indicate the quality of potential sexual mates. Research has also shown that CHCs are used by insects to identify whether the individual insect is a member of the colony. A study on *Hymenoptera, Vespidae*, showed paper wasps discriminated against individuals that did not match the CHC profile of the colony. Even slight changes in the profile can be detected (Tannure-Nascimento et al., 2007). This is hypothesised to allow the colony to detect invaders and defend against usurpation. CHCs are also used in social insects to inform task decisions (Greene & Gordon, 2003). Red harvest ants, *Pogonomyrmex barbatus* have no central control and hence must organize tasks such as nest construction and foraging using CHCs. Therefore, ants carrying out different tasks will have a slightly different CHC profile (Greene & Gordon, 2003).

It has also been shown that diet can have an impact on CHCs (Fedina et al., 2012). Fedina et al showed that this is because of the diets impact on aging and the activity of nutrient sensing pathways in Drosophila (2012), showing that dietary yeast and sugar can drive changes in CHCs. For instance, yeast in the study was shown to increase short-chain CHCs by decreasing insulin signalling.

CHC's can also be used as a form of chemical camouflage (Whitehead et al., 2014). One study on the beetle *Piezogaster reclusus* found they either use chemical camouflage, chemical deterrence via metathoracic dense glands or behavioural traits to deter ants from attacking them. The study concluded that only cuticle compounds appeared to be essential in allowing *P. reclusus* to feed on Bull-horn acacia trees undisturbed. In a further study Endo and Itino showed that *Myrmecophilous* aphids, a species of aphids that are farmed by ants, produce CHCs that resemble those of their tending ants (2013). This CHC mimicry is an antipredation strategy stopping the ants from preying on them and allowing the symbiotic relationship to continue.

Time of day and social environment can impact CHC combination with the latter involving the direct transfer of CHCs between individuals. A study by Kent *et al* showed CHC profile of the *D. melanogaster* males is directly affected by social interaction, with flies that have more social interaction together having more similar CHC profiles (2008). Whether they were kept in constant light or dark was also shown to have an impact on chemical cues (Kent et al., 2008).

A good model for conditions of drought or reduced water will be the use of water with increased salinity mimicking the natural evaporation of water during dry seasons. A study on the water beetle, *Nebrioporus Enochrus* found there were longer chained CHCs in the species that showed a higher

salinity tolerance (Botella-Cruz et al., 2019). Higher salinity tolerance was also associated with increase in the relative abundance of branched alkanes and with a lower proportion on n-alkanes and unsaturated compounds (Botella-Cruz et al., 2019), suggesting that reducing cuticle permeability was one of the key mechanisms to adapt to higher salinity waters. Saline species also demonstrated the ability to adjust CHC profiles to changing salinities in a relatively short time (Botella-Cruz et al., 2019). As of yet there have been no robust studies on the relationship between CHCs and water salinity in mosquito larvae or pupa.

From an experimental point of view cuticular hydrocarbons are also incredibly stable (Martin *et al*, 2009), so much so that CHCs have been shown to maintain their structure and species-specific profiles after 20 years (Martin et al., 2009). This stability allowed us to handle CHC samples with relative ease without the need for them to be stored in climate-controlled environments or under specialised conditions.

CHC in mosquitos

A lot of research has taken place on the role CHCs play in the desiccation of Drosophila. However, far less research has taken place with the relationship between CHCs and desiccation in mosquitos despite their huge impact on human health in developing countries. However, studies have been conducted on the composition of cuticular hydrocarbons in *An. gambiae* by age and sex (Caputo et al., 2005). The study found forty-eight cuticular hydrocarbons on the epicuticular surface on *An. gambiae* using GC-MS analysis. The study further identified 14 n-alkanes, 16 monomethyl alkanes, 13 dimethylalkanes and 5 alkenes with the main chain lengths ranging from C (17) to C (47). These results were consistent with results from other mosquito species such as Culicidae (Horne & Priestman, 2002). It was also shown that CHC profiles change between sex. Both sexes of *An. gambiae* undergo strong CHC profile changes with age and individuals aged between 0-2 having a remarkably different CHC profile in comparison to older individuals (Carlson & Service, 1980). The study also managed to use CHCs to correctly assign sex more than 85% of the time with females and 75% with males. Differences in CHC profiles between *An. gambiae* complex were also found, when age and sex were controlled (Carlson & Service, 1980). Nineteen-day old female *An. gambiae*, have on average, 1.5 times more CHCs than newly emerged females, again highlighting the impact of age on CHC profile (Wagoner et al., 2014a) and indicating that gas chromatography can be used to identify species, for example determining the difference between *An. gambiae* sensu stricto and *An. arabiensis* (Carlson & Service, 1980).
CHCs can also be use forensically in mosquito populations predicting age and hence derive malaria transmission rates (Brei et al., 2004). Nonacosane (C29) and hentriacontane (C31) change significantly with respect to the mosquito's age. This ratio of C29 to C31 can be calculated and is a good predictor of whether the mosquito is old enough to transmit the malaria parasite (Brei et al., 2004). These studies centre more around the use of CHCs as a form of forensic taxonomy.
The role of CHCs as pheromones during mosquito courtship has also been. Mating alters the cuticular hydrocarbons of female *An. gambiae* investigated (Everaerts *et al*, 2010). Analysing the mosquitos before and after mating found that the proportions of two hydrocarbon components n-heneicosane and n-tricosane were significantly reduced after mating. However, there were no changes in CHC profiles after the males mated. When CHCs were extracted from individuals that had mated and were applied to unmated females a decrease in rate of insemination was noted (Everaerts et al., 2010). Inseminated females were also shown to have 58% more CHCs than virgin females (Wagoner et al., 2014a). Indicating that CHCs may play a role in chemical communication during courtship as well as desiccation.
Arcaz *et al* investigated the tolerance of *An. gambiae* and *Anopheles coluzzii* to desiccation (2016). Six CHCs accounted for 71% of the variation in desiccation tolerance and three accounted for 72% of variation in the rate of water loss. Wild *An. coluzzii* also showed increase desiccation tolerance

during dry seasons, indicating CHC composition in *An. coluzzii* changes depending on the season. The study by Arcaz *et al* concluded that the primary mechanism of desiccation tolerance is the combination and amount of CHCs, with spiracle size playing a less important role in desiccation tolerance (2016). In female adult *An. gambiae* it was noted that mosquitos subjected to dry conditions had a greater quantity of CHCs. However, total amount of CHCs alone did not increase desiccation tolerance. This was required in tandem with other factors to increase tolerance. The study also showed that mosquitoes subject to desiccation beforehand survived longer in high humidity condition suggesting mosquitos maybe able to alter their CHC composition rapidly in response to sudden changes in climate (Arcaz et al., 2016).

Introduction conclusion: hypotheses and design of experiments

The literature that has been reviewed for this project outlines various gaps in knowledge, which I aim to address within this research. It is clear from the literature that there are very few papers that explore the impact of different variables on the pupal stage of *An. gambiae*. Hence, investigations on pupae will be the pivotal for these experiments. From the literature reviewed, age and sex play an important role in CHC expression (Caputo et al., 2005). The tested hypothesis therefore seeks to see if age and sex equally play a critical role within *An. gambiae* pupae like it does with adults. The literature review shows that age plays one of the biggest roles in CHC expression (Cuvillier-Hot et al., 2001). Therefore, from an experimental design perspective, it will be important to test the relationship between age and CHC expression in *An. gambiae* pupae before testing the impact of other variables on CHC expression. Further to this, the literature also indicates as well as age, sex plays a crucial role. Hence, by sexing pupae I will aim to observe if this difference in CHC expression based on sex occurs to the same extent in pupae**.** Much of the published research on how desiccation impacts CHC expression in the Anopheles genus focuses on one form of desiccation in terms of low humidity, high temperature and absence of water to induce the desiccation stress. However, it may also present a novel area of research to see how other forms of orthogonal desiccation, such as water salinity, impact CHC expression. This is possible because unlike the adults the pupal stage is an aquatic life stage. This may add to our understanding of how adaptable and resilient every life stage of *An. gambiae* is to different forms of desiccation. As of writing, there are no papers concerning *An. gambiae* pupae and how desiccation impacts their CHC profiles. Therefore, an investigation on how age, sex and different forms of desiccation impacts CHC expression within the pupal stage of *An. Gambia* may provide a fertile area for novel research.

2. Materials and Methods

2.1 Mosquito rearing

Laboratory specimens used were *An. gambiae* G3 kept in an insectary at Durham University. The insectary incubator (FitoClima 5.000, Aralab) had the following conditions: T=27 ± 1 °C, relative humidity=70% (night) - 75% (day), 12 h dark: 12 h light. The lights turned on at 7am and off at 9pm with no progressive light reduction. Larvae were reared at a density of 100 larvae per 1000 ml of water in plastic boxes with deionised (DI) water with added Blagdon tonic salt at a ratio of 10 g per 10 liters ('standard rearing solution'). The standard rearing solution used was prepared in 10 litre batches and stored overnight in a sealed plastic bucket in the incubator to climatise. The larvae were fed with a food pellet of the Nishikoi Staple Food Small Pellet, 1 per day per tray, given at 1pm. Adults were provided with a 10% sucrose solution continuously. Mated adult females were fed sheep blood using temperature-controlled membrane-feeding devices. Eggs were collected from the resulting gravid females by providing them with a cup of water containing wet filter paper on which to deposit their eggs. Freshly deposited eggs were collected by providing mated, gravid females with wet filter paper as an oviposition substrate for 15-20 min. All mosquitos were from the same genetic line, the *An. gambiae* M-form strain Ngouss. This was done to reduce the degree to which genetic diversity could have an impact on variability in the mechanism of CHC production and therefore the profiles produced. Further to this, the laboratory colony was derived from a small population size. Therefore, they are subject to founder effects upon establishment, minimising the pool of genetic variation within the lab colony (Ranford-Cartwright et al., 2016). Inbreeding leads to reduced variability between CHC profiles. This has been previously observed in the ant species *Camponotus aethiops* and *Hymenoptera opacior* (Menzel *et al.*, 2016; van Zweden *et al.*, 2009). Therefore, it can be assumed that genetic variance plays a negligible role in determining CHC profiles within this investigation compared to the variables that were investigated.

2.5 Experiment 1: effect of age on cuticular hydrocarbon profile of *An. gambiae* pupae

This experiment was designed with the aim to identify whether CHCs change with the age of the pupae.

Pupae were collected from 11am to 9pm Monday to Friday. Newly formed pupae were collected from the rearing trays every hour, giving a maximum uncertainty of the age of the pupae of 1 hour. The pupae were collected with a plastic Pasteur pipette and transferred to a 100 ml plastic cup. Afterall the pupae for that hour were collected, an additional 100 ml of water was added to the cup, the cup was labelled with the date and time of collection and left in the insectary in the same conditions as in the rearing trays. The collected pupae remained in their cups in the insectary for a given amount of time ranging from 1 hour to 30 hours. The pupae were then taken out of the cups and were placed on filter paper to dry. They then were transferred to another filter paper, until there were no traces of water left on the filter paper itself. The pupae were dried thoroughly because residual water may have interfered with the hexane extraction of cuticular hydrocarbons at a later stage. The pupae were then transferred from the dry filter paper to a 1.5 ml Eppendorf tube, placed into the -20 °C freezer and stored there until enough samples were collected. The samples were then taken from the freezer and placed on ice for sorting. The frozen pupae were organised into 15 groups as shown in **Table 1**.

Sample Name	Age	Number of Pupae	Sample number
KHR001	1 hour	20	1
KHR002	1 hour	20	2
KHR003	1 hour	12	3
KHR004	1 hour	20	4
KHR005	1 hour	20	5
KHR006	2-3 hours	20	6
KHR007	4-5 hours	18	7
KHR008	6 hours	16	8
KHR009	19-20 hours	20	9
KHR010	21-23 hours	21	10
KHR011	24-25 hours	17	11
KHR012	26 hours	16	12
KHR013	27 hours	21	13
KHR014	28 hours	23	14
KHR015	29-31 hours	20	15
KHR016	control	0	

Table 1. Samples for Experiment 1: effect of age on the CHC profiles. Colours indicate grouping for **Figure 15**.

2.6 Experiment 2: effect of salinity on the cuticular hydrocarbon profiles of *An. gambiae* pupae

The purpose of this experiment was to investigate the effects of desiccation on the CHC composition. The desiccation was achieved by keeping pupae in water with increased salinity (Riedl et al., 2016).

For this experiment I used solutions of sodium chloride (10428420, Fisher Scientific) at 0, 50, 100, 150 and 200 g/L in DI water. The solutions were kept in 1l bottles in the rearing incubator to allow the bottle to reach 27 °C and for the salt to fully dissolve. The salt fully dissolved at all concentrations used.

Once the pupae had been collected (as described above for **Experiment 1**), they were immediately placed in a 100 ml cup with a given salt solution and left there for 24 hours. I ensured that only a minimal amount of water from the rearing trays was used to transfer pupae into the salt solution cups, thus minimally changing the concentration of the salt solution. The pupae were taken out of the salt solution after 24 h and placed into an Eppendorf tube using the same technique to remove water as in **Experiment 1**, and frozen at -20 °C. If some pupae died during the 24 h in the solution, this was recorded, and the dead pupae (completely uneclosed, meaning the pupa case was still sealed) were collected and stored separately from the live ones. Once enough samples were collected, the pupae were sorted into groups of 10 pupae for GC-MS analysis as the GC-MS results for **Experiment 1** indicated that the sizes of groups could be reduced from 20 to 10 individuals and still yield good quality data. **Table 2** shows the sample groupscollected for **Experiment 2.**

As one can see from **Table 2** there is a significant imbalance between the number of samples from the intermediate ranges, (50 – 150 g/L) compared to the extremes, (0 g/L & 200 g/L). This is because the initial aim of the experiment was to see if in fact salinity had an impact on CHC expression, and not the rate to which changes in salinity concentrations impacted CHC profiles. It was already shown that if salinity did impact CHC expression, the greatest degree of impact would be at the most extreme salinities (Botella-Cruz et al., 2019). Therefore, many more samples were submerged in concentrations of 200 g/L to allow for the results to conclusively show whether or not salinity does impact CHC expression. This was done in case salt concentrations of <200 g/L were not high enough to trigger noticeable changes in CHC profiles. Preliminary experiments on *An. gambiae* showed that the maximum salinity the pupae could tolerate was around 200 g/L, anything much above this led to the mortality rate being too high, decreasing the number of samples that could be collected. Even at 200 g/L, 77 pupae, died which therefore meant this experiment could not be directly compared to the data from the live pupae from other salinities.

A

Sample name	Salinity (g/L)		Number of pupae
C1	0		10
C2	0		10
C3	0		10
C4	0		10
C5	0		8
C6	50		10
C7	50		10
C8	100		10
C9	100		10
C10	150		10
C11	150		10
C12	200		10
C13	200		10
C14	200		10
C15	200		10
C16	200		10
C17	200		10
C18	200		10
C19	200		10
C20	200		10
C21	200	Dead	10
C22	200	Dead	7
C23	200	Dead	10
C24	200	Dead	10
C25	200	Dead	10
C26	200	Dead	10
C27	200	Dead	10
C28	200	Dead	10
C29	Control		

B

Sample number	Salinity (g/L)	Alive or Dead	Number of pupae per sample
1	0	Alive	10
2	0	Alive	10
3	0	Alive	10
4	0	Alive	10
5	0	Alive	8
6	50	Alive	10
7	50	Alive	10
8	100	Alive	10
9	100	Alive	10
10	150	Alive	10
11	150	Alive	10
12	200	Alive	10
13	200	Alive	10
14	200	Alive	10
15	200	Alive	10
16	200	Alive	10
17	200	Alive	10
18	200	Alive	10
19	200	Alive	10
20	200	Alive	10
21	200	Dead	10
22	200	Dead	7
23	200	Dead	10
24	200	Dead	10
25	200	Dead	10
26	200	Dead	10
27	200	Dead	10
28	200	Dead	10

Table 2: (A) Samples analysed for **Experiment 2**: effect of desiccation by increased water salinity on the CHC profiles. (B) Samples for **Experiment 2**. Conditions that each sample was kept at, the number of pupae per sample and whether they died. The colours correspond to **Figure 17**.

2.7 Experiment 3 & 4: effect of desiccation on the cuticular hydrocarbon profiles of *An. gambiae* pupae

The purpose of **Experiments 3** and **4** was to investigate how the CHC composition of a pupa changes when the pupa is fully removed from water and desiccated in air.

Experiment 3 was a precursor to **Experiment 4**. **Experiment 3** differs from **Experiment 4** in that the age of the pupae was determined approximately, by taking the average time of the pupae life stage **(Table 3)** subtracted by the age of the pupae when desiccation began. However, in **Experiment 4**, I was able to use a video camera to record the exact time of eclosion more precisely, which gave a more exact length of time the pupae spent desiccating before eclosion.

Length of pupal stage experiment

Before the start of **Experiment 3**, I carried out an experiment to identify the average length of the pupal life stage of *An. gambiae* in our lab. The recording was set up in the main insectarium incubator. The time that the larvae pupated was recorded by using the same method as for **Experiment 1**.

Figure 7. Images of how the pupal period was recorded.

The pupae were left for 24 hours in the normal 100 ml plastic cups as described above. After 24 hours the pupae were sexed and split into mini petri dishes based on their time of pupation (See **Figure 7**). Enough water was added to each petri dish to give a 1 cm water depth. A USB camera (B089D6DBCX, DEPSTECH) was set-up above the pupae and the recording was started using *SharpCap* image capturing and processing software. The software was set to run for 12 hours, recording in intervals of 30 minutes, the recording lasted for 5 minutes for each interval. 30 minutes was chosen as it provided the highest frequency of intervals over the 12-hour time frame without filling up the memory capacity of the computer used for this experiment. After the experiment was completed, the recording was watched back to determine the time of eclosion (the process of moulting of the cuticle layer birthing the adult *An. gambiae*) for each pupa.

Age, h	Female	Male
30	1	1
30.5	1	0
31	2	2
31.5	0	0
32	1	2
32.5	1	3
33	1	0

Table 3: Age of pupae at time of eclosion. After 33 hours all pupae had eclosed.

The data in **Table 3** supports the estimate that the pupal lifecycle lasts between 30-33 hours. This was also seen in **Experiment 1**. The average length of the pupal stage for females was 31.5 h. The average length for males was 31.7 h. Running a multiple regression on these results yields a P-value of 0.68, where P values about 0.05 were not significant ($P<0.05$) showing there is no significant difference between the length of the male pupal stage compared to the female pupal stage. The total average length of males and females togetherwas found to be 31.6 hours in water at 27 °C.

Experiment 3

Pupae were collected from trays, as described for **Experiment 1**, and were then transferred to a 100 ml plastic cup filled halfway with water from the standard rearing solution from the bucket in the incubator. The cup was then labelled with the time that the pupae were collected and was left in the rearing incubator. Trays were checked every hour on the hour, collection started at 11am and lasted until 8 pm. Once all the pupae for the day had been collected, they were all sexed together using the technique outlined below (**Figure 8**) with the Zeiss SteREO Discovery V20 microscope.

Sexing of pupae was done in 3 stages to reduce the time which the pupae spent out of water. This was done to stop the pupae from desiccating before the experiment had begun. The 100 ml plastic cups containing pupae were carried out of the incubator and placed near the microscope for sexing. The pupae were kept in water next to the microscope while sexing them, which could last up to an hour. The room where they pupae were sexed had T=24 ± 1 °C, relative humidity = 32.1%, but the low humidity is unlikely to pose an issue as the pupae were kept submerged in water throughout the sexing process. The pupae were moved from one plastic cup and placed into a clean petri dish. The excess water was then removed from the petri dish, but a sufficient amount was left surrounding the pupa in order to prevent desiccation and erratic jumping behaviour, which makes pupae difficult to sex. Water removal was done to reduce the movement of the pupae while sexing, making them easier to handle under the microscope. The pupae were placed underneath the microscope lens using a fine tip brush. The pupae were gently turned until the paddles on the tail of the pupa were visible. It could then be discerned whether the pupa is male or female with a high degree of certainty using the criteria in **Figure 8**. However, there were instances where the sex could not be discerned from the tail due to developmental defects or damage to the paddle. In this case the pupa was discarded.

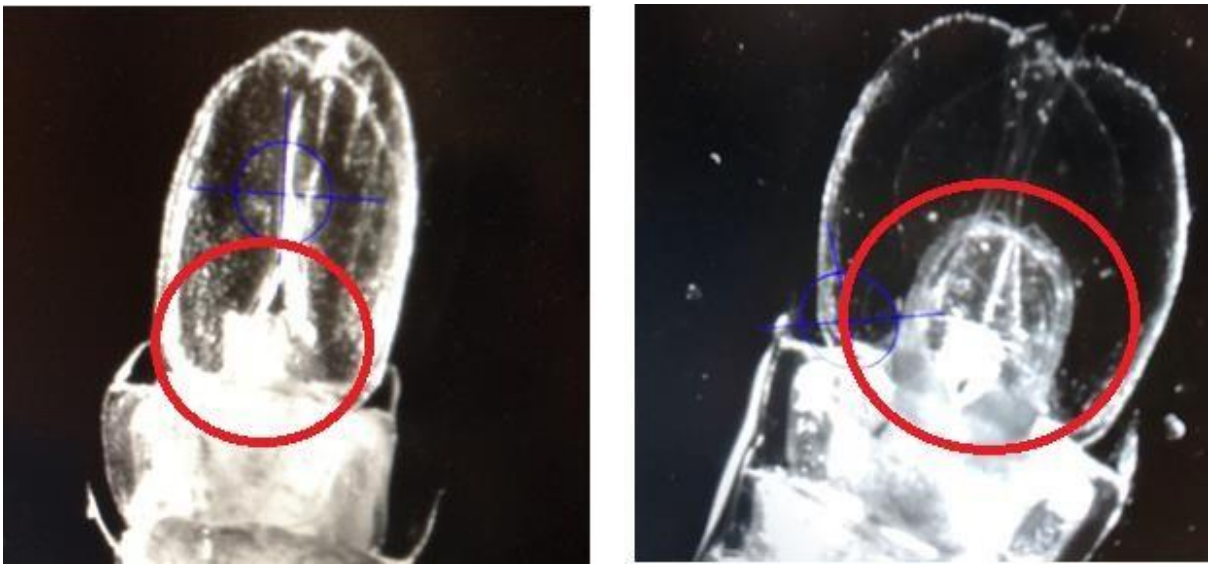


Figure 8. Sexing pupae. Left: a female tail with a much smaller middle paddle than the male (right).

After the pupae had been split into males and females, they were placed into two separate mini-Petri dishes (35x11mm) (0619197005172, ADFEN) labelled with the sex and time of pupation. They were then re-submerged in water until all samples for the day had been sexed. Once sexed, the individuals were desiccated. Pupae were removed from the mini petri dish using a pipette, excess

water was removed from the pipette until only the water surrounding the pupa was left. These pupae were then deposited onto filter paper, allowing the water to soak into the paper. Once all pupae were added, they then were moved to another filter paper where the same drying technique as mentioned in **Experiment 1** was used. Timing was important as the pupa should be left long enough for all excess water from the surface of the pupa to be removed but not left too long as the pupa will begin jumping, making the handling of them more difficult, as mentioned previously.

For **Experiment 3**, the pupae were dried (using steps mentioned above) and placed into dry mini petri dishes labelled M and F to distinguish between sexes. They were then placed into a larger plastic box 20 x 5 x 5 cm (Really Useful Box) closed with a lid to stop adult mosquitos from escaping. Four holes were made in the lid to allow for air flow. The lid was later changed in **Experiment 4** to a net covering (B09FJ1QHWW, Generic) to allow for more air flow and better equilibration between the air in the incubator and the air in the box containing the mosquitos. Once the lid or net was fastened on the plastic boxes, the boxes were placed in the incubator (IPP30, Memmert). The humidity in the incubator was maintained at around 99% RH, using a tray with a saturated solution of potassium sulphate in water. This was selected following readily available information on different salt solutions yielding different levels of humidity (Greenspan, 1977). It was discovered that humidity below 83% RH (see **Figure 31**) led to a dramatic increase in the mortality rate, leaving few live pupae to collect. Hence 99% was selected as it was the humidity with the lowest mortality rate of the desiccating pupae. Throughout the experiment there were slight changes in humidity and temperature and hence humidity and temperature were recorded in real time using a sensor (HT1, SensorPush). Humidity normally fluctuated between 96% and 99% with the majority of experiments staying constantly at 99%. However, for 3 samples the average humidity was 83.5%, where the mortality rate was 100%. These samples were not included in the final analysis.

The time that the pupae were placed into the incubator was recorded and the age of pupae when placed in the incubator for desiccation was also recorded. The pupae were left overnight and dead pupae or eclosed adults were collected the following day. Time of collection was recorded. The numbers of adult mosquitoes, dead pupa, half-hatched, and cases (or exuvia, which are the remains of an exoskeleton after eclosion) was also recorded. All samples including adults and pupa were moved immediately from the incubator to the -20 °C freezer.

Experiment 3 estimates the age of the pupae from when they were placed into the incubator for desiccation. However, it was not possible to record inside the Memmert incubator due to the parameters of the incubator. It was impossible to keep temperature and humidity constant and allow cables for both cameras and a light source within the incubator. It is hence assumed that the older the pupae were when placed into the incubator, the less time the pupae spent desiccating. That being said, due to the slight range in pupal stage length between individuals (see **Table 3**), this will not be true for every sample. The preliminary experiment (see section on “Pupation timing”) was conducted to allow us to subtract the age of pupae when placed in the incubator from 31.6 hours (which is the average lifespan of the pupal phase, see **Table 3**) in order to yield an estimate for the time spent desiccating. However, it should be noted that due to the information from **Table 3** being gathered in water and a relatively small sample size these estimates will have a large degree of uncertainty attached to them.

It is also important to note that deciding to control for sex was an improvement made in **Experiment 3** during the experiment and hence 132 samples are unsexed, 105 were sexed from adults and rely on antennae sexing and 308 were sexed as pupae as described above. The data from unsexed samples was not mixed with data from adult sexing or pupal sexing. Each sample for **Experiment 3** was comprised of 3 pupae as shown below.

Female pupa grouping	Age (hours)	Number of pupae per group
F1P	19	3
F2P	20	3
F3P	21	6
F4P	23	12
F5P	24	33
F6P	25	6
F7P	26	9
F8P	27	3
F9P	28	6

Male pupa grouping	Age range (hours)	Number of pupae per group
M1P	21-24	3
M2P	24	3
M3P	25-26	3
M4P	27	3
M5P	29	3

Table 4. The number of individual pupae in each sample for **Experiment 3**

The quantity of pupae that were collected at each age group was not constant as seen in the column, "number of pupae per group" (**Table 4**). this is because pupations were more common at certain times of the day (see **Figure 15**). However, each group that was extracted had a maximum of 3 pupae in each vile, the rest were stored in the freezing in case repeat were needed for a particular age. For example, sample F5P had 10 backup samples. **Table 4** only includes data from **Experiment 3** where age of the pupae is record but that exact time in pupa form in the incubator is not unlike **Experiment 4**.

For experiment 3 & 4 the status of the pupa after it had been desiccated was categorized into 3 main groups as shown in **Figure 9**. Half-hatched (**Figure 9A**)**.** Here the case had opened, however, the adult mosquito could not completely emerge from the case and normally it was found dead with the head outside the case but the abdomen remaining stuck inside the pupal case.; 2) Pupal cases (**Figure 9B**). Here the pupa survived, and the adult successfully emerged from the case; 3) Dead pupa (no image). Here the pupa has died during the desiccation experiment and did not hatch. They tend to be slightly darker on collection but for the most part were very similar to live pupae.

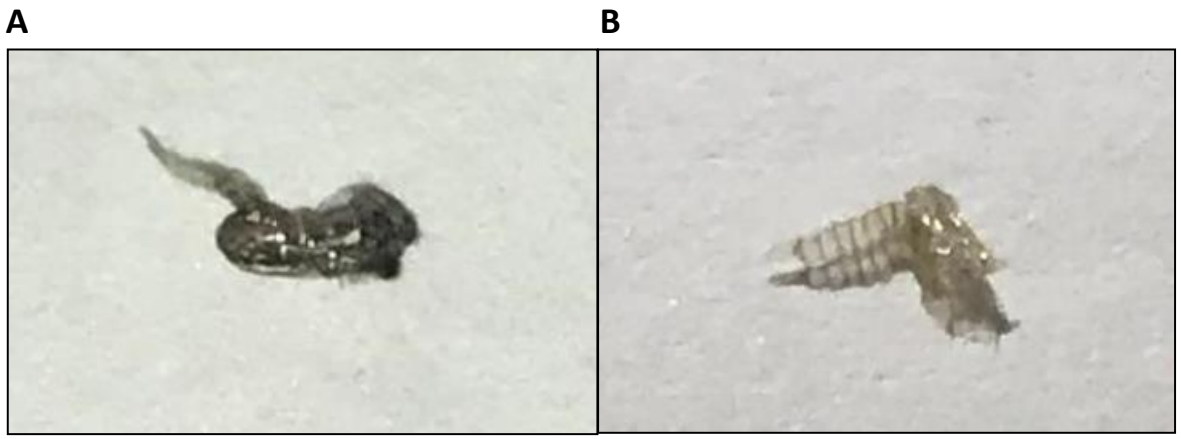


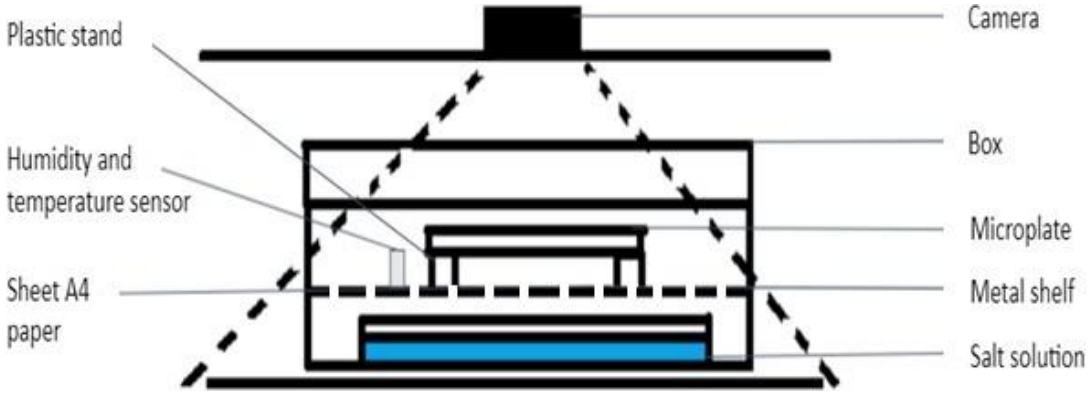
Figure 9. Shows the difference between a half-hatched case, where the adult has only partially emerged (A), and an empty case where the adult has successfully emerged completely (B).

Experiment 4

Experiment 4 follows a very similar format to **Experiment 3**, the crucial difference is that during **Experiment 4** I was able to more accurately record the time that the pupa spent desiccating.

As mentioned before, I used the insectarium incubator (FitoClima 5.000, Aralab) to allow sufficient space for the recording setup as shown in **Figure 10**.

A



B

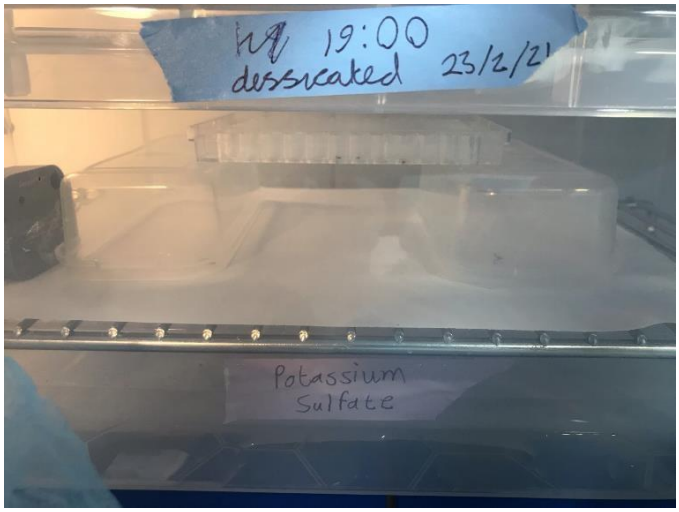


Figure 10. Recording setup for the **Experiment 4**. The pupae were contained within their own sealed microenvironment within a plastic box within the larger incubator. The pupae were placed on two boxes on top of a metal grate to allow for air flow within the box.

The incubator temperature could not be changed (because the change would affect mosquito lines reared in the same incubator), and hence remained at constant 26.8 °C. However, humidity for the experimental pupae could be controlled using a 10-litre plastic box. A solution of potassium sulphate was placed into the box to regulate humidity. An A4 piece of paper was inserted below the stand to allow for better contrast between the pupa and background and hence make it easier to identify when the pupa had eclosed. A 96-well microplate (655094, Greiner) was also placed onto a stand to ensure the pupa were not in contact with the paper and hence no water droplets formed inside the well stopping the desiccation. The stand also allowed for better air circulation making it more probable that the humidity within the wells was the same as which the sensor was recording. Before

any samples were recorded, the box was left overnight to allow the humidity to reach a stable level of 99%.

The pupae were collected using the same method as for **Experiment 1**, the age and sex of the pupa was noted. The pupae were dried in the same manner as for **Experiment 3**. After being dried, the pupae were placed into a 96-well microplate with one individual pupa occupying one well as shown in **Figure 5**.

Figure 11. Individual pupae are confined to one well of the microplate.

This allows for eclosion of an individual pupa to be more easily noticed and to ensure the sex and age of individual pupa are not confused with another.

Once the pupae were placed into the well, plastic film (8011529145600, ES-pack) was added to the top of the microplate to stop adult mosquitos from escaping. Two small holes were then made per well in the film to allow air flow. The microplate was then quickly placed into the box and the lid of the box was closed. A light (B08613S3TD, SLATOR) was switched on near the box and shone onto the microplate. Before starting the recording, the position of the light and box were both adjusted to ensure the best image quality for the video recording. Once the pupae were clearly visible, the recording began. The pupae were left overnight in the climate-controlled box. In the morning the microplate was removed from the box and immediately placed into the -20C freezer. The time at which the samples were frozen was noted.

Once enough samples were collected, they were sorted and grouped. The frozen adults, pupae and exuvia were sorted into groups composed of 3 individuals, categorised by proximity in time spent desiccating. Time spent desiccating is defined from the start of the recording to when the pupa eclosed. For example, in sample F3P all pupae spent the same time desiccating, 6 hours. However, for other samples individuals grouped together had slight variances in the time each individual spent desiccating i.e., 7.5 h, 6.5 h and 9.5 h were grouped together in sample M2P.

2.2 Cuticular hydrocarbon extraction

After **Experiments 1-4** were conducted on the pupae, the pupae were usually frozen directly after the experiment under conditions of -20 °C and humidity 22.8% in the laboratory freezer (Lec Medical,LSFSF232UK). Freezing reduces rate of decay of samples and better preserves chemical composition (Gunn, 2019). Freezing the CHC samples at -20 °C is a common practice in the literature even though CHCs are already incredibly stable (Martin et al., 2009). Despite this freezing the CHCs is a practice still used in studies as recent as 2022 (Golian et al., 2022). The frozen pupae were sorted into groups of similar experimental conditions (ages, salinity, etc.) before extraction. Grouping of samples was done to increase the concentration of CHC extracted, giving a better signal to noise ratio of the chromatography data. The pupae were transferred from plastic Eppendorf tubes into glass screw neck 1.5 ml vials (CZT, 451100011). Hexane (VWR, 1.04371.2500) was added into the glass tubes using glass Pasteur pipettes (Fisher, 747720), enough to completely submerge all pupae in the vial. The extraction was conducted for 10 mins (Menzel et al., 2017), after which the hexane was pipettedout of the vial and into a secondary glass vial, leaving the pupae behind. For each extraction a new Pasteur pipette was used. The hexane solution was then allowed to evaporate, leaving the extracted CHCs as dry residue on the sides of the vial. When no liquid was remaining in the vials, they were capped with screw top caps (CZT, 3111C3010) and placed in a -20 °C laboratory freezer. The samples were transported by air at ambient temperature to the University of Würzburg, Germany, for furtheranalysis.

2.3 Cuticular hydrocarbon processing

Samples in the form of evaporated hexane residue were brought over from the UK to Germany in sealed CZT screw neck vials held within a neoBox 10 X 10 cm (NeoLab, 2-1940). The samples were first extracted by reintroducing hexane to re-dissolve the CHC residue in the vials. The hexane was reintroduced by using 230 mm Pasteur Pipettes (Best Nr Brand, 747720), adding around 1 cm^3 of hexane to each vial. The vials were then swirled, ensuring that the hexane completely dissolved the CHC residue on the sides of the vial. The hexane was then pipetted from the vial into 6x30 mm glass inserts (Artikel-Nr u-Einsatz, 501106012) and the insert was placed back into the vial. For each sample a fresh pipette was used to ensure no cross contamination. To increase the strength of the CHC signal when analysed, the concentration of CHCs in comparison to the solvent (hexane) in each sample was increased, by placing the insert under a CO_2 pump to increase evaporation of hexane before it is placed in the GC-MS machine. The ideal concentration of hexane left within an insert is usually 0.5cm^3. However, the amount reintroduced could depend on the concentration required: If previous runs of the GC-MS machine indicated a greater concentration will yield a clearer GC-MS spectrum, then volume of hexane added can be reduced. The same GC-MS machine (Agilent Technologies, 7890A GC system) was used for all experiments. GC screw-top vials (Agilent Technologies, G4513-68001) were placed into the sampler (Agilent Technologies, 7693 autosampler). Hexane and dichloromethane vials (DCM) (SupraSolv, 1.00668) in the sample injector were filled with the minimum amount (200 ml) according to the GC-MS settings before each run. The DCM, when used in conjunction with hydrogen carrier gas, can improve sample resolution due to faster throughput compared to helium. DCM also is used alongside hexane to automatically clean the instruments before each sample, preventing cross contamination. The majority of samples were injected automatically. However, when the sample volume was too little, then manual injection was required. Manual injection was carried out by initially rinsing the syringe (Hamilton, 1705) with DCM, by drawing up six-ten microliter volumes of DCM into a 10-microliter syringe and discarding the DCM

washes into a waste beaker. Residual DCM was cleared by pumping air through the syringe, to evaporate it. The syringe was then rinsed with the sample. Then 1-microliter of sample was drawn up slowly to avoid air bubbles and injected into the inlet. The GC-MS machine was equipped with a HP5-MS capillary column (Agilent, 19091S-111LTM) (30 m × 0.25 mm ID; d.f =0.25 µm). The GC-MS run program was: 60 °C for 2 min, then increased by 60 °C/min up to 200 °C and subsequently by 4 °C/min to 320 °C, where it remained constant for 10 min. Helium was used as carrier gas with a constant flow of 1.2 ml/min. Analyses were run in spitless mode with an inlet temperature of 250 °C. Electron impact mass spectra were recorded with an ionization voltage of 70 eV, a source temperature of 230 °C and a quadrupole temperature of 150 °C. Using this set-up from Menzel *et al*, substances up to C41 can be detected (2017). It took the machine 1 hour to complete the running of one sample. After the samples had finished running, the spectrum was automatically saved onto a desktop computer connected to the GC-MS machine.

To detect larger molecules (up to C50), the samples were additionally injected into the GC-MS equipped with a high temperature column (Agilent DB-1 HT and Phenomenex ZB-5HT; column dimensions as above). Temperature was raised from 60 °C by 5 °C min−1 up to 350 °C and then kept constant for 10 min. The interface had a temperature of 350 °C. All other settings were as above. To detect even larger samples, some samples were sent to The University of Muenster for further testing using MALDI-TOF, Matrix-Assisted Laser Desorption to vaporise samples (Hillenkampet al., 2012).

2.4 Cuticular hydrocarbon data analysis

The automatically saved data files were opened in Agilent MSD Productivity 'ChemStation (version 01.03.2357)' for GC and GC.MS System Data Analysis Application – Agilent Technologies, revealing the spectrums. Each spectrum was integrated, meaning the area under the hydrocarbon peak was quantified individually based on the spectrum. The integration was done in a manner to reveal all relevant peaks whilst simultaneously ensuring that the area of integration represents the true area of the peak and is not over or under valued (see **Figure 12**). During the analysis, appropriate integration parameters were selected such as outlined in **Figure 12** to obtain accurate estimates for the areas of peaks without missing relevant compounds.

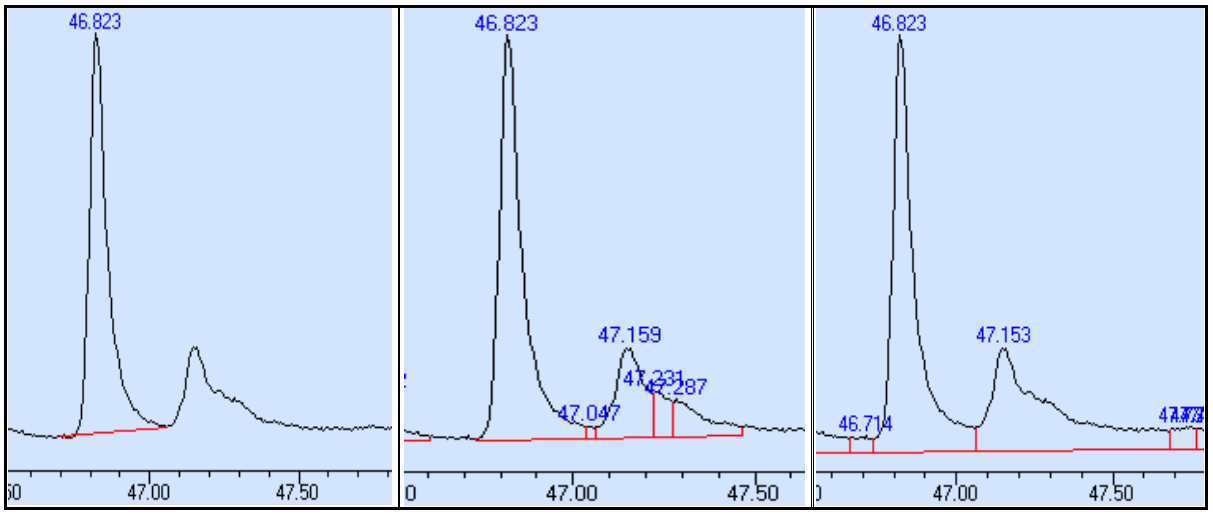


Figure 12. Integration of GC-MS peaks. Left: Overintegration, integration threshold (11) is too low. Area of the peak is overestimated. Middle: Underintrgration, integration threshold (18) is too high. Relevant peaks are missing. Right: Integration threshold (14) adequately estimates peak size.

Once an appropriate integration threshold was selected, the spectrum was integrated, and the results were copied into an Excel file together with the integration parameters used for each sample that was integrated.

All samples for a given experiment were combined into the same Excel sheet, with "Peak Number", "Retention Time (RT)", "%" and "Area" data columns retained for each peak. This yielded a data set with each sample containing peaks with their respective retention time, area, and ratio of the area of that peak in comparison to the sum of all areas within a given sample (see **Equations 1** & **2**). Retention times were then sorted across samples, so that all retention times that corresponded to the same peak were aligned together. A Python script was built to iterate over the columns and automatically align them. After the script was run, the alignment was checked again with the GC-MS spectra to ensure all retention times were grouped correctly with their corresponding peak. Aligned peaks were identified manually, based on molecular weight and MS spectrum.

Even though many of the samples contained the same compounds with differing abundances, there were some compounds that were present in some samples that were not present in other samples of the same experiment. E.g., different hydrocarbons may feature in younger pupae compared to older pupae. This means that each sample was inspected individually to see if there are any compounds that are present in some samples that were absent in other samples.

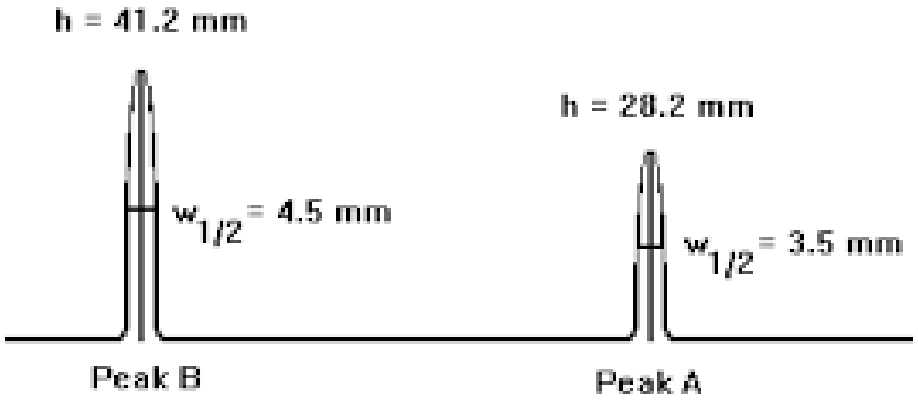


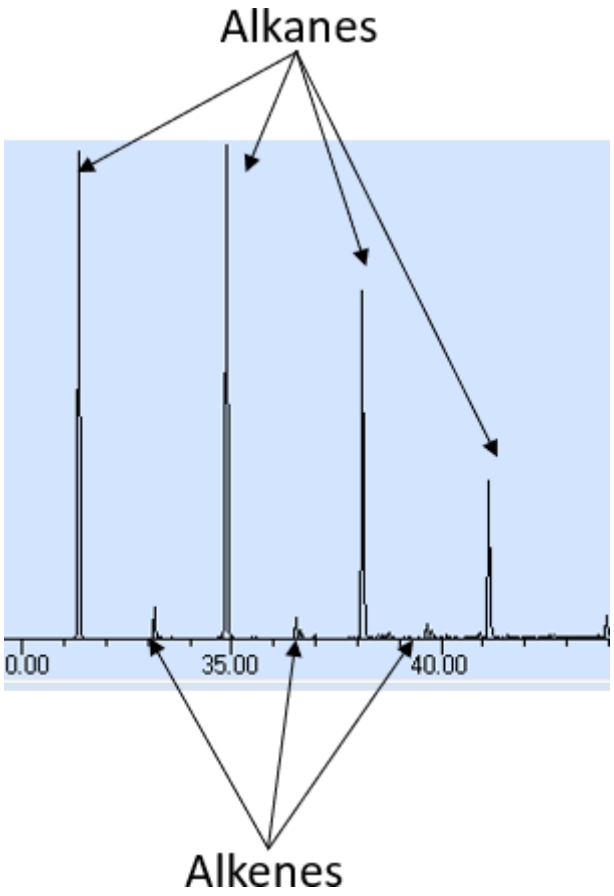
Figure 13. Diagram to show how areas are calculated. Width (w) is equal to the difference in retention time between the start of the peak and the end of the peak. Height (h) corresponds to the abundance of the peak. Image source: ChemPages, Laboratory (ChemPages *Gas Chromatography: Calculating the Area*).

$$Area = h \times W_{1/2} \quad (1)$$

$$Percent\ A = \frac{Area\ of\ A}{\sum Areas\ in\ sample} \times 100 \quad (2)$$

Equation 1 & 2. These equations show how both the area of individual peaks, and the area ratio was calculated.

A



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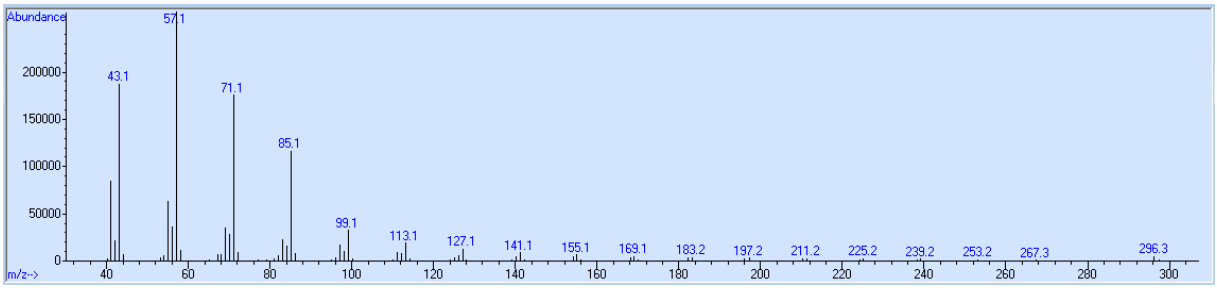


Figure 14. (A) Indicates the position of where alkanes and alkenes are typically found. (B) The typical MS spectrum of an alkane.

Once all the peaks in a spectrum had been identified, the principal component analysis (PCA) scaling graphs were produced.

I used MetaboAnalyst (http://www.metaboanalyst.ca) to analyse and plot the majority of my data (**Figures 16, 17, 19, 20 & 23**). A PCA plot converts the correlation (or lack thereof) among all the samples into a 2-D graph. Hence, samples that correlated strongly in terms of having the same CHCs with similar abundances cluster closer together. It also should be noted that the axes of a PCA plot are ranked in order of importance. Differences along the first principal component axis (PC1) are more important than differences along the second principal axis (PC2) (Jackson., 1991). For example, in **Figure 16**, PC1 accounts for 60.6% of the variation and PC2 accounts for 17.7% of the variation.

3. Results

Introduction to results

The main aim of this project was to understand the impact of desiccation stress on CHC profiles of pupal *Anopheles gambiae*. I.e., is there a noticeable change in their CHC profiles when the pupae are removed from water? However, information was also gathered on the impact of aging and changes in salinity on the CHC profiles. Further information was also recorded on changes in eclosion outcomes, such as if the pupae would eclose successfully or become trapped in its exuvia as a result of desiccation stress. I was also interested to see if there was a difference between male and female pupae in the way they reacted to desiccation stress.

Pupation timing

Throughout **Experiment 1** pupae were collected on an hourly basis. The main reason of collecting pupae in this format was to control for the age of the pupae with high temporal precision. A secondary result of collecting pupae in this manner and recording the time at which they pupated, yielded information about frequency of pupation at certain times of day, **Figure 15**.

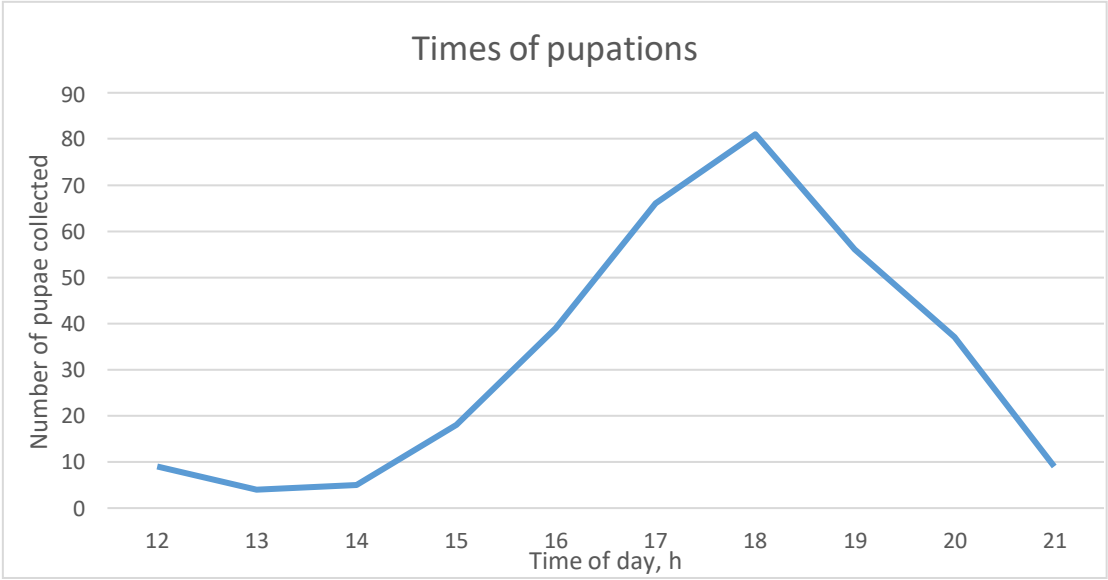


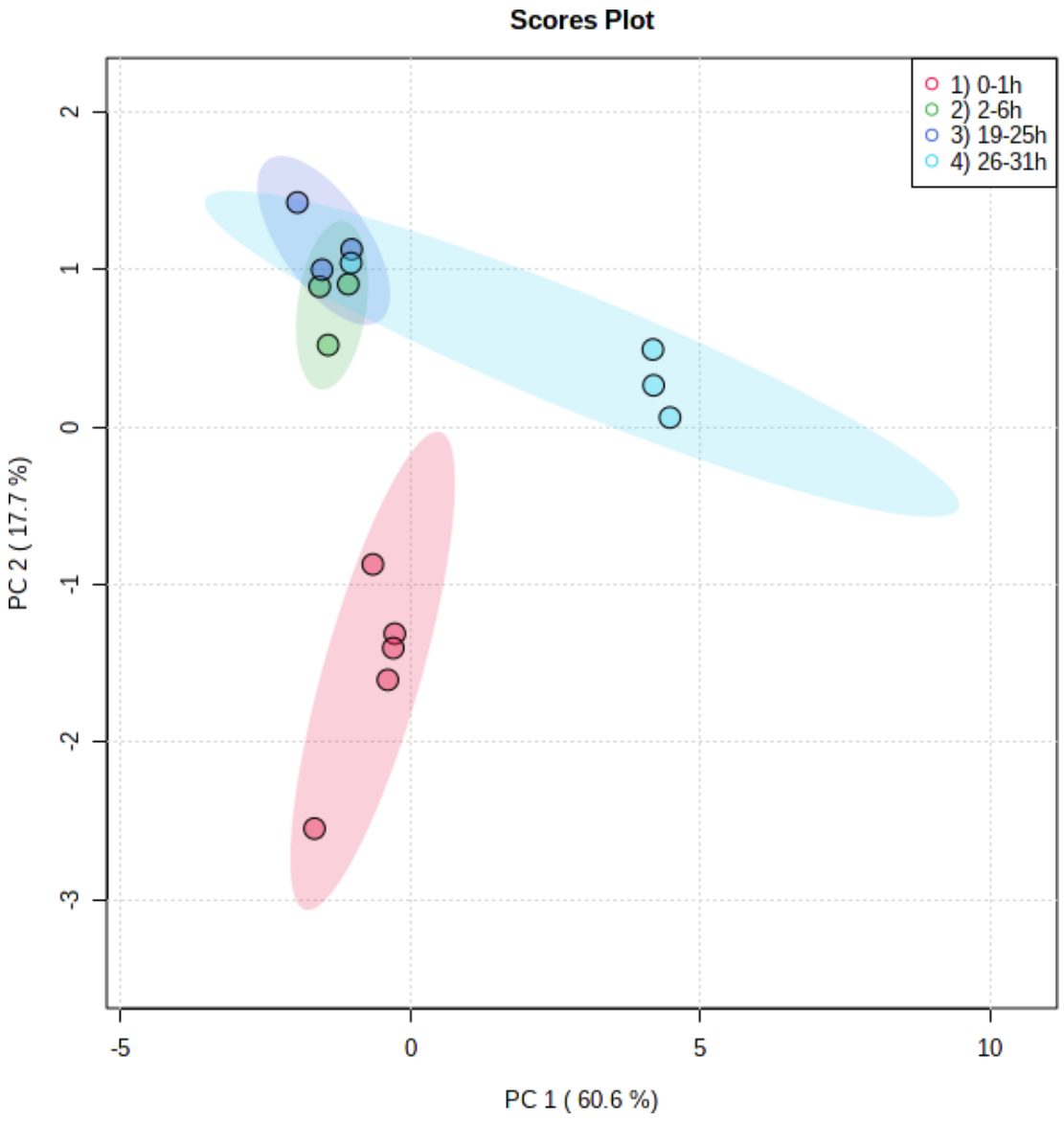
Figure 15. The total number of pupae collected throughout **Experiment 1** and the time of day they were collected. In total the number of pupae collected, N=324.

Pupation occurs predominantly within certain times of the day (**Figure 15**). Therefore, it could be inferred that pupation is under some sort of circadian control. From **Figure 15** we can clearly see that pupation of larvae is far more likely in the evening, increasing from 14:00 to 18:00 and then decreasing from 18:00 to 21:00. The trays were unmonitored overnight (21:00 – 10:00). However, there were very few pupae during the morning collection (when the trays were cleared of all pupae) before the start of the day's collections (See **Method, Experiment 1**). This infers that limited pupation occurred from 21:00 to 10:00.

Experiment 1: impact of pupal age on cuticular hydrocarbon profiles.

The first experiment that was conducted was to understand the impact of age on CHC profile compositions.

A



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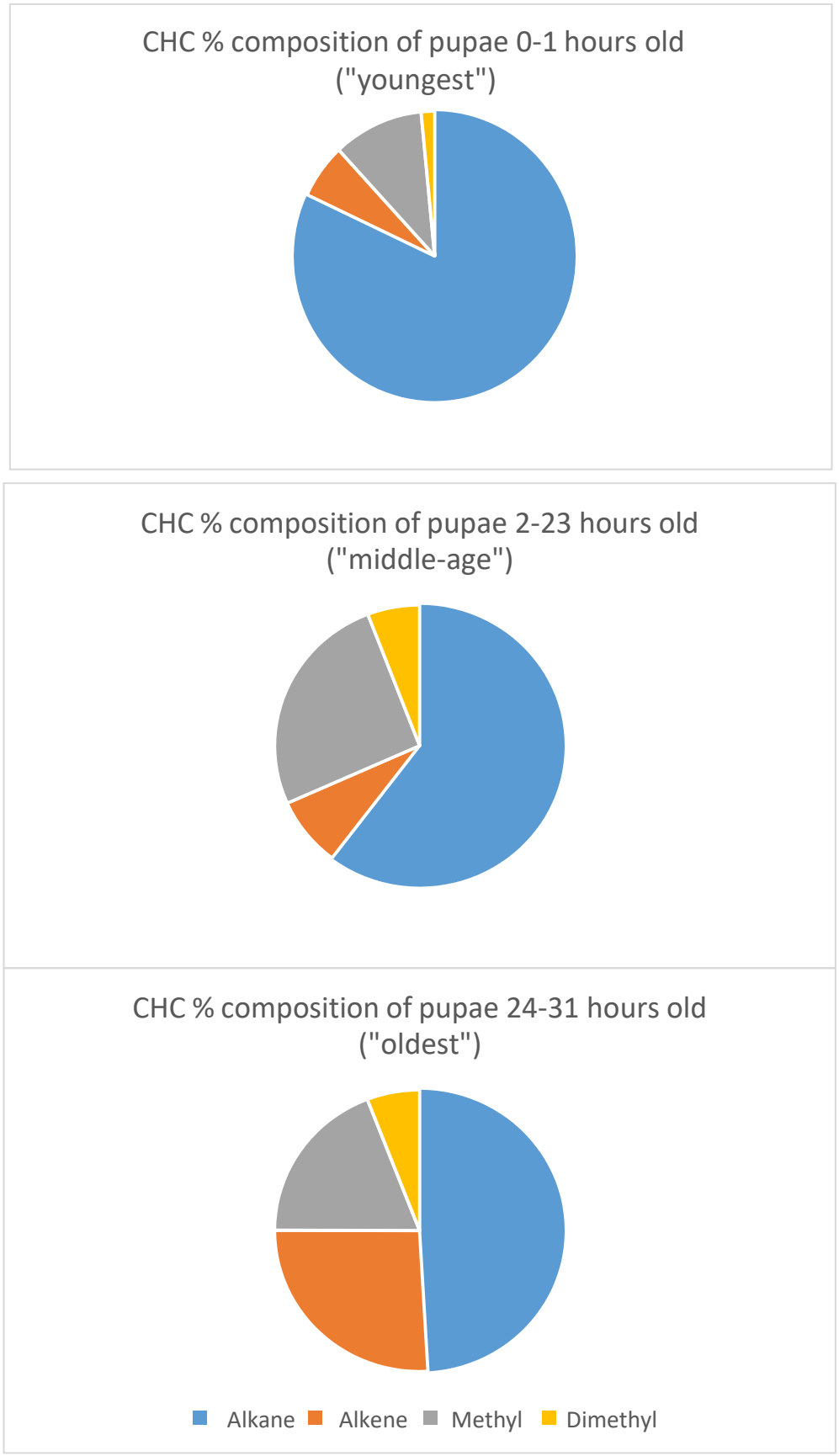


Figure 16. (A) Principal component analysis showing the similarities of the relative compositions of CHCs in the context of age. Red dots represent pupa samples that are 0-1 hour old (h) (N=92), green dots are 2- 6 h (N=54), purple dots 19-25 h (N=58) and blue dots are 26-31 h (N=80). The coloured ovals surrounding the dots display a 95% confidence region. Each datapoint corresponds to a sample consisting of 12-23 pupae, as indicated in Table 1. The x-axis, PC1 corresponds to 60.6% of the variance, PC2 corresponds to 17.7% of the variance. (B) The compositional change in the proportions of hydrocarbon classes between 3 age groups of pupae. Youngest (N=92), Middle-age (N=95) and Oldest (N=97). (5 samples per group).

These results (**Figure 16** & **Table 1**) show how age impacts the CHC profiles. The samples, for the most part, tended to group into 3 distinct age groups. Oldest (26-31 h), youngest (0-1 h) and middle ages (2-25 h). This shows that age has an impact on CHC profiles in *An. gambiae* pupae, especially between the youngest and oldest samples. However, there is an exception, sample 13 (mean age of 27 h, **Table 1**) shown overlapping with group 2-6 h and 19-25 h. The youngest 4 samples are shown to have very similar CHC profiles in comparison to other age groups, as 1-0 h samples all group together. There is also a lot of clustering seen between ages 2-25 h, thus, it can be inferred that CHCprofiles do not change dramatically between 2-25 h post puparium formation. The last group, the oldest pupa samples: 12, 14 and 15 with ages between 26 and 31 h show the greatest departure from the rest of the samples (when factoring in that greater variation is shown on the x-axis). This indicates that towards the later stages of the pupa life cycle that the pupa begins to experience sizable changes in its CHC profile. This rapid change in the CHC profile could be in preparation for eclosion.

The anomalous result seen with sample 13, may have been caused by several different factors. Perhaps contamination during hexane extraction occurred. For example, a pupa rupturing during the extraction process, resulting in the insides of the pupa being dissolved in the hexane. An even more likely reason is the pipette used for hexane extraction could have mistakenly already been used between samples. Hence, hexane residue from other pupa samples (likely one of the samples between 6-11) contaminated it. This may also go towards explaining why it is positioned between the two groups. Another suggestion is that the pupae in this sample, despite their age, may not yet be ready for eclosion. Hence, it may naturally take longer than normal for the pupae in sample 13 to eclose and therefore they experience the chemical changes associated with eclosion (as seen in **Figure 16**) later.

Alkane length	Youngest group's Abundance	Chain length (CH) x Abundance		Alkane length	Oldest group's Abundance	Chain length (CH) x Abundance
21	20.2390771	425.0206191		21	6.87556258	144.3868142
22	1.89041078	41.58903716		22	7.3654679	162.0402938
23	20.73658912	476.9415498		23	6.2484334	143.7139682
24	1.4424483	34.6187592		24	0.46330558	11.11933392
25	17.02042686	425.5106715		25	5.411332	135.2833
26	1.15656468	30.07068168		26	1.04694942	27.22068492
27	7.5484849	203.8090923		27	4.46314996	120.5050489
28	0.47288566	13.24079848		28	0.19266774	5.39469672
29	2.18098346	63.24852034		29	7.72405736	223.9976634
31	8.38597558	259.965243		31	8.09768572	251.0282573
	$\sum$ (CH x Abundance)	1974.014973			$\sum$ (CH x Abundance)	1224.690061
	Average chain length	24.3483572			Average chain length	25.57372241

Table 6. The percentage abundance of different chain lengths of alkanes and the average chain length of the alkanes from the youngest 5 groups and the oldest 5 groups.

Experiment 2: impact of salinity on cuticular hydrocarbon profiles.

This experiment was designed to mimic desiccation not via removal of pupae from water, but via increasing the salt content of the water. I expected that high salt concentrations will be equivalent to low humidity conditions as both increase the osmotic gradient (Beyenbach, 2016; Salt, 1956). Thus, changes in salinity were expected to lead to changes in the pupae CHC profiles. A coloured version of **Table 2** is included below to better demonstrate changes in salt concentration between treatments (**Table 2B**).

The results from **Experiment 2** shows no relation between salinity and CHC profiles (**Figure 17**). This does not necessarily indicate that desiccation has no impact on CHC composition but perhaps changes in salinity are not a mechanism to trigger changes in CHC profiles of the pupae. It is also interesting to note that the CHC profiles of pupae found dead after the 24 hours are only slightly different from those which were found alive after the 24 hours under saline conditions. This Elsewhere demonstrates that the CHC profiles of the exuvia experience no noticeable change after the pupa dies.

There is very little plasticity in *An. gambiae* with regards to changes in CHC composition with changes in salinity concentration. **Figure 18** shows that there is virtually no change between the CHC profiles of pupae left 24 hours in 0 g/L NaCl solution (standard DI water) to pupae left in 200 g/L NaCl solution. For example, both 0 g/L and 200 g/L had 65% of their CHC profile composed of alkanes. **Figure 18** also shows no noticeable difference between the CHC composition of alive and deadpupae under these conditions.

Experiment 3: Impact of desiccation stress on cuticular hydrocarbon composition (Time spent desiccating is based on the age of the pupa when desiccation began)

As described in **Methods**, data collected for **Experiment 3** contains only estimations about the time each pupa spent desiccating. This is based on the age the pupae were when placed into the incubator, when desiccation began.

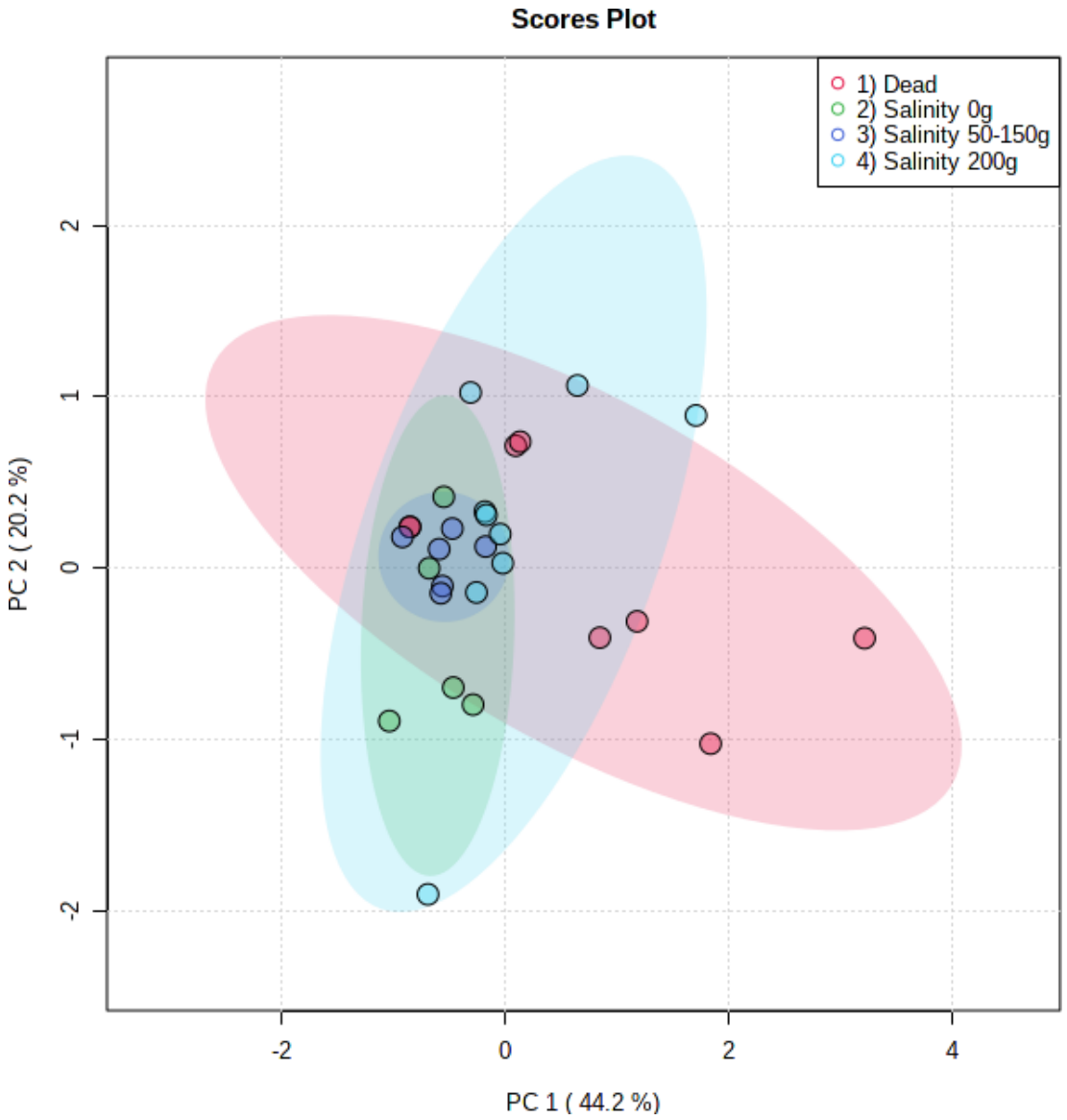


Figure 17. Principal component analysis showing the similarities of the relative compositions of the CHC profiles in the context of salinity. Green dots represent pupa samples left 24 hours in 0 g/L salinity water, purple dots represent all pupae left in 50-150 g/L for 24 hours, blue dots represent thepupae left under 200 g/L and lastly red dots show the pupae that were left 24 hours under 200 g/L but died during the 24 hours. Each sample in this figure contains CHCs extracted from 7-10 pupae, asindicated in **Table 2**.

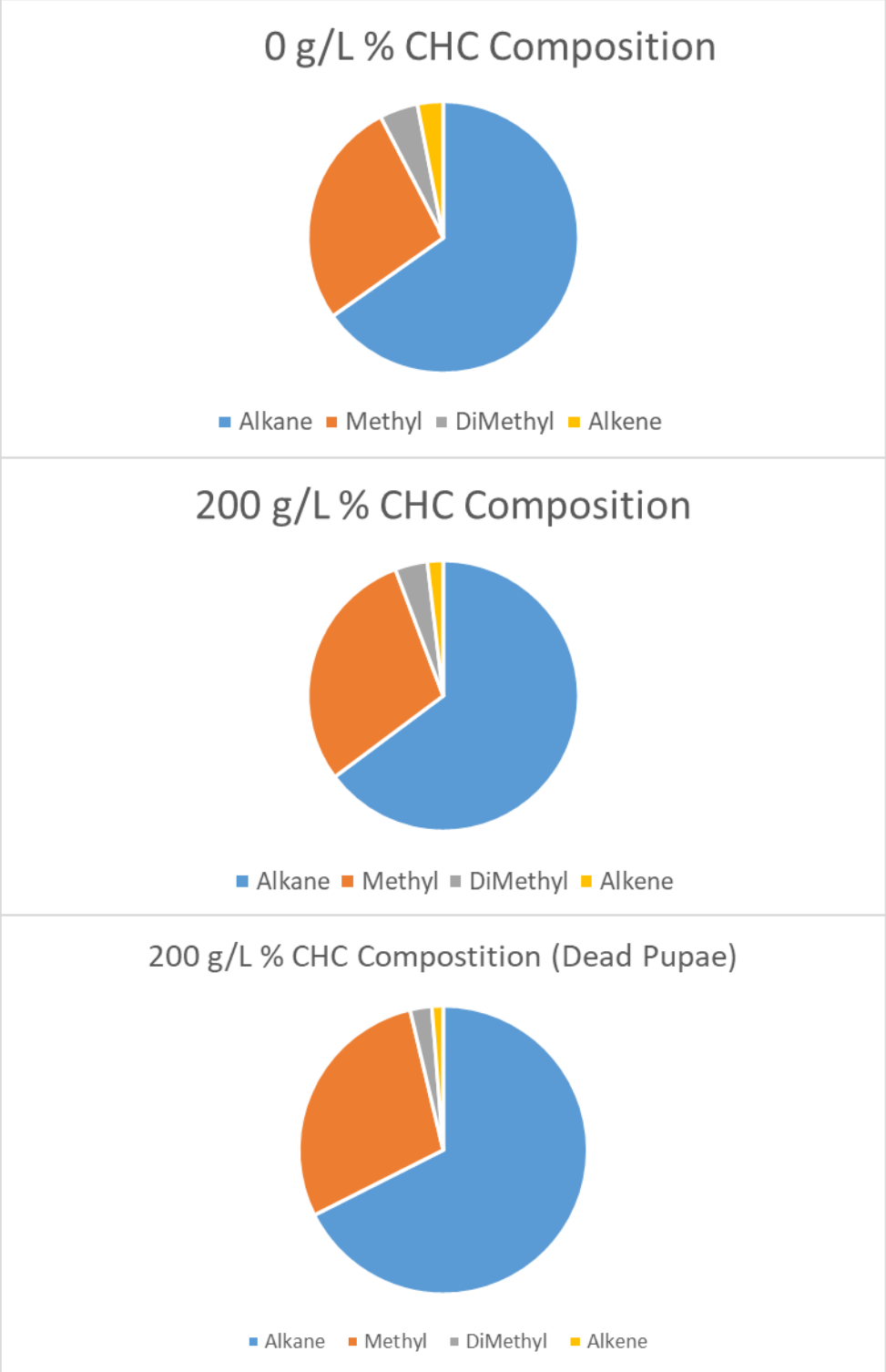


Figure 18. The ratio between alkanes (blue), methylalkanes (methyl) (orange), dimethylalkanes (grey) and alkenes (yellow) at differing salinities. The graph shows only the chemical compositions of pupae left 24 hours in 0 g/L salt waterand 200 g/L salt water. The graph for 0 g/L represents the combined CHC abundances of 48 pupae. The graph for 200 g/L represents the combined CHC abundances of 80 pupae. The bottom graph shows the combined CHC abundance of all 77 pupae that were found dead at 200 g/L.

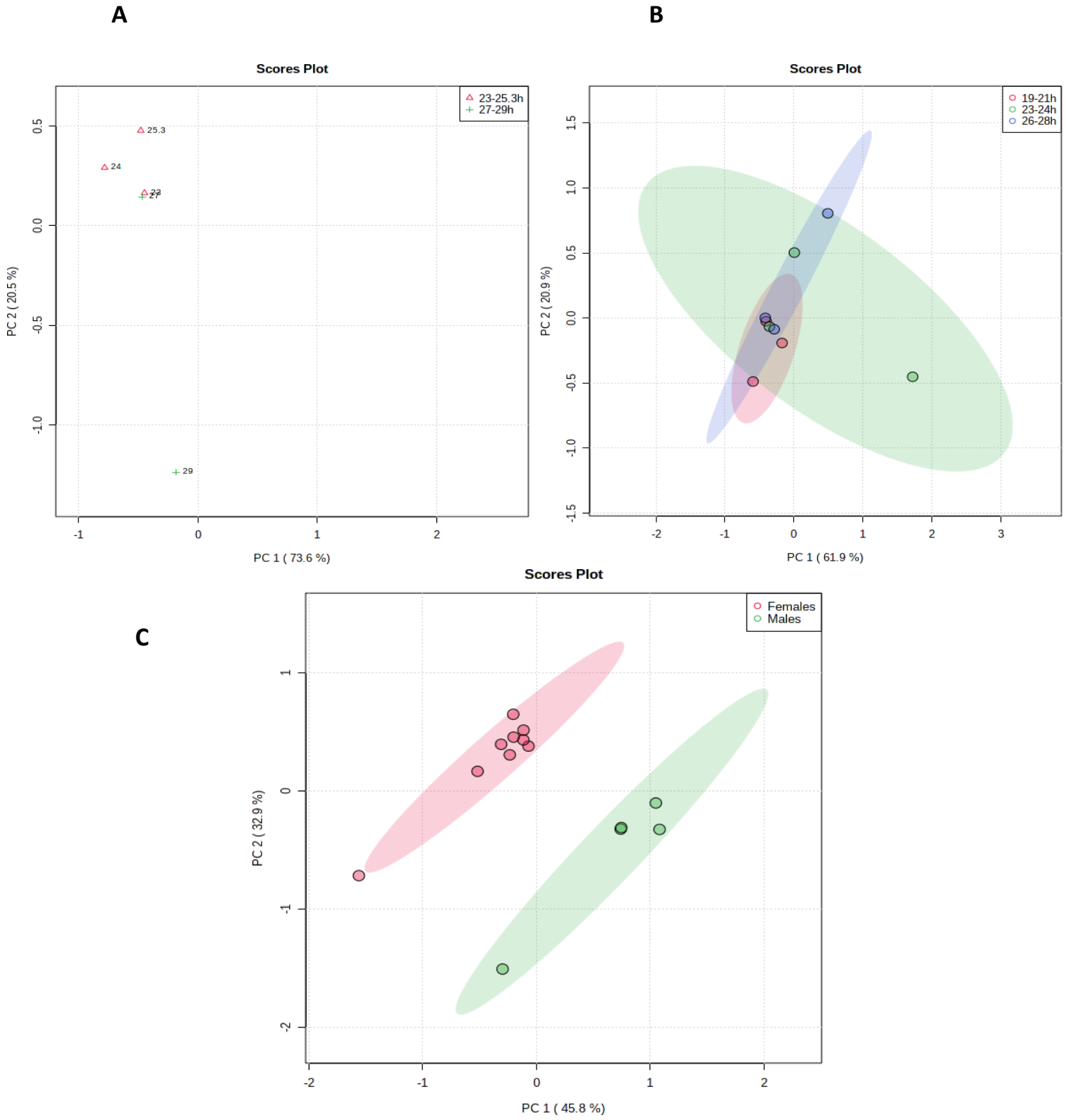
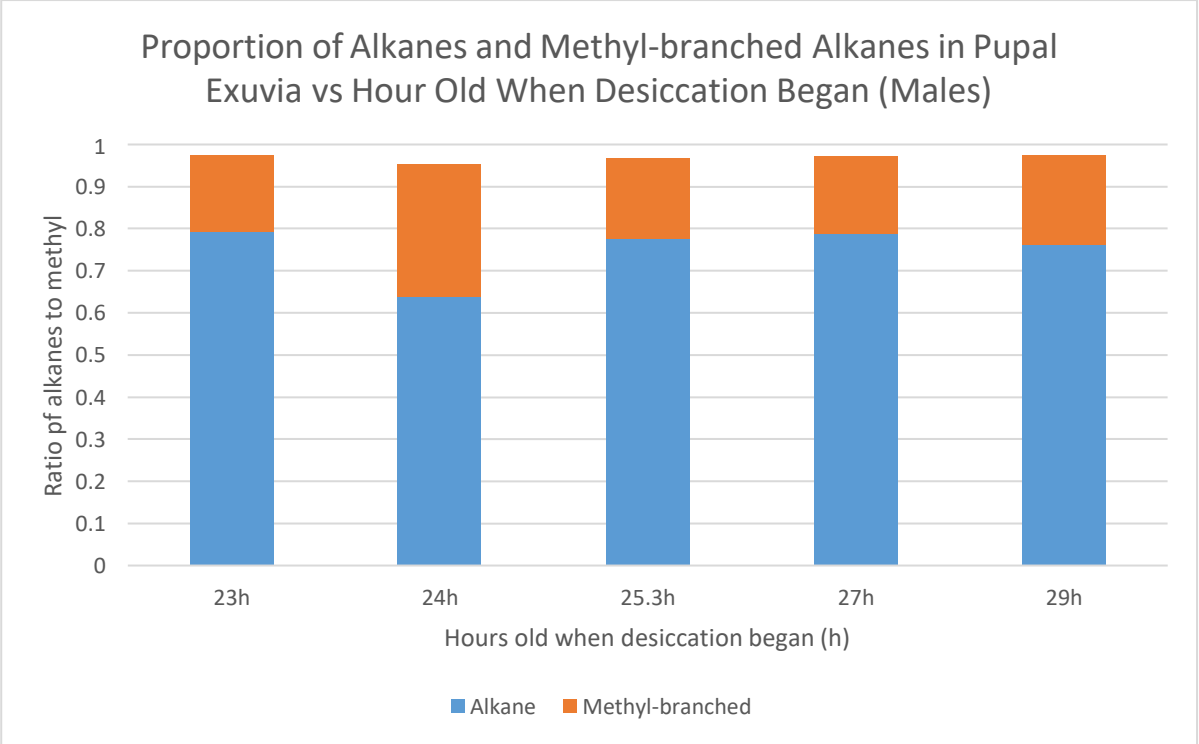


Figure 19. Principal component analysis showing the similarities of the relative compositions of CHCs for male and female pupae in the context of the age of pupae when desiccation began. (A) Is a PCA of only male samples. The green crosses show pupae aged 27 hours old and 29 hours old (h) when desiccation began. Red triangles represent pupae aged 23, 24 and 25.3 hours old when desiccation began. There are only 5 male samples and hence there was insufficient information to display the 95% confidence regions and hence the individual points are labelled with their age upon desiccation and the 95% confidence enclosing oval is absent. (B) A PCA of only female pupae grouped within 3 separate ages when desiccation began. Red dots represent pupae in the age group 19-21 h, green dots 23-24 h and blue dots 26-28 h. Each group contains the same number of pupae. (C) A PCA where male samples from (A) (green dots) and female samples from (B) (red dots) are plotted on the same PCA graph together.

A



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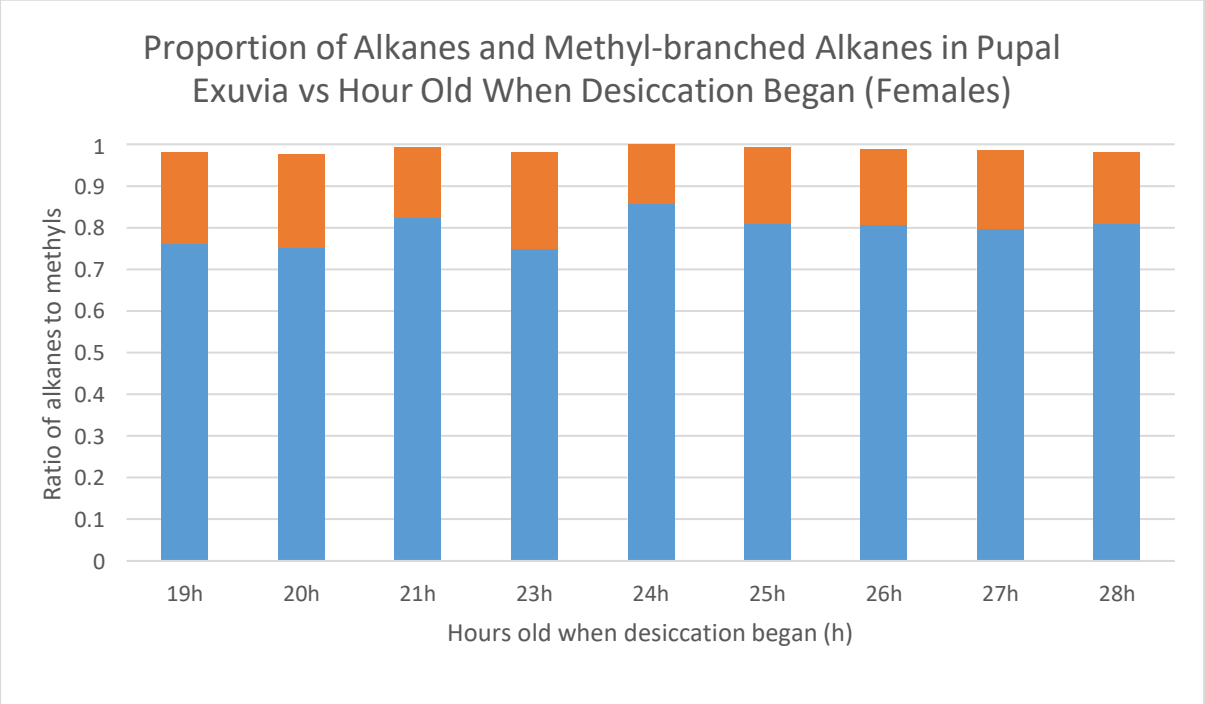


Figure 20. The ratio of methyl-branched alkanes (orange section) to alkanes (blue section) and how they change with respect to the mean age of the pupae when desiccation began. Each mean age group is comprised of the CHCs extracted from 3 individual pupa exuvia. (A) The ratio of alkanes to methyl-branched alkanes in male samples from 23 to 29 hours old when desiccation began. (B) The CHC ratios in female samples from ages 19 to 28 hours old when desiccation began.

There is no grouping of data points with regards to age of the pupae when desiccation began (**Figure 19**). I.e., with in the PCA graph there is no clustering of datapoints within the context of age at the start of the desiccation. This is true for both male and female samples. If the age of the pupae at the start of the desiccation had an impact on CHC expression, you would expect that pupae with similar ages upon desiccation would be closer together, however this is not the case (**Figure 19A** & **Figure 19B**). However, there is a distinct difference between CHCs from male and female pupae (**Figure 19C**). There are two distinct clusters, with females occupying exclusively the top left of the PCA plot and males the bottom right (**Figure 19C**). This indicates that sex has a significant impact on the CHCs that *An. gambiae* pupae produce.

The age at which the pupae were placed into the incubator for desiccation has no impact on the ratio of methyl-branched alkanes and alkanes present in the exuvia after eclosion (**Figure 20**). The majority of compounds that were identified during the analysis of **Experiment 2** were alkanes and methylalkanes. However, a very small amount of dimethylalkanes were identified in the female samples for **Experiment 3**, only 1.17% and hence were not included in **Figure 20**. **Figure 20** further supports the notion that there is no relationship between the CHC composition and the age of the pupae upon desiccation. For males, **Figure 20A** shows that the ratio of methyl-branched alkanes to alkanes stays relatively the same regardless of age upon desiccation. For **Figure 20A**, although methylalkanes range from 18% to 31% the majority, 4 out of the 5 age samples, stay more or less constant at 23% methylalkanes. This indicates that for male pupae the CHC composition is independent of their age upon desiccation. Females also show a relatively constant ratio of methylalkanes to alkanes regardless of age upon desiccation, methylalkanes stay relatively constant at around 20%.

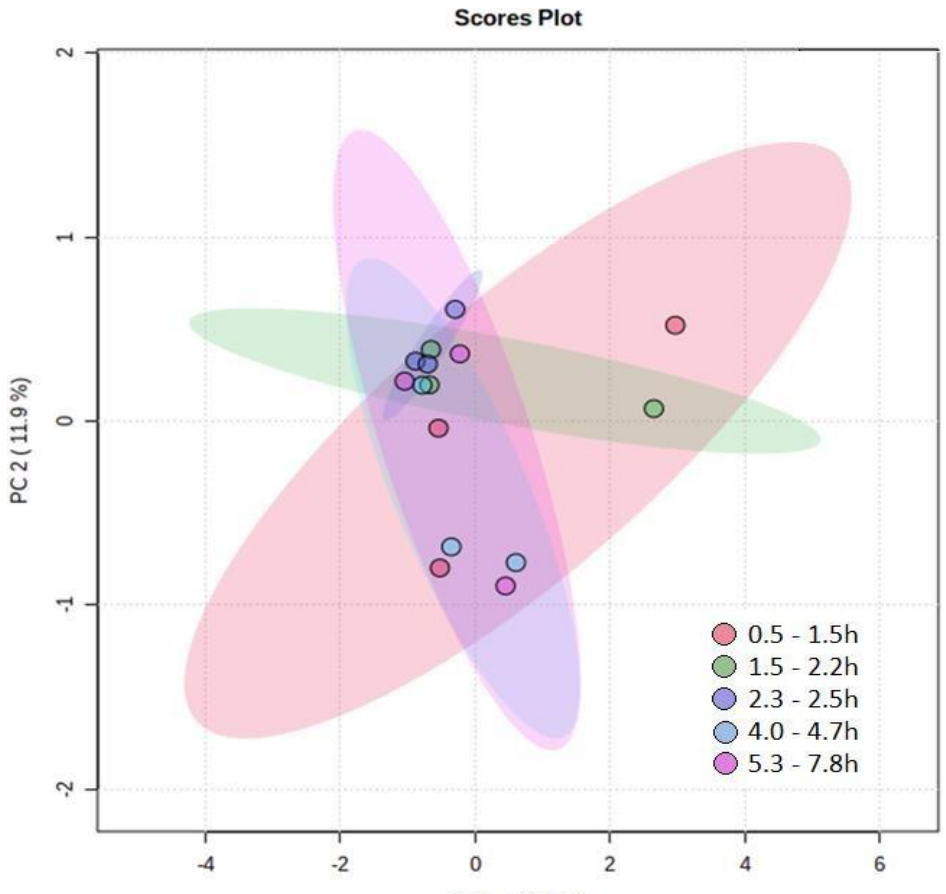
Experiment 4: impact of desiccation on cuticular hydrocarbons (exact time the pupae spent desiccating is known)

Experiment 4, as mentioned before, set out to investigate if there was a correlation between changes in CHC profiles and the length of time pupae were left desiccating. The sex of the pupae was recorded, and length of time spent desiccating was also recorded as the time (in hours) the pupa spent in the incubator without water at 96-99% RH, at the pupal stage.

Throughout the experiment in order to obtain the maximum number of pupae, all pupae that were pupated under the conditions outlined in the **Method** were used in the experiment. The number of females and males produced in total was roughly equal. However, this is not the case at every age group. Although age was a controlled variable, the number of pupae within each age group varied. Therefore, in **Figure 21** the age ranges between male and females are not set at exact intervals but instead group in a way to allow for the most even distribution of pupae between age groups. Meaning that each sample is roughly comprised of the same number of pupae and hence CHC profiles between samples are of a similar validity.

The results in **Figure 21** indicate that there is no discernible difference between the length of time the pupae spent desiccating and the CHCs expressed on the shells (or exuvia) of the pupae. This is true for both males and females. Even between the extremes in both groups: 0.5-1.5 h to 5.3-7.8 h in the male samples, and 0.5-1.3 h to 7.2-8.5 h in the female samples, show no obvious difference between them. These results indicate that time in which a pupa is left desiccating (at ≈ 99% humidity and at a temperature of 26.8 °C) has little impact on the CHCs expressed within the shells once extracted.

A



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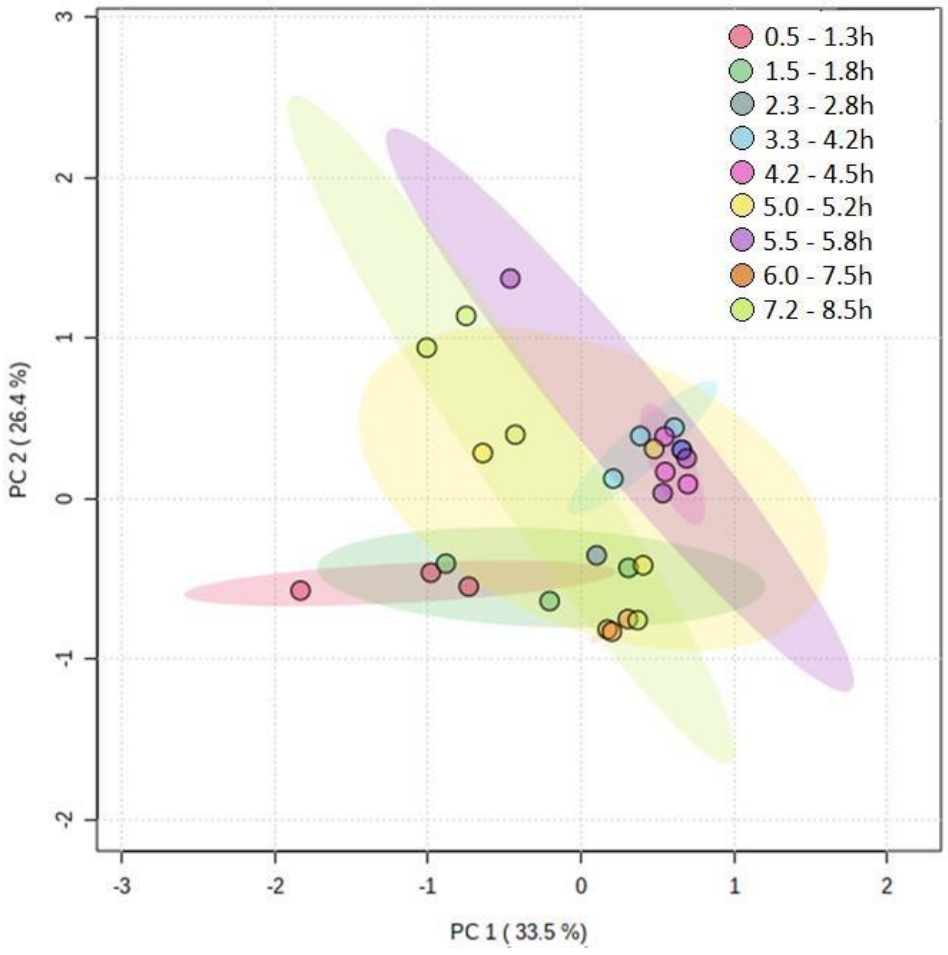
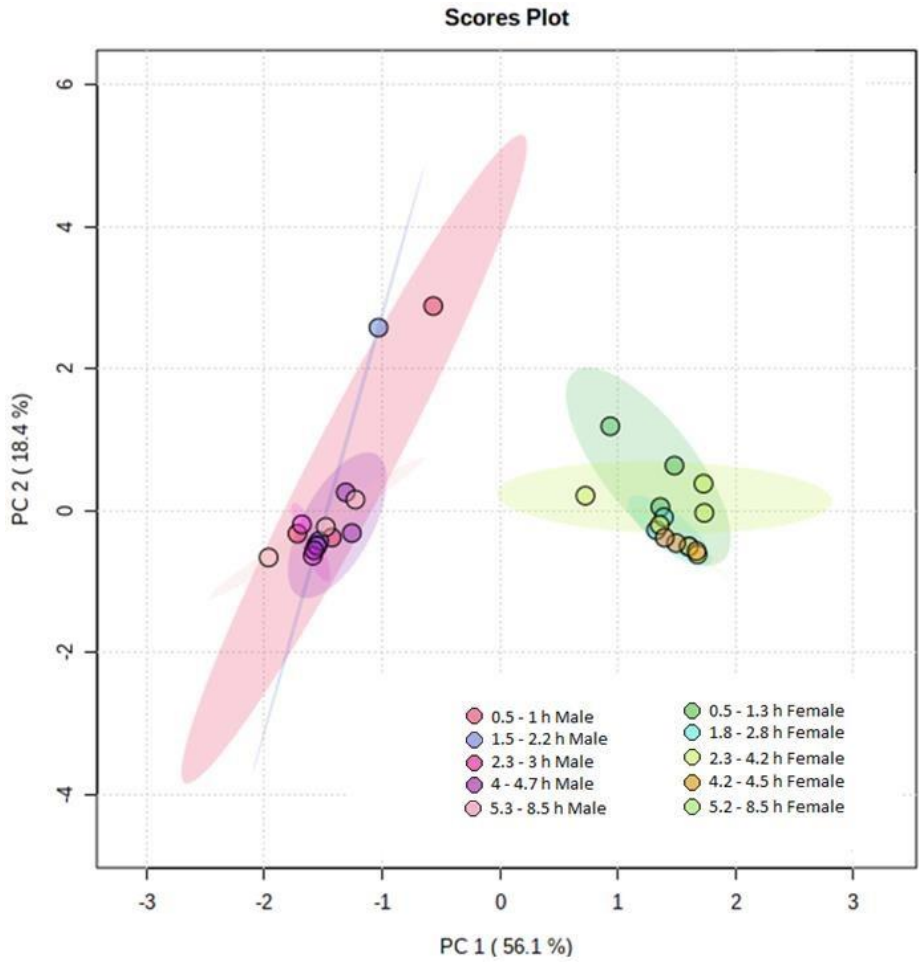


Figure 21. Principal component analysis showing the similarities of the relative compositions of CHCs for male and female pupae in the context of time spent desiccating (h, hours). (A) Male samples were split into 5 distinct time groups with red dots representing pupae that spent between 0.5-1.5 hours desiccating, green dots show males that spent 1.5-2.2 hours desiccating, purple dots 2.3-2.5 hours, blue dots 4.0-4.7 hours and lastly pink dots that spent 5.3-7.8 hours desiccating. (B) Female samples were split into 9 distinct time groups, ranging from 0.5-1.3 hours spent desiccating (red dots) to 7.2-8.5 hours desiccating (light green dots).

A



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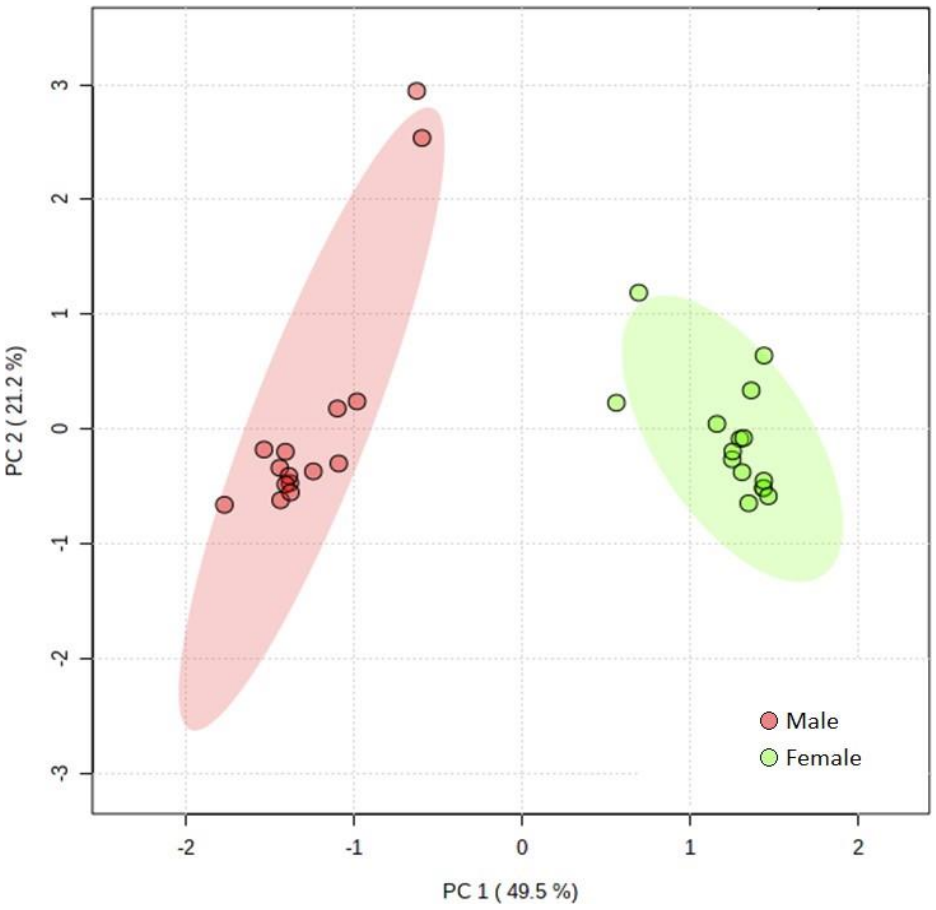


Figure 22. Principal component analysis showing the similarities of the relative compositions of the CHC profiles in the context of the sex of the pupae and time spent desiccating. This PCA graph represents data collected from **Experiment 4**. Male sample datapoints cluster on the left, shown in a red scale and female sample datapoints cluster on the right, shown in a green scale. (A) The similarity in CHC compositions between samples of differing sex as well as time spent desiccating. For example, light pink dots on the left-hand side represent male pupae that spent 5.3-8.5 hours (h) desiccating, whereas the orange dots on the right-hand side represent female pupae that spent 4.2-4.5 hours desiccating. (B) Simply shows relative composition of CHCs in the context of only sex, to highlight the sex difference.

Male and female pupae's CHCs are shown to be significantly different from one another (**Figure 22**). The red and green circles enclosing the samples displays a 95% confidence region. The difference between sexes is more pronounced once the time variable is removed (**Figure 22B**).

Like **Figure 21**, there is no strong correlation between time spent desiccating and CHCs produced (**Figure 22A**). However, there is a strong difference between males and females in terms of the CHCs they produce when desiccated (**Figure 22**). The combination of CHCs produced from the desiccated male pupae are far more similar to one another than any of the CHC combinations from their female counterparts. This demonstrates that whilst the length of time spent desiccating seems to have little impact on the combination of CHCs produced, sex appears to play a pivotal role in the CHCs expressed.

To investigate the sex differences of CHC profiles further, I investigated the abundances of CHCs produced by male and female pupae. **Figure 23** shows that the proportions of alkanes and methyl-alkanes are roughly the same in their percentage abundance, with alkanes being the most common, then methyl-alkanes between male and females. However, the stark difference between the sexes is the sizable presence of dimethylalkanes within the female pupae, which could be the compound responsible for the different eclosing characteristics (see **Figure 26**) and the overall difference in CHC profiles between male and females shown in **Figure 22.**

The length of time spent desiccating has no significant impact on the CHC composition in female and male pupae (**Figure 24**). In **Figure 24A,** there is a slight increase in methyl-alkanes between 0.5-2 h and 2.17-4 h in the male pupa groups (26% to 37%). However, there is an almost negligible change between groups 2.17-4 h and 4.3-8.5 h, 37% to 39% increase in methyl-branched alkanes. The same is true for females. For example, in **Figure 24B** for female pupae, although there are slight fluctuations (ranging between 7% -14%) the percentage abundance of dimethylalkanes remains relatively constant. Furthermore, the abundances of both alkanes and methyl-alkanes also stay relatively the same between female age groups. 58%, 63% and 57% for alkanes and 31%, 30%, 29% for methyl-alkanes in age groups 0.5-2.8h, 3.3-5.2 h and 5.5-8.5 h respectively. This indicates that for both male and female *An. gambiae* pupae, time spent desiccating has minimal impact on their CHC profiles.

Finally, I wanted to see the correlation between CHC profiles between different experiments (**Experiments 1, 2** & **4**). This was done to see if there was a notable difference between the pupae CHC profiles under different conditions (normal, saline, desiccation). **Figure 25** shows the difference in CHC profiles between pupae that were left to age normally under normal rearing conditions in water ("AGE"), saline conditions ("SALT") and lastly desiccation conditions ("DESSICATION"). **Figure 25** shows that pupae from the age and salt experiment contain more similar CHC combinations than those from the desiccation experiment. This is expected as the physical conditions between **Experiment 1** (aging experiment) and **Experiment 2** (salt experiment) are more similar to one another than **Experiment 4** (desiccation experiment). In **Experiment 1** & **2** the pupae were still contained within a body of water. All datapoints from **Experiment 4** on the other hand are shifted over to the right of the graph. This indicates a stark difference in there CHC profiles when compared to pupae from **Experiment 1** & **2**. Especially considering that the x-axis, PC1 is weighted at 60.8% of the variance. However, an important point to mention when combining these 3 experiments, is the methodology is similar but not identical. This may have an impact on the differences shown in **Figure 25** between **Experiment 1** & **2** compared to **Experiment 4**. For example, with **Experiment 1** & **2** pupae where frozen before eclosion, however in **Experiment 4** only pupae exuvia were frozen. Hence, **Experiment 1** & **Experiment 2** are CHCs extracted from the whole pupae whereas in **Experiment 4** CHCs were only extracted from exuvia. This may also be a factor in explaining the sizable difference seen in CHC profiles from **Experiment 1** & **2** when compared to **Experiment 4.**

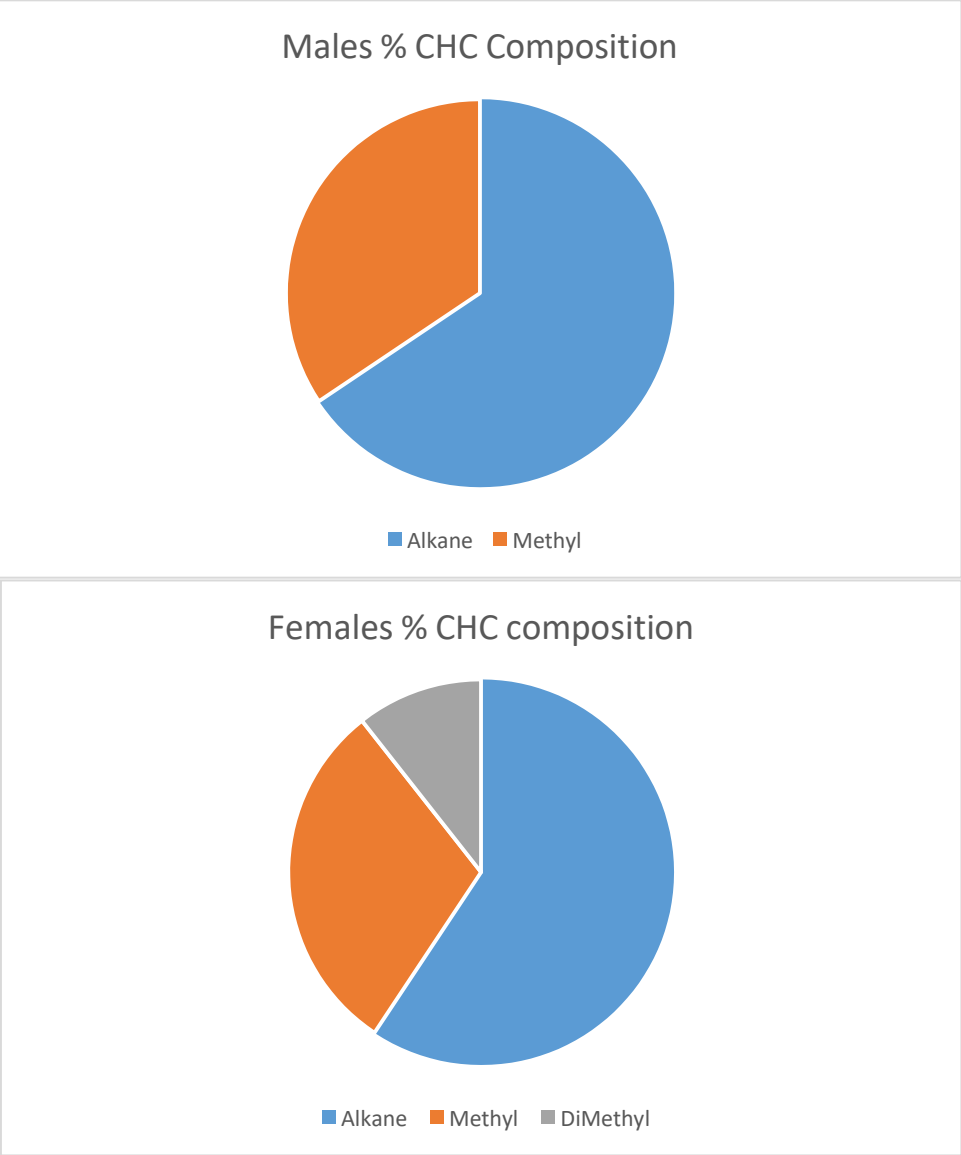


Figure 23. The difference in the composition of CHC compounds between males and females. The orange section shows the proportion of methyl-branched alkanes. The blue section shows the proportion of alkanes. The grey section shows the proportion of dimethylalkanes. The male pie chart is the total CHC data from 16 extractions with each extraction comprising of 3 pupal exuvia (N=48). The female pie chart is the total CHC data from 28 extractions with each extraction comprising of 3 pupal exuvia as well (N=84). The data is the combined CHC abundances from all samples regardless of time spent desiccating.

A

B

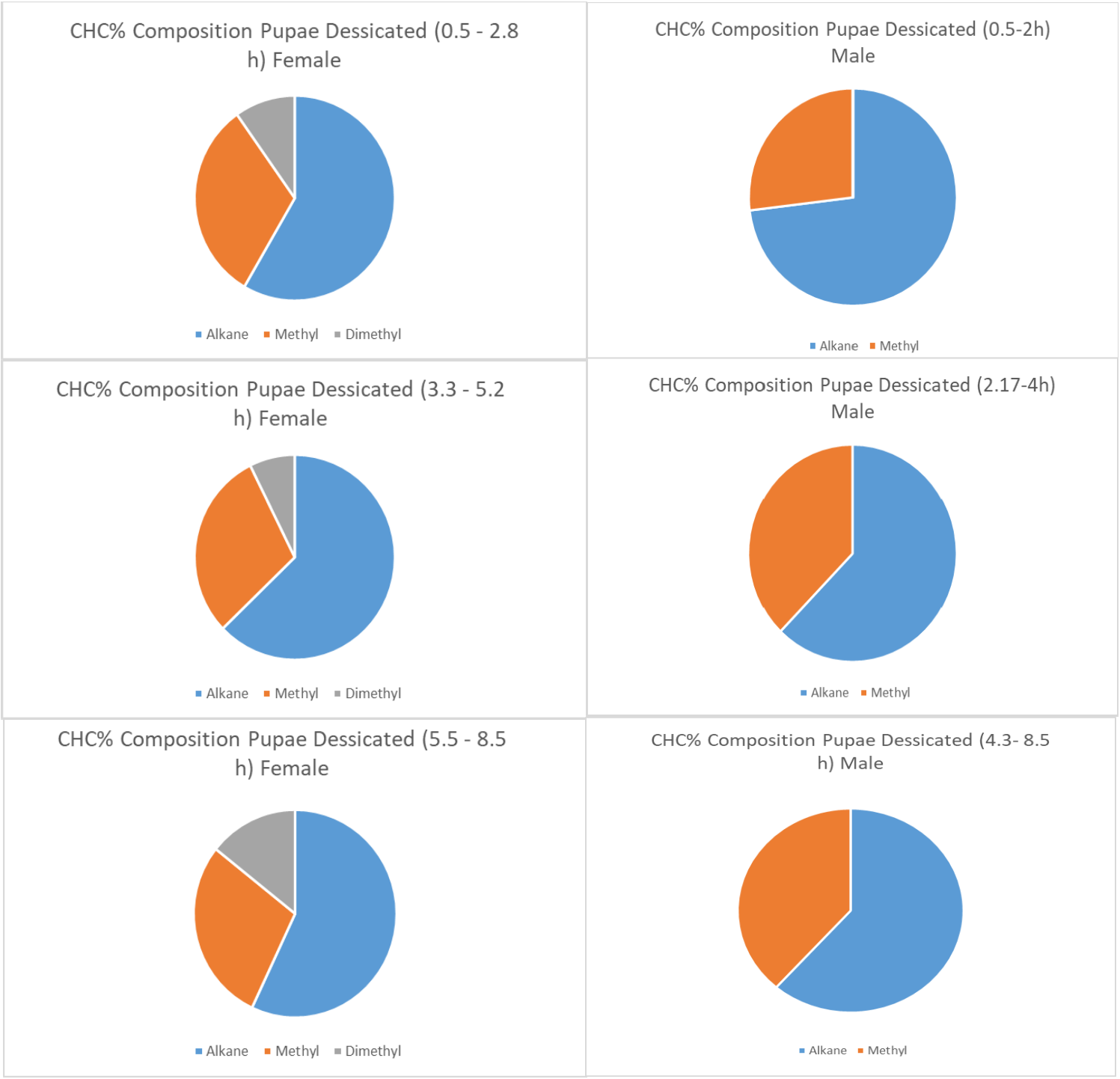


Figure 24. The composition of CHC profiles with respect to sex and time spent desiccating. (A) Changes in CHC composition of female pupae with respect to time spent desiccating. The first graph represents CHCs extracted from female pupae exuvia that were under desiccation conditions for 0.5-2.8 hours (N=27) (h), the graphs below show pupae that spent 3.3-5.2 hoursdesiccating (N=27) and 5.5-8.5 hours desiccating (N=30). (B) Changes in CHC composition of male pupae with respect to time spent desiccating. The first graph shows the CHCs extracted from male pupal exuviae that were left desiccating for 0.5-2 hours (h) (N=15). Graphs below that show groups 2.17-4 hours (15) and 4.3-8.5 hours males spent desiccating (N=15). As previously mentioned, the blue section Shows the proportion of alkanes, grey sections show the proportion of dimethylalkanes, and the orange part shows methyl-branched alkanes.

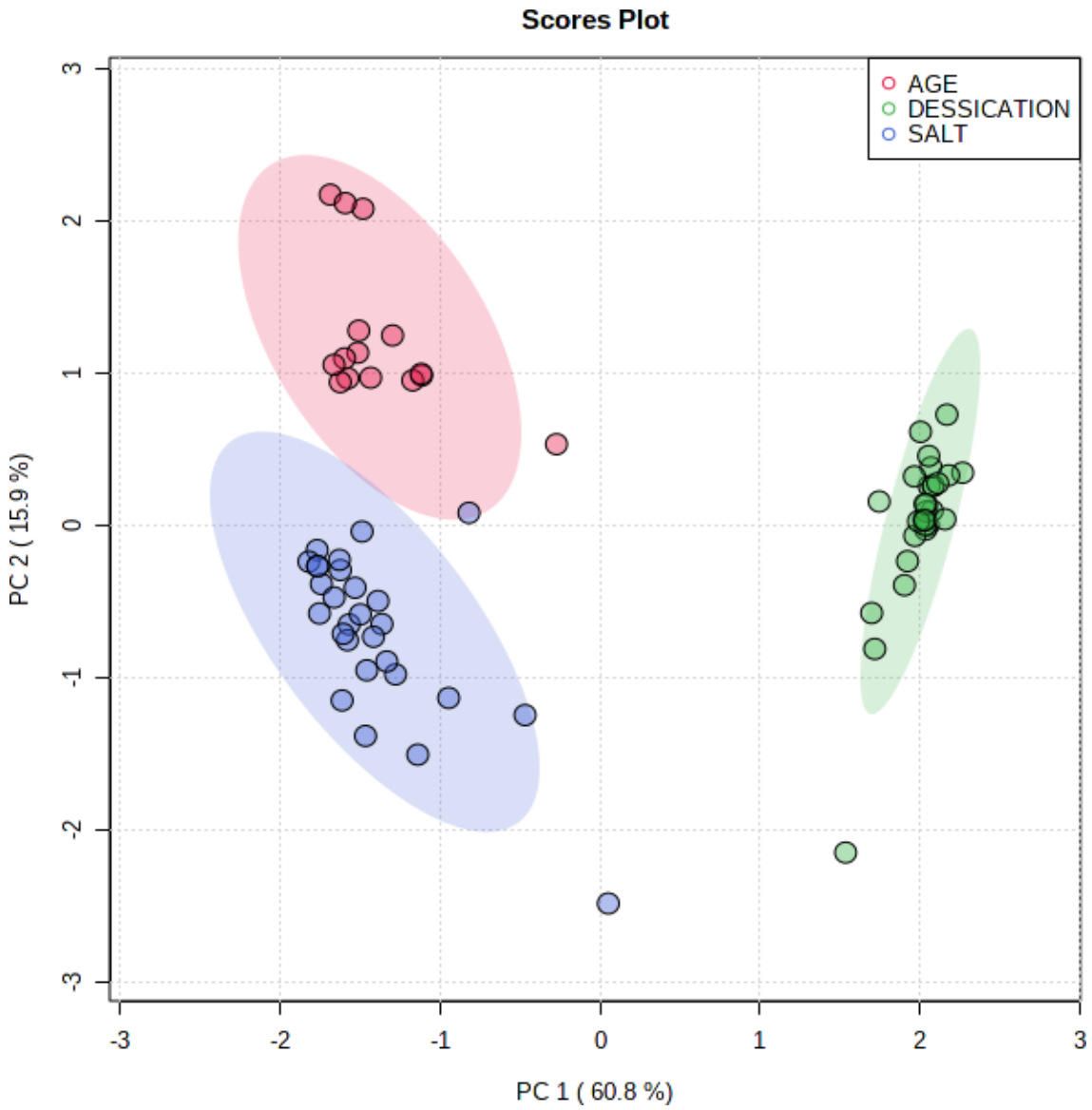


Figure 25. Principal component analysis showing the similarities of the relative compositions of CHCs for samples from 3 distinct experiments. "AGE" (**Experiment 1**), where pupae were left under normal rearing conditions and then frozen at different ages (red). "SALT" (**Experiment 2**), where pupae were placed in different water salinities (blue). "DESSICATION" (**Experiment 4**), where pupae were left out of water (green). All datapoints from all three experiments were used in producing **Figure 25**

Impact of age and sex on outcomes of eclosion after desiccation

As mentioned in the **Method** physical characteristics were recorded after desiccation such as the outcomes of eclosion (half-hatched, pupae case or dead) (**Figure 9**).

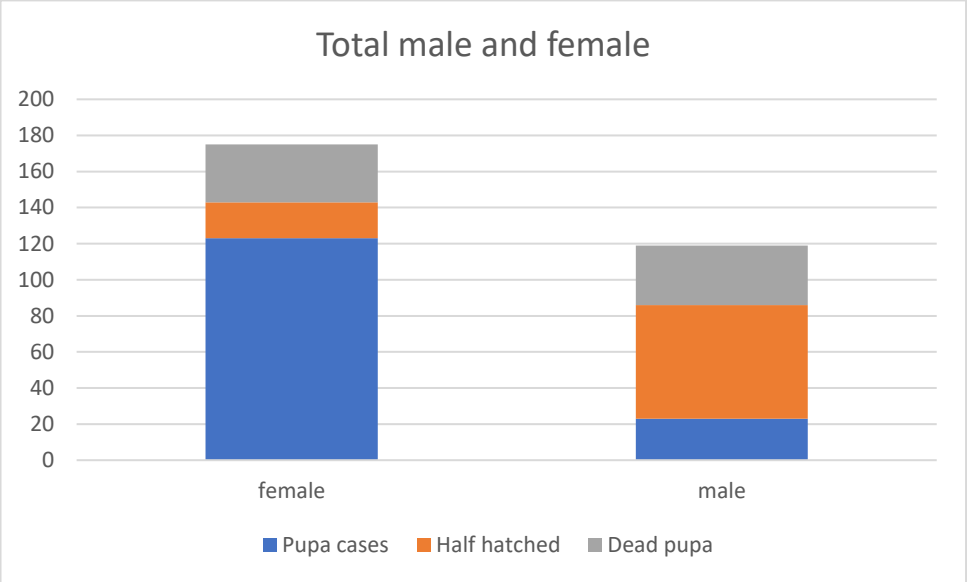


Figure 26. Survival of male and female pupae during desiccation. (A) The proportion of different outcomes after desiccation of all the male samples (B) shows the proportion of different outcomes after desiccation of all the female samples.

Female pupae when desiccated are far more likely to have a successful eclosion in comparison to their male counterparts (**Figure 26**). There is a far higher ratio of females emerging fully and leaving empty cases behind shown in **Figure 26**. The desiccated males also had an overall greater mortality ratethan the desiccated females, 18% of desiccated females compared to 27% of desiccated males.

If the pupae that are dead and will eventually die (half hatched) are compared to the proportion of *An. gambiae* that successfully eclose, the sex differences become even more drastic. Half-hatched pupae will eventually die due to starvation/dehydration. Hence half-hatched pupae and dead pupae can be grouped together and compared to the rest of the samples. Only 30% of female pupae die (half- hatched and dead) after desiccation whereas 81% of male pupae die after desiccation. This is an immense difference between the *An. gambiae* sexes.

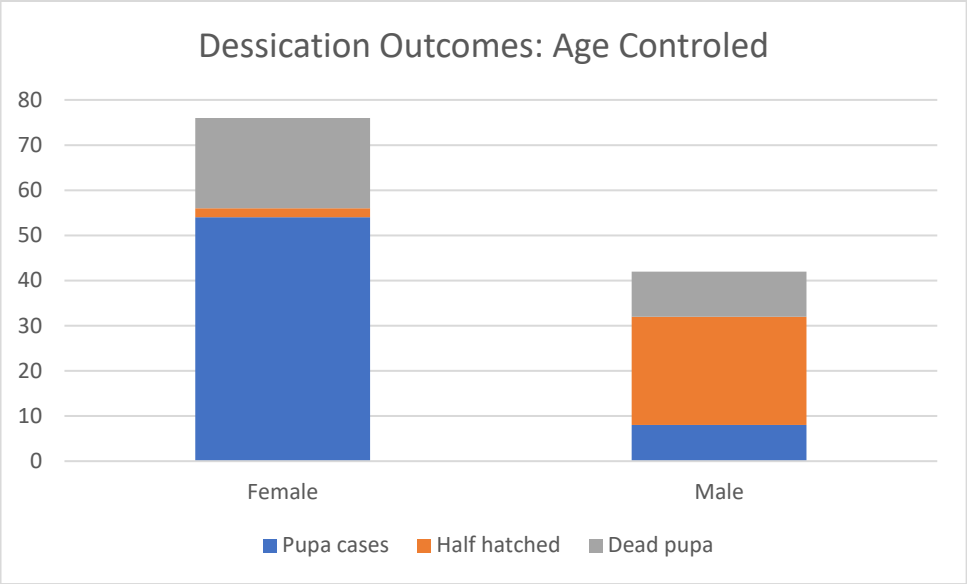


Figure 27. Sexual dimorphism of desiccation outcomes for pupae that were desiccated at 24 h. (A) Outcomes for female pupae that started being desiccated 24 h after puparium formation. (B) Outcomes for male pupae that started being desiccated 24 h after puparium formation. Data shownhere is a subset of data shown in **Figure 26**.

Figure 27 depicts the data from pupae that were desiccated at the same age, 24 h after puparium formation, and hence time spent as a pupa in the incubator is reasonably constant too. The data in **Figure 27** is from **Experiment 3**, where the exact time pupae spent in the incubator desiccating is unknown. It is instead based on the age of the pupae when desiccation began (See **Method, Experiment 3**). 24 h was chosen as it was the age group with the greatest number of samples. The graph shows an even greater difference between the percentage of half-hatched pupae outcome between sexes when compared to **Figure 26.** However, there is a relatively similar difference in overall mortality rate (half-hatched & dead pupa) between sexes when the variable of age upon desiccation is removed.

To investigate the nature of the half-hatching phenotype further, I split the pupae into 6 categories based on their age at the start of **Experiment 3** (3 age groups for both males and females, with each group containing roughly the same number of pupae). For the females, the age group 1 contained pupae between 19-21 hours old (h) at the start of desiccation **Experiment 3**, group 2 were 23-24 h and the female group 3 were 25-28 h. The male groups were 21-23 h (group 1), 24-26 h (group 2) and27-29 h (group 3) at the start of the desiccation experiment.

It should be noted that the oldest pupae (i.e., the 25-28, 27-29 groups) spent the least amount of time as pupae before hatching under the 99% humidity condition in the experimental box.

Females		
Group Age (hours)	Out comes	Number of pupae
19-21	Cases Half hatched Dead	15 13 4
23-24	Cases Half hatched Dead	66 6 21
25-28	Cases Half hatched Dead	42 1 7

Males		
Group Age (hours)	Outcomes	Number of pupae
21-23	Cases Half hatched Dead	2 11 15
24-26	Cases Half hatched Dead	13 44 16
27-29	Cases Half hatched Dead	8 8 2

Table 5. Shows the raw data of collection used to produce **Figure 26** and the samples sizes used

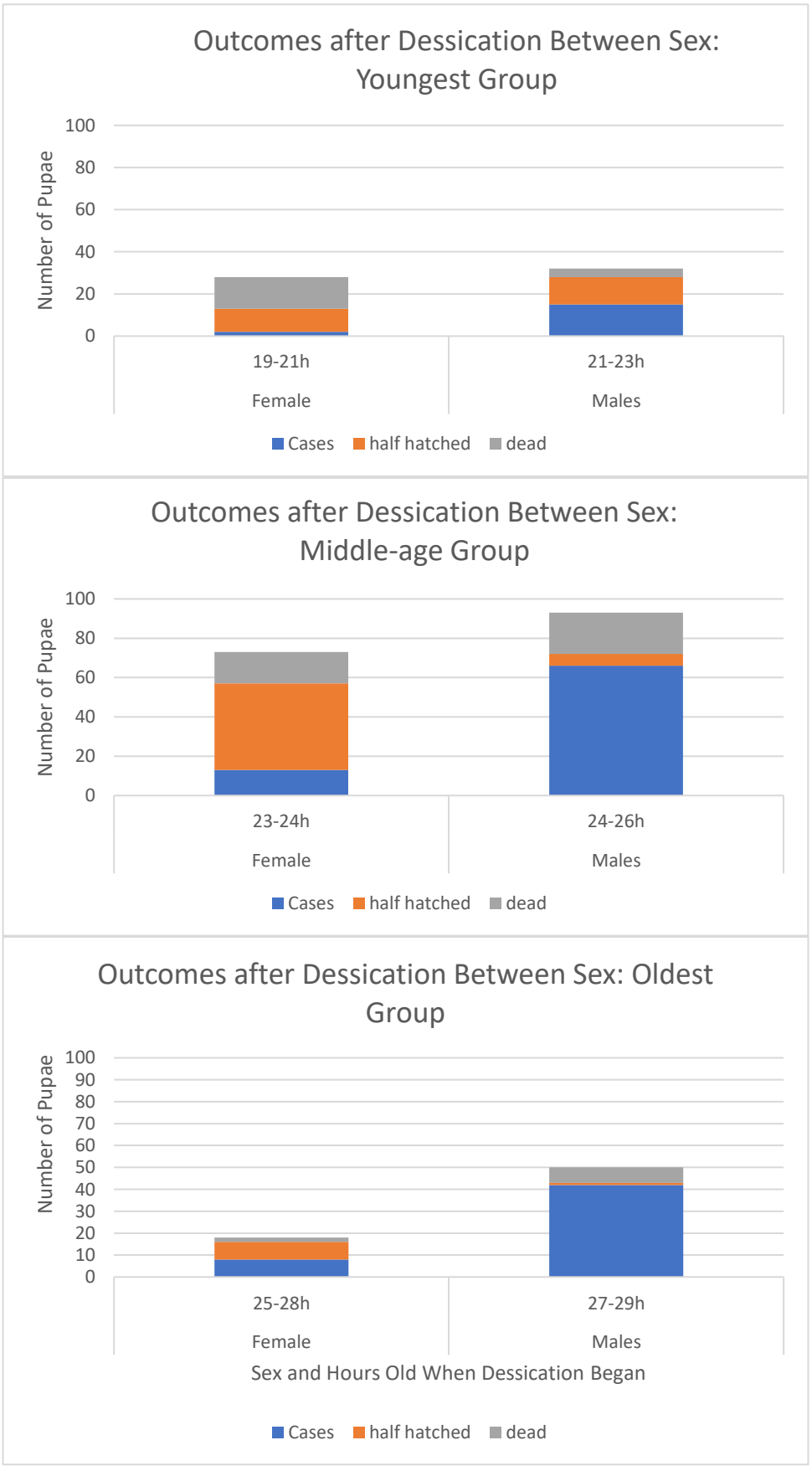
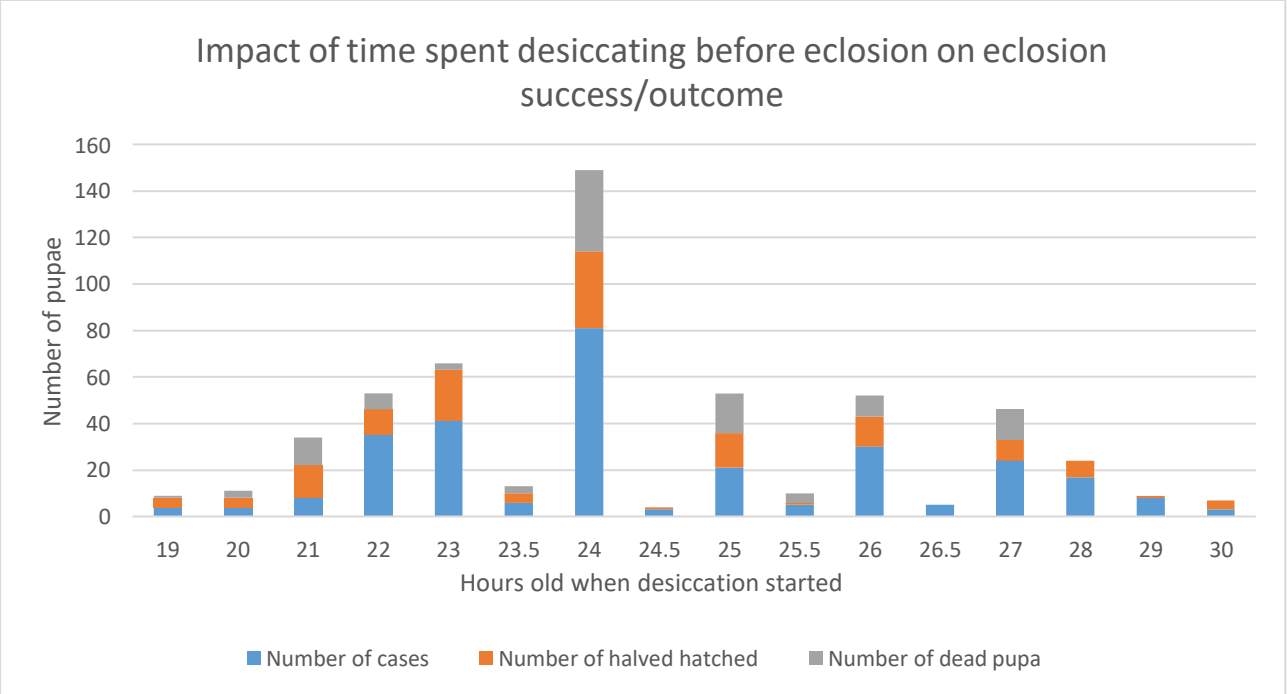
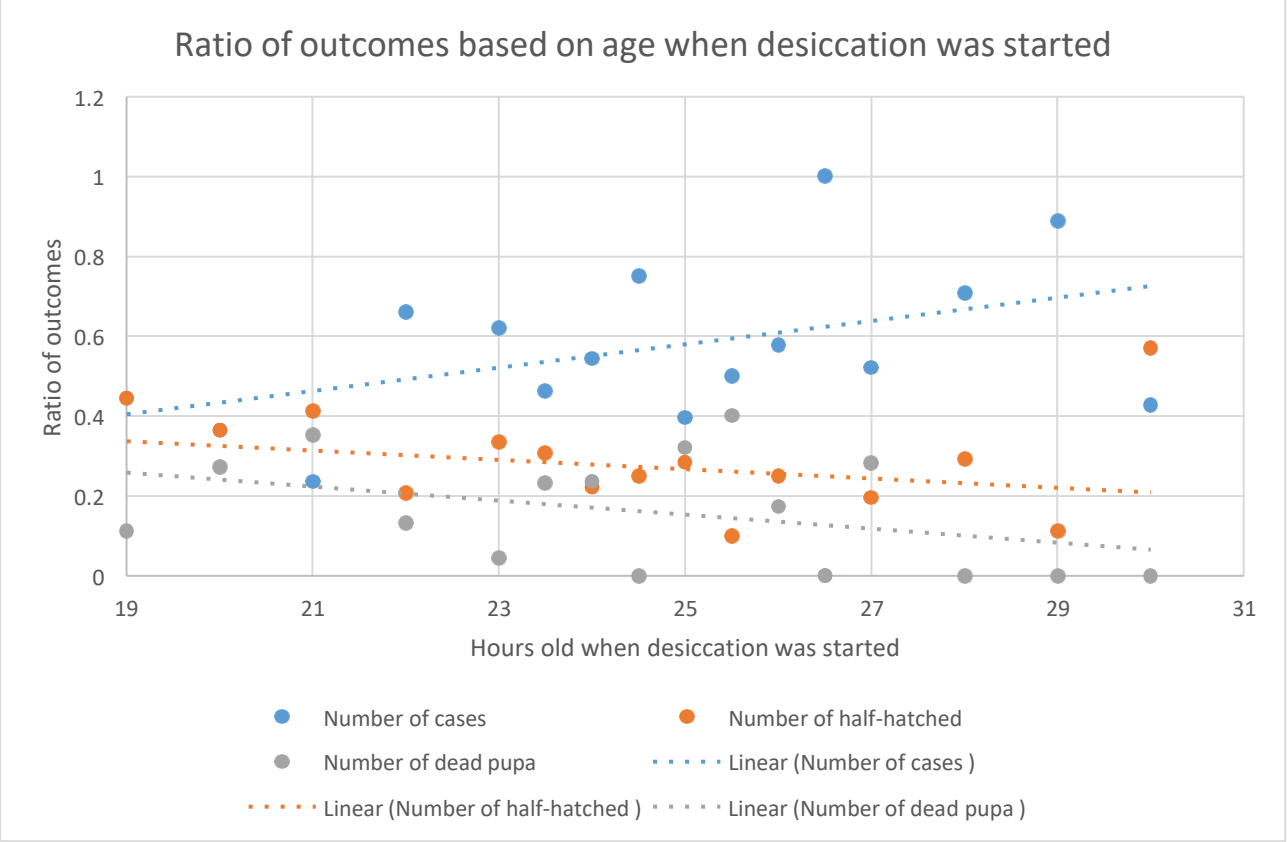


Figure 28. The eclosion outcome status not only differs between sex but also age of the pupae when desiccation began.

A


Impact of time spent desiccating before eclosion on eclosion success/outcome
Number of pupae
160
140
120
100
80
60
40
20
0
19
20
21
22
23
23.5
24
24.5
25
25.5
26
26.5
27
28
29
30
Hours old when desiccation started
Number of cases
Number of halved hatched
Number of dead pupa

B


Ratio of outcomes based on age when desiccation was started
Ratio of outcomes
1.2
1
0.8
0.6
0.4
0.2
0
19
21
23
25
27
29
31
Hours old when desiccation was started
Number of cases
Number of half-hatched
Number of dead pupa
Linear (Number of cases)
Linear (Number of half-hatched)
Linear (Number of dead pupa)

C

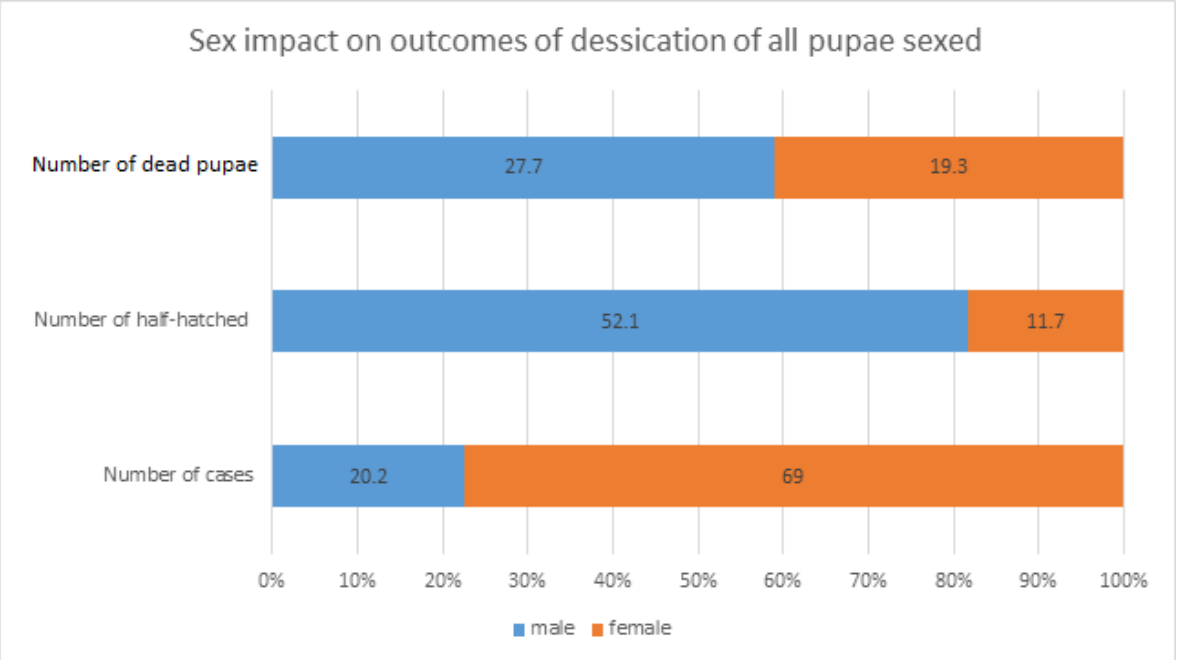


Figure 29. The different outcomes that occurred per age group for all the 545 pupae. (A) The proportions of each outcome relative to how many pupae were in each group. () The impact of desiccation depending on sex of all 316 pupae (N males=119, N females=197) that were sexed before desiccation. This is shown as a proportion of each population regardless of age upon desiccation. Average age of desiccation for female samples is 24.5h and for males is 25.2 h.

A trend can be seen within **Figure 28** and **Figure 29,** that the older the pupae are when desiccation begins, then the lower the mortality rate is. **Figure 29** shows that when every pupa is included, the correlation between age upon desiccation and outcome (cases, half-hatched or dead) is less obvious. I ran a multiple regression analysis on all 545 pupae to determine the significance of the relationship between the number of hours spent desiccating and eclosion outcomes (**Figure 29B**). The test showed that P = 0.504 (P<0.05). This means that there is no statistical significance between age and outcomes (for males & females combined). This is despite the rough trend that can be seen in **Figure 29B**. According to the trendline, the older the pupae were, the more cases they left, showing more eclosion successes. The trendlines for both half-hatched pupae and dead pupae seems to decrease with age, again indicating greater likelihood of eclosion success the older the pupa was when desiccation began; however this trend is not statistically significant. Regression statistics were performed for each individual outcome with regards to age upon desiccation. The P-values for the correlations between each of the variables to one another (cases, half-hatched and dead pupae) were 0.829, 0.448 & 0.622 (P<0.05) respectively. This indicates that when a multiple regression is performed on all samples male and female (combined) the correlation between eclosion outcomes and time spent desiccating is not significant for any of the three outcomes. I then performed a multiple regression analysis on both male and female pupae individually as well as calculations on correlations between the variables. Although a regression analysis was done for female age and number of pupa cases (i.e., the number of pupae that survived desiccation) showing 0.0865, which may indicate the older the female pupae were when placed in the incubator the better their chances of survival. For female pupae that half-hatched it was -0.665, a negative correlation. Hence, on the surface indicating the older the female pupae were when desiccation began the lower the likelihood of half-hatching occurring. The same is true for dead pupae the older the female pupae were when

desiccation began the less likely they would be found dead (-0.084). However, again it is important to note that a regression analysis of female pupae on outcomes showed that only the correlation scene from half hatched pupae was significant (P=0.0005) the outcome of number of pupa cases and dead pupae were not significant (P=0.76 & P=0.33 respectively). Comparing correlation results, it is notable that age upon desiccation has the biggest impact on the half-hatched outcome. Regression statistics on only female pupae, yield a R-squared value of 0.4697 showing that 47% of the variance seen can be explained by the age the pupae were when desiccation began. Further to this the significance F results of the regression analysis was 0.004678 meaning there is a 0.47% chance that this output was obtained by chance. For male pupae, the correlation is even stronger. For pupacases, half-hatched and dead pupa vs age, the correlations were 0.172, -0.378 and -0.805 and the R squared 0.668, showing that age accounts for 67% of the variance seen in eclosion outcomes for males. However, for male pupae for pupa cases, half hatched and dead pupae outcome, none were significant (P=0.3, P=0.81 & P=3.04 respectively). Despite similar coefficients between male and female pupae most were not significant and hence further testing is required to prove these correlations.

The younger the pupae were when placed in the incubator, the more time they spent in the incubator, the higher the mortality rate and the greater frequency of pupae not successfully eclosing (when successful eclosion is defined as leaving an empty case) (**Figure 28**). The older the pupae were and hence the less time they spent in the incubator as a pupa the more likely they were to eclose successfully. For example, the mortality rate of the male pupae (half-hatched + dead pupae) (**Figure 28**), that spent the longest time in the incubator desiccating (21-23 h group) was 61%, whereas the males that spent the shortest amount of time in the incubator as pupae (27-29 h group) had a mortality rate of only 50%. For females, the percentage of pupae that died directly after desiccation (grey section of the graph, **Figure 28**) did not fluctuate as much between the age groups, only 12.5-22%. The success rate and mortality rate for females however fluctuated greatly. For females successful eclosion increased for the groups that spent less time in the incubator as pupae. Successful eclosion of females went from 47% for 19-21 h group to 84% for 25-28 h group. A similar increase of successful eclosion was also seen in males: from 7% in the youngest group to 44% in the oldest group. This indicates that although the ratios of successful eclosion between females and males are very different, with females being far more likely to successfully eclose, the impact of desiccation seems to have an equal influence on disrupting the eclosion progress with a 37% decrease in success found between the oldest and youngest females' groups and a 37% decrease in success within the male groups. These results suggest that at these conditions (pupae without water but at high humidity levels ≈99% RH) females fare better. However, both sexes see increases in mortality as the time spent desiccating in pupal form increases.

The results indicate for both sexes that the more time the pupa spends desiccating the greater the mortality rate and less chance the pupa will successfully eclose into an adult. Males required less time under desiccation conditions to begin having problems, such as difficulty leaving the case or dying as a pupa.

It is important to note as well that group sizes vary greatly, as seen in **Table 5**. For example, female group 23-24 contains 93 pupae, whereas male group 27-29 has only 18 pupae. Hence, the reliability of the results will vary between groups.

The percentage of successful outcomes i.e., the pupae successfully eclosing and leaving their cases behind, heavily favours females, 69% compared with males 20.2% (**Figure 29C**). The same is true for pupae that did not eclose successfully or died during desiccation with the majority of these outcomes belonging to the male pupae. This is even more prominent when you consider that the

average female pupa age is 24.5 h and for males it is 25.2 h and therefore females would have spent a longer time on average desiccating as a pupa.

Further analysis was conducted on survivability between male and female *An. gambiae.* All samples from **Experiment 3** that were sexed were used. To calculate survivability, half hatched pupae and dead pupae were summed together, forming the group of pupae that did not survive desiccation. The number of pupa cases (pupae that did survive into adulthood) where then divided by the total number of pupae per age group.

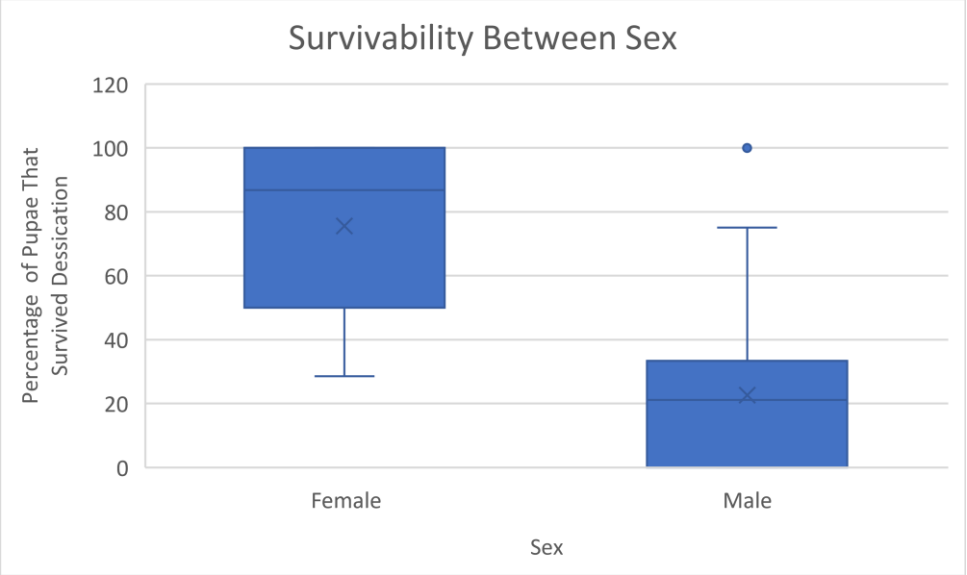


Figure 30. Comparison between the percentage of pupae that survived under desiccation conditions between males and females.

It can be seen clearly from **Figure 30** that females have a higher survivability than males. A linear regression was then performed to see if the difference in survivability between males and females was statistically significant. This test yielded a P-value of $9.71e^{-16}$ well below 0.05. This indicates that sex has a statistically significant impact on survivability of pupae when desiccated.

Humidity vs mortality

Through preliminary experiments it was noted that once the pupae were removed from water, relative humidity (RH) was a determining factor in eclosion success, up to a certain RH threshold. The majority of pupae died at RHs less than 85%. This is shown in **Figure 31** with 100% mortality rate at a RH of 83.5%.

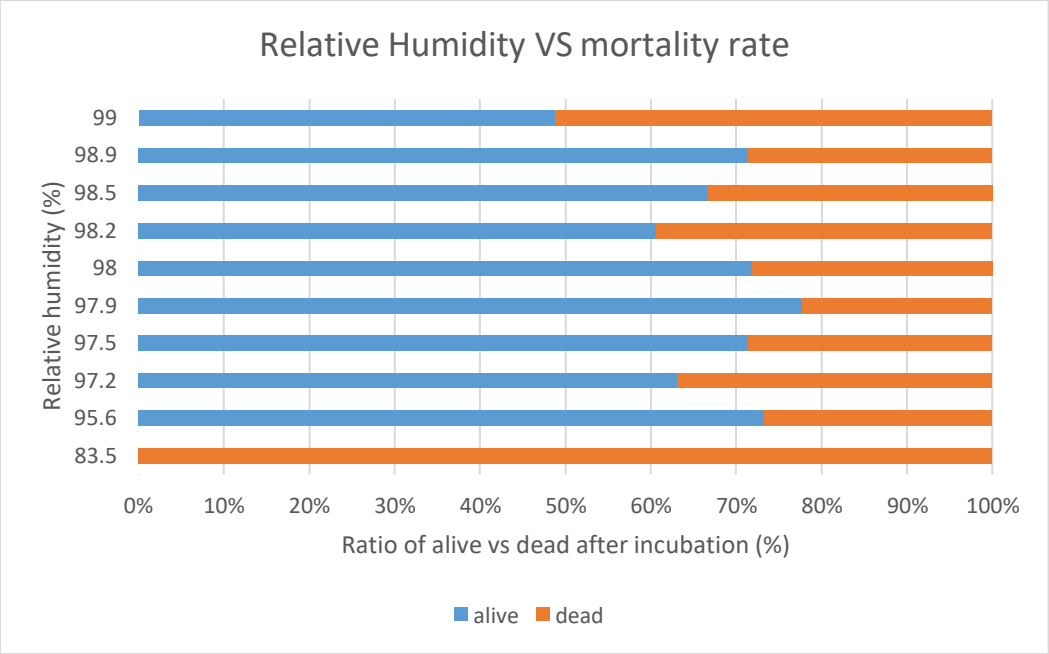


Figure 31. The relationship between relative humidity and mortality rate i.e., how many pupae successfully emerged as adults after the desiccation time ("alive"), and how many died during the time spent desiccating in the incubator ("dead"), at a given humidity.

ANOVA statistical analysis of PCA graphs

Although the PCA graphs already display a 95% interval zone, further analysis was conducted on the PCA graphs using built in functions on MetaboAnalyst. ANOVA tests were run on all PCA graphs from **Experiments 1 to 4**. This was to test whether the differences seen between more than two groups is statistically significant, such that the mean of each of the groups significantly differs from one another. An ANOVA test for **Figure 16**, age experiment, was conducted. The ANOVA test shows that the changes seen between the age groups, in 23 out of the 30 hydrocarbons identified, were statistically significant. Only 7 compounds did not change significantly between age groups ($P<0.05$). **Figure 32** shows this in greater detail and how this is calculated for each compound. 15;14;13MeC30, shows a highly significant change in percentage abundance between each age group ($P=1.48e^{-7}$), whereas the percentage abundance of C33en does not change significantly between age groups ($P=0.356$). These boxplots (**Figure 32**) were carried out for every compound within a PCA, determining whether a given compound's abundance changed significantly between groups. **Figure 17**, salinity experiment, out of the 22 compounds analysed none were significant. This shows there is statistically no correlation between the salinity groups and changes in abundances between any of the identified hydrocarbons. **Figure 19A,** males under desiccation conditions, an ANOVA test could not be performed due to the sample size being too small. **Figure 19B**, the ANOVA test performed on the female group showed that none of the 13 compounds analysed showed a significant change between age groups when desiccated. **Figure 19C**, this PCA graph contained only two groups (males & females) and therefore a t-test was performed in lieu of the ANOVA test. Out of the 12 compounds tested only 5 were significant showing sex differences have a significant impact in changing abundance on some compounds but not others. Lastly, statistical analysis was conducted on the PCAs from **Experiment 4**. **Figure 21A,** statistical analysis shows that no compounds significantly changed between times spent desiccating, even when the

data was grouped into two larger groups, 0.5-2.3h and 3.8-5h, none were significant. The same was true for **Figure 21B**, none of the 16 compounds changed significantly between different times spent desiccating. A t-test performed on **Figure 22B**, showed that 11 out of 19 compounds change significantly between female and male desiccated pupae. Again, showing sex plays a statistically significant role in hydrocarbon profiles of *An. gambiae* pupae.

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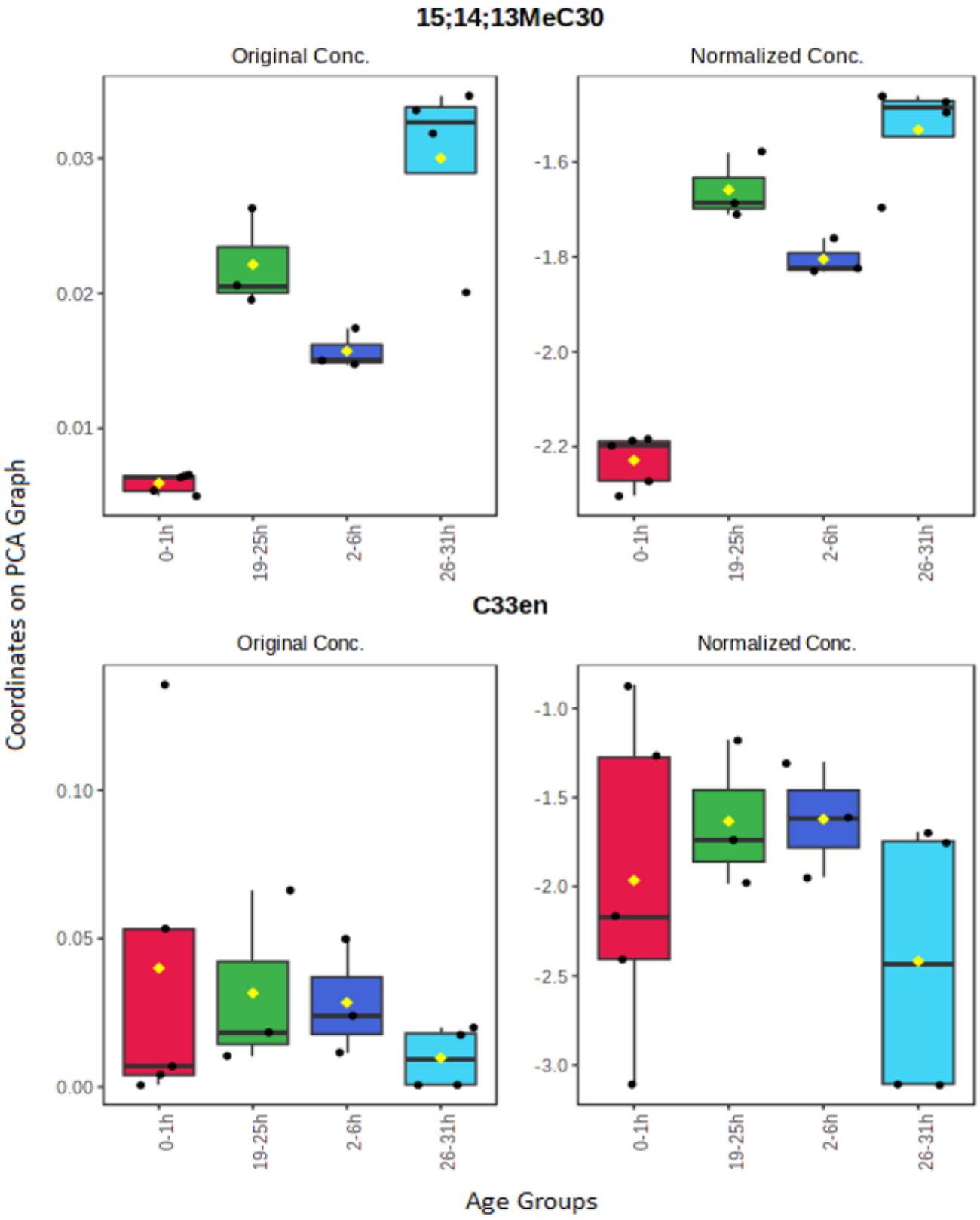

Figure 32. Two boxplot graphs show how the significance can vary greatly between compounds in the PCA graph. The top boxplot (15;14;13MeC30) each groups' mean varies significantly by age group. However, for C33en (bottom boxplot graph), the means between each age group do not vary significantly for this compound between age groups and are noticeably much closer together.

Figure 33. Comparison of the CHC compositions from samples from **Experiments 1 to 4.**

4. Discussion

General findings

Throughout this study we begin to understand how CHC expression in pupae of *An. gambiae* changes depending on a variety of factors, including age, water salinity and presence of water. Furthermore, I also investigated to what extent these variables impact the success of eclosion and mortality rates for both male and female *An. gambiae*.

Age experiment discussion

PCA age graph

The first experiment conducted was to study the impact of age on the CHC profiles of *An. gambiae* pupae. This experiment was initially conducted as it was known that age had a significant impact on CHC expression within insects (Braga et al., 2016). For example, a study on CHCs of *Chrysomya albiceps* found that older larvae tended to have higher molecular weight (longer-chain) hydrocarbons than younger larvae (Alotaibi et al., 2021). From the literature we also know that age impacts the CHC profiles for adult *An. gambiae* (Caputo et al., 2005c). It is thought that age impacts the abundance of CHCs, as the oenocytes which biosynthesise CHCs tend to have greater coverage within the epidermis the older the insect is (Holze et al., 2020). Further to this, the biological processes that produce CHCs within *An. gambiae* are slightly different depending on the life stage of the mosquito (Grigoraki et al., 2020). According to Grigoraki et al, the pathway starts with a fatty acid synthase (FAS) which utilises malonyl-CoA to generate a fatty acyl-CoA (2020). In the case of methyl-branched hydrocarbons propionyl-CoA groups (as methyl-malonyl-CoA) are also incorporated in the growing fatty acyl-CoA chain. The fatty acyl-CoA chain is further extended by elongases. This extension of hydrocarbons occurs more readily in older *An. gambiae* with a greater proportion of longer chain hydrocarbons (Grigoraki et al., 2020). Shortening of the carbon chains is catalysed by P450 enzymes, which are more prolific in younger *An. gambiae* (Qiu et al., 2012). However, despite the essential role the pupal life stage plays in the life cycle of *An. gambiae*, few studies have explored in detail about it. One paper by Lyimo et al discuss the effect of temperature on the age at pupation, showing an increase in pupation age with a decrease in temperature from 30 °C to 24 °C (1992). Balabanidou *et al* discuss CHCs in the pupal life stage of *An. gambiae*, describing how the biosynthesis of CHCs is less active in adult oenocytes and that large deposits of CHCs are generated during the late larval and pupal stages (2016). However, it falls short of analysing these CHCs from the pupae.

To quantify the impact of various environmental changes on the pupae, one must first understand how aging impacts the CHCs of *An. gambiae* pupae. As shown in **Figure 16, Experiment 1**, there is an obvious difference between the compositions of CHCs, collected from pupae of different ages. The different age groups, 0-1 hours old, 2-6 hours old, 19-25 hours old and 26-31 hours old, separate approximately into 4 groups with slight overlap between the 19-25 and 2-6 groups. Due to this overlap, for the calculations below, groups 2-6 h and 19-25 h were combined into one cluster, 2- 25 h and categorised as the middle age. The results indicate that, like *An. gambiae* adults, the

pupal CHCs' also change as a factor of ageing (Caputo et al., 2005c). The exact changes in percentage abundance of compounds are shown in **Figure 28**

Figure 16 shows a PCA, where differences along the first principal component axis (PC1) are responsible for more of the variance than differences along the second or third principal component axis (PC2 & PC3). This means that the oldest group (26 -31 hours) is the least similar to any of the younger pupa groups in terms of their CHC profiles. The coordinates of the midpoint of the oldest cluster (26-31 h), excluding datapoint 13, are: PC1: 3.61 (56%), PC2: 0.35 (19%) & PC3: 0.48 (8.5%), the middle age cluster (2-25 h) coordinates are: PC1: -1.21 (56%), PC2: 1.05 (19%) & PC3: 0.48 (8.5%) and the youngest cluster (0-1 h) coordinates are: -0.68 (56%), PC2: -1.48 (19%) & PC3: 0.13 (8.5%).Using **Equation 3** the distance between the cluster's midpoints can be calculated.

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$$

Equation 3. The distance between two points, in a 3-dimensional plane. The distance between points (X_1, Y_1, Z_1) and (X_2, Y_2, Z_2) is given by **Equation 3**.

Oldest to middle cluster d=4.87, oldest to youngest cluster d=4.67, and middle to youngest clustered=2.608. This quantitively shows that CHCs from group 0-1 h are more similar to 2-25 h than group 26-31 h.

From the calculations above and **Figure 16**, it can be inferred that towards the end of a pupal life stage the *An. gambiae* pupae experience the greatest compositional change in their CHC profiles. Research conducted on *Vespula germanica*, shows similar changes in CHCs between early-stage pupae compared to late-stage pupae, with greater amounts of n-nonacosane present in younger pupae, and greater quantities of n-heneicosane and n-tricosane in older pupae, which are closer to eclosion (Brown et al., 1991; G. H. Zhu et al., 2006). Nonacosane, n-heneicosane and n- tricosane are all known to be volatile compounds used as pheromones (Brei et al., 2004; Funaro et al., 2018). Nonacosane and tricosane are also known to exhibit antibacterial properties (Wang et al., 2012). However, most important for this research nonacosane, n-heneicosane and tricosane have molecular formulas: $C_{29}H_{60}$, $C_{21}H_{44}$ and $C_{23}H_{48}$. This shows a greater presents of certain shorter chain hydrocarbons (n-heneicosane & tricosane) in older pupae compare to younger pupae, that have a greater abundance of nonacosane. These compounds contradict the conclusion from Braga etal., and **Table 6**, which suggest chain length increases with age (2016). However, this maybe an exception for nonacosane, n-heneicosane and n-tricosane, a required trade-off for their useful pheromonal properties. Research has also been conducted both on how CHCs in *An. gambiae*and *Aedes aegypti* adults change with age (Caputo et al., 2005; Hugo et al., 2006). However, this is the first study to quantify the CHC changes within the pupal life stage of *An. gambiae*.

The more radical changes in CHC composition happen towards the end of the pupal life stage. This may be due to different CHCs required for the adult's stage and hence a ramping up in abundance of certain CHCs, most notably alkenes according to **Figure 16B**. This finding is supported in the literature(see **Figure 34**, reproduced from (Zhang et al., 2021). Although the paper by Zhang et al focuses on *Sarcophaga peregrina* it concludes that: "the difference of CHCs of late pupa was more obvious than early pupa", which is exactly consistent with the findings in this study.

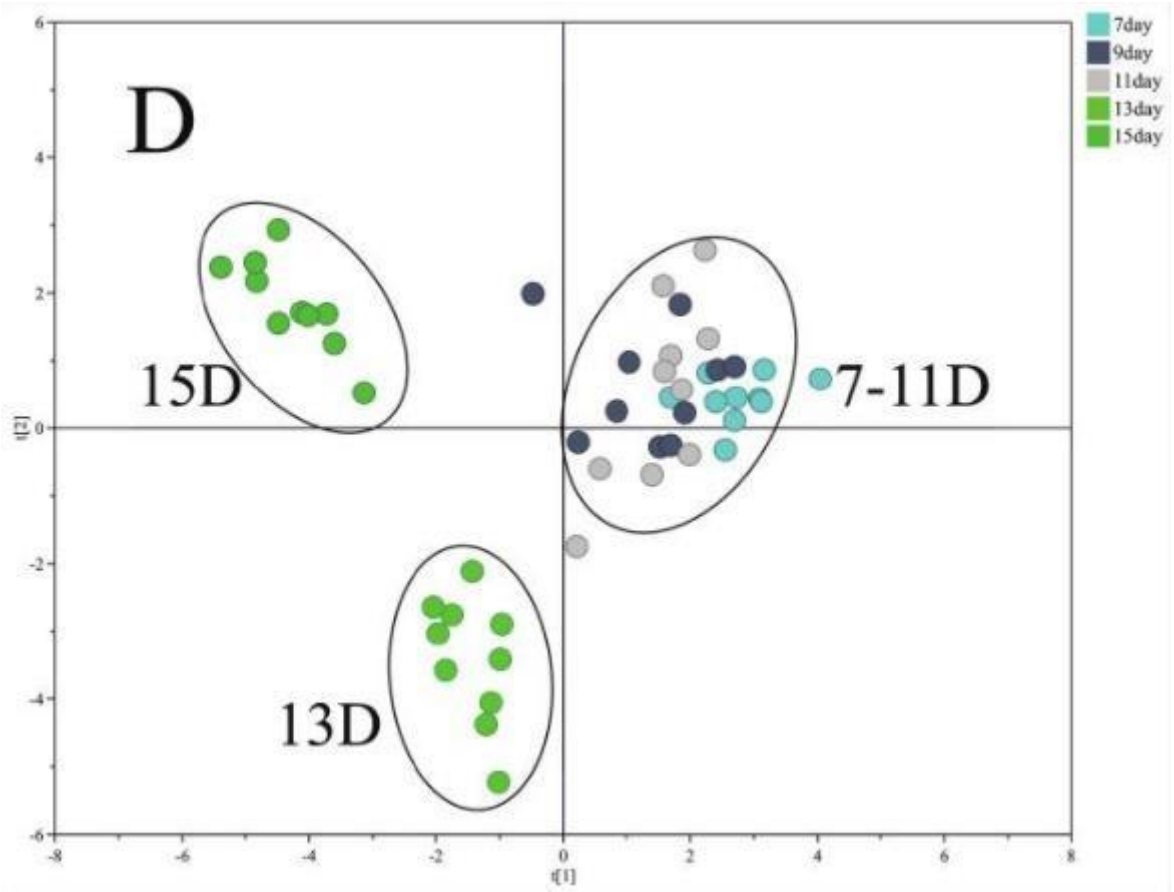

Figure 34. CHCs of pupal *Sarcophaga peregrina*. The CHCs change as a factor of aging and, much like the results for *An. gambiae* in **Experiment 1,** the difference of CHCs between older pupae were more obvious than younger pupae (Image reproduced from Zhang et al., 2021).

This indicated that this ramping up of CHCs towards the end of the pupal life stage is not exclusive to *An. gambiae* pupae but may occur in many different types of species.

The percentage abundance of alkanes within the cuticular layer seems to decrease with age (**Figure 16B**) shows that. The CHC abundance of the youngest pupae show that alkanes comprise by far the majority of the profile (82%) but decrease to less than half in the oldest pupae (49%). Alkenes increase slightly from the youngest to middle-age pupae, and then dramatically from middle-age pupae to the oldest pupae. Dimethylalkanes stay more or less constant, despite a slight reduction in the youngest pupa group, the same is also true for methylalkanes. The decrease in percentage abundance of alkanes and the percentage increase in alkenes with age is rather surprising, as mentioned before alkanes pack the tightest and alkenes the least and hence alkanes offer the greatest waterproofing and desiccation resistance and alkenes the least (Ferveur et al., 2018). **Figure 16B** implies that pupae have the least resistance to desiccation towards the end of the pupal stage and the most at the start of the pupal stage. This may be due to early stage pupae needing this tighter combination of CHCs to withstand the osmotic stress during this aquatic period, compensating for a smaller amount of total CHCs (Botella-Cruz et al., 2017; Hugo et al., 2006). However, this is not entirely at odds with the literature, as one study by Falcon *et al* found that the proportion of unsaturated CHCs, such as alkenes, increases with age in *Apis mellifera* (western honeybees) (2014). The study surmisesthat this increase in the proportions of alkenes compared to n-alkanes in later stage pupae and newly emerged adults may be because alkenes are more informative in insect sociality (Falcón et al.,2014)., which is more useful in later stages of development (Krasnec & Breed, 2013).

Analysis of the youngest and oldest pupae samples shown in **Table 6**, show the oldest pupae have an average chain length of 25.5 carbon atoms whereas young pupae have an average chain length of 24.3. Chain length is a factor in increasing lipid melting point and hence increasing desiccation resistance (Menzel et al., 2019). The importance of chain length in determining desiccation resistance however is still outweighed by the shape (I.e., number of branches and double bonds) of the compound, giving more weight to the type of compound I.e. if it is an alkane or an alkene insteadof the length (Gibbs & Rajpurohit, 2010). Meaning, even with the older pupae having a slightly greater average chain length they will still have a lower lipid melting point which infers reduced resistance to insecticides and desiccation (**Figure 4**) (Balabanidou et al., 2018).

Understanding why it is the case that younger pupae, according to **Figure 16B**, have a more tightly packed lipid layer which is more resistant to desiccation, according to the literature, than older pupae, hints at areas for further research. An idea to explain the surprising results seen in **Figure 16B** would be that according to the research CHC composition is a balancing act (**Figure 4**) (Menzel et al., 2019). The cuticle is a multifunctional device with CHCs playing an important role in chemical communication as well as waterproofing (Moussian, 2010; van Wilgenburg et al., 2011). Chemical communication can only occur if the CHCs are sufficiently liquid to diffuse and equally are able to be recognised by the perceiving insect via CHC binding to olfactory binding proteins on the olfactory sensilla (Blomquist & Bagnères, 2010; Hansson & Stensmyr, 2011; Maitani et al., 2010). Hence, reducing the overall CHC melting point, by increasing the proportions of alkenes, makes diffusion easier and improves chemical communication or signalling (Menzel et al., 2019). Therefore, chemicalcommunication could be more important to older pupae than their younger counter parts. Another theory for why average CHC melting point decreases with age, is that CHCs are an adaptive characteristic, and many studies have highlighted the plasticity of CHCs depending on the environment, over time adjusting to certain environmental stresses (Otte *et al*., 2018). Therefore, considering in this experiment the pupae were raised under optimal laboratory conditions, there may have been little need for such a tightly packed, alkane filled, CHC defensive layer against optimal climatic conditions. Therefore, more weighting could have been given to chemical communication and therefore a greater abundance of alkenes with lower melting points. Perhaps this is put best by Menzel, stating that insects may have to trade off waterproofing and perceptibility of communication signals at a given temperature (or climatic conditions), hence at every temperature the CHC composition must be adjusted to fulfil both functions (2019). This may explain why species exhibit temperature dependent CHC changes leading to profiles that may not necessarily appear optimal in terms of protection against desiccation at a given temperature (Sprenger et al., 2018). Considering the dual functionality of CHCs, future investigations should be conducted on pupae communication via CHCs to better understand this trade-off within *An. gambiae*.

Application of age experiment

Experiment 1 can be used to show compositional changes of CHCs with age. One would expect that as a pupa is preparing for eclosion, it would produce longer chain CHCs withfewer alkenes and branched CHCs such as dimethylalkanes. This is because unlike pupae, adult mosquitos do not reside in water, which increases the rate of evaporation from the cuticle (Church,1959). However, analysis of **Experiment 1** in terms of CHC compounds expressed with regards to age is rather surprising (**Figure 16B**).

The composition of the pupal CHC profiles changes with respect to their age (**Figure 16B**). It is shown quite clearly that as the pupae age, the CHC profile changes as well (**Figure 16B**). The older the pupae are the smaller the percentage abundance of alkanes. Older pupae also tended to have a greater percentage abundance of alkenes (**Figure 16B**). Changes in CHC profiles due to ageing has been extensively researched in flies, as well as a range of adult mosquito species, including *Ades aegypti*and *Anopheles farauti* (Braga et al., 2016; Hugo et al., 2006). Braga et al showed that mosquitoes tend to have CHCs that include somewhat shorter chain length components than most other insects and the older insects are, in general, the longer the CHCs are (2016). Furthermore, individual studieshave been conducted on CHC changes with respect to age, specifically with *An. gambiae* but mainly in terms of absolute abundance of CHCs and only on adults (Wagoner et al., 2014a). Hence, **Figure 16B** shows us conclusively that the dynamic nature of CHCs is not exclusive to the adult stage as suggested by Wagoner et al, but changes within other life stages as well (2014).

This improved understanding of how CHC profiles change throughout the pupal stage could have implications for the application of insecticides. This is because CHCs play a vital role in defending mosquitos from insecticides, in addition to reducing water loss in scenarios of extreme heat and/or a scarcity of water (Balabanidou et al., 2019; Qiu et al., 2012). Therefore, depending on the CHC profile, mosquitos can be more or less susceptible to insecticides. Changes in the dry weight of CHCs in mosquitoes (*Culex quinquefasciatus*), specifically the mean amount in nanograms of CHCs, has been proposed as a way of predicting the differences in levels of resistance between different populationsof *Cx. quinquefasciatus* towards changes in climate and insecticides. The more CHCs per nanogram, the more resilient the population was to higher temperatures & insecticides (Talipouo et al., 2021). Furthermore, populations of *An. gambiae* with a greater total amount of CHCs tended to survive better in areas with lower relative humidity, in comparison to those with less CHCs (Wagoner et al., 2014a). According to **Figure 16B**, CHCs change throughout the pupal life stage. Thus, it can be assumed that younger pupae with a greater abundance of alkanes are less vulnerable to insecticides or drought than pupae towards the end of this life cycle. However, it is important to note this is based only on optimal laboratory conditions and hence to reasonably confirm this, further field studies are necessary.

Pupal and larval stages are often touted as the most vulnerable part of the mosquito life cycle as they remain in still bodies of water and are less mobile than adult mosquitos, as well as being more visible than when they were eggs (*Mosquito Life Cycle | Portsmouth, VA*, 2022)(Ross et al., 2020). This could present an opportunity to exploit a weakness in an already vulnerable stage of life for *An. gambiae*. For example, known pupae within a stagnant pool of water could be targeted with insecticides towards the end of their pupal life stage which correlates to when the CHC layer is most permeable to insecticides due to more alkenes and less alkanes. This could maximise the effectiveness of the insecticide used on this pool of water meaning less insecticide use, which are known to cause damaged to other non-targeted taxa (Brühl et al., 2020).

Salinity experiment discussion

Experiment 2 investigated the effect of water salinity on CHC profiles by placing pupae in differing concentrations of salt solutions (0 g/L – 200 g/L). **Figure 17** indicates that changes in salinity have little impact on the CHC profiles expressed in *An. gambiae* pupae. A study by Botella-Cruz et al (2019) showed that certain species of aquatic beetles (*Nebrioporus, Adephaga: Dytiscidae and Enochrus, Polyphaga: Hydrophilidae*) demonstrate changes in their CHC profiles to increase water proofing depending on salinity conditions (**Figure 35**). The study showed that species from a more saline habitats, as a default, had less branched CHCs compared to species from less saline waters (Botella-Cruz et al., 2019). Furthermore, these authors noted that saline species also displayed an "extraordinary" ability to adjust their CHC profiles in a relatively short amount of time.

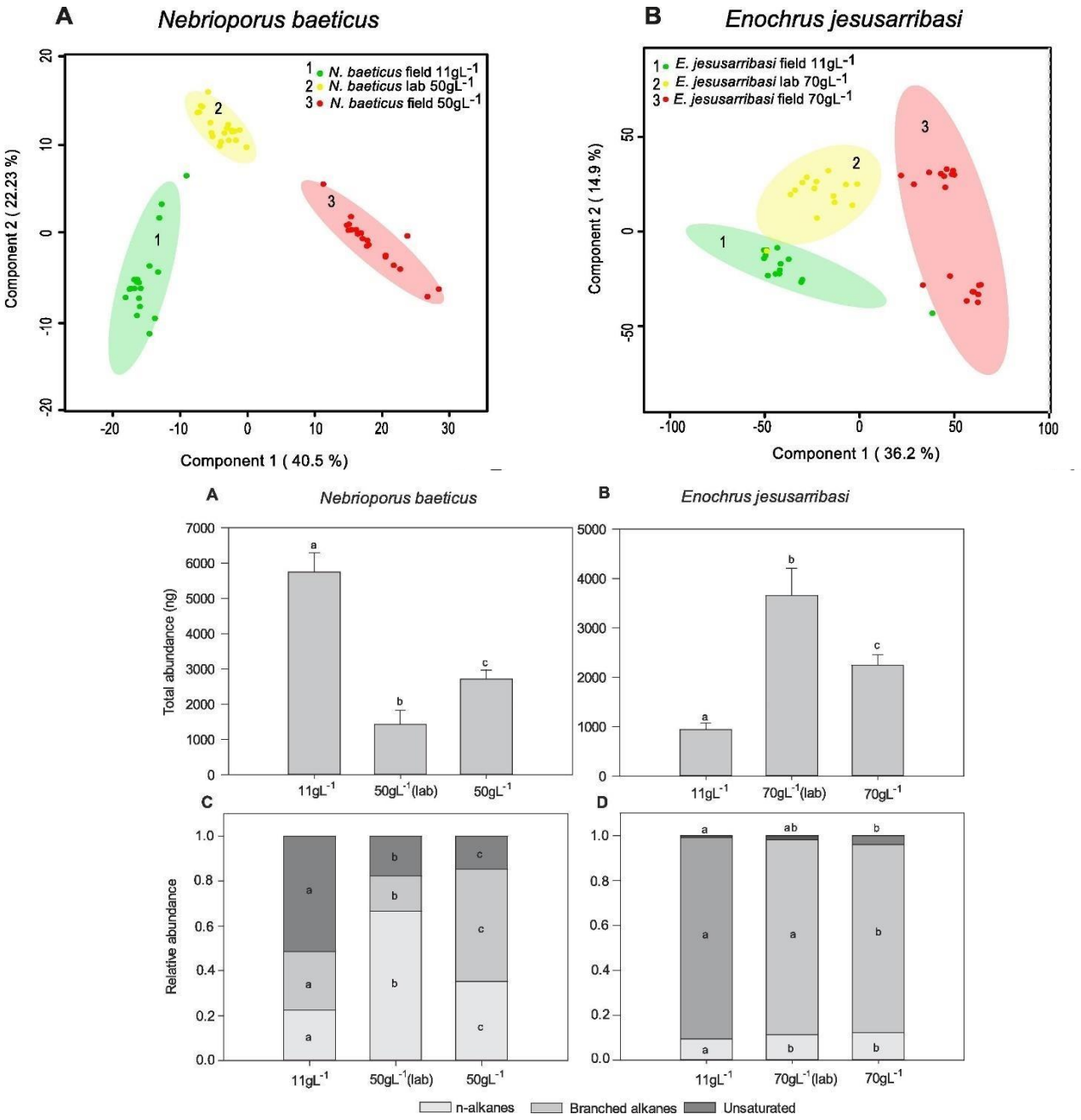

Figure 35. CHC composition changes of *Nebrioporus beeticus* and *Encochur jesusamrribasi* species and how different levels of salinity also alter the abundance of CHCs and the ratio of branched, saturated, and unsaturated CHCs. Graph from Botella-Cruz et al., 2019.

This is very much in contrast to the results from **Experiment 2,** where no such plasticity in *An. gambiae* was seen. **Figure 17** shows that there is virtually no change between the CHC profiles of pupae left 24 hours in 0 g/L NaCl solution through to pupae left in 200 g/L NaCl solution. This begs the question why salinity impacts CHC profiles of species in other studies but seemingly had no impact on the CHC profiles of *An. gambiae* pupae in this study. In the Botella-Cruz study, each species was maintained for 48 h at a salinity corresponding to their collection site and then was placed into a high salinity environment for testing, such as the 50 g/L for *Nerbioporus beaticus* and 70 g/L for *Enohcris,* for 1 week. This may indicate that simply the time frame for **Experiment 2** of only 24 h for *An. gambiae* pupae was insufficient to induce changes in their CHC profiles. Hence, perhaps CHC profile changes may have been more apparent if the pupae were left in more saline conditions for the maximum observed duration of the pupal life cycle, which according to **Table 3**

is around 31 hours. However, it is possible that even 31 h is insufficient to induce changes in their CHC profiles, and, like Botella-Cruz's study, perhaps a time frame of multiple days is required to observe changes. It is impossible of course to run this type of experiment for the pupal stage of *An. gambiae* for multiple days, considering that the maximum pupa age before eclosion was 33 h (**Table 3**) under these conditions. That being said, the salt concentrations used in this study were still considerably higher than even the most extreme saline conditions that *An. gambiae* can be found in. For example, *An. gambiae* larvae have been recorded surviving in conditions of 75% seawater, albeit with a high mortality rate (Ribbands, 1944). This shows that if high salinity concentrations do change the CHC profile, the concentrations used in this study would likely be sufficient to reach whatever threshold there would be.

It has been suggested that the main process by which osmoregulation occurs in anopheline species is via ion regulation by using non-dorsal anterior rectum cells to excrete and resorbs ions, instead of changing CHCs concentrations (Smith et al., 2008). This process depends upon alternative localization patterns of membrane energizing proteins. As such, the level of salt tolerance in a given anopheline species is dependent upon the degree to which that species is capable of shifting the location of these proteins (Smith et al., 2010; White et al., 2013). If this is the principle mechanism of *An. gambiae* osmotic regulation than it would go towards explaining the lack of correlation between CHC profiles and changing salinities.

Surprisingly, the dead pupae from the 200 g/L samples are not noticeably different to the live pupae (**Figure 18**). This indicates that perhaps when pupae die, if CHC compositional changes do occur after death, it is not directly evident in their CHC profiles straight away. This may be due to the chemically stable nature of CHCs once produced (Martin et al., 2009).

Desiccation experiment discussion

At RHs above 95% it seems that mortality rate is not correlated to increases in humidity. This finding is also supported in the literature with Roca & Lazzari's investigation on *Triatoma infestans*, stating there was "no difference between groups; eclosion success ranged from 86 to 94% RH" with the maximum humidity in the investigation being 95%. However, some insects such as *Dinoderus minutus* had an increase in "hatchability" (from egg to first instar larvae) up to a given humidity and then a decrease when humidity became too high (**Table 7**) (Norhisham et al., 2013). However, none of the life stages of *D. minutus* are in water, which may explain this difference between them and *An. gambiae* (Norhisham, 2015).

Influence of relative humidity on egg eclosion

Relative humidity	Egg development	
	Incubation period (day)	Hatchability
20%	4.63±0.25c	8%
40%	5.79±0.13b	66%
56%	5.49±0.18b	74%
75%	5.44±0.14b	86%
85%	10.43±0.32a	48%

Table 7. The relationship between hatchability and relative humidity. Table reproduced from Norhisham et., 2013

Unlike *An. gambiae* towards a certain point (≈75% RH) increases in RH can be detrimental to hatchability. Studies specifically on the effects of humidity on *An. gambiae* show that for adult mosquitos under periods of extremely low levels of RH, which is defined as levels <10% RH, were found to be fatal (Yamana & Eltahir, 2013). This is in stark contrast to the relatively high levels of humidity that are fatal to pupae (outside of water) according to **Figure 31**. One extreme study even showed that female *An. gambiae* could survive up to 30 hours under <10% RH (Gray et al., 2009). Again, this is in great contrast to the experiment performed in this study with a number of fatalities still occurring athumidity levels of 99% and >=1 hour of time desiccating. This shows that, unsurprisingly, adult mosquitos are far more resilient to lower levels of humidity than during their water-bound pupal stage. However, **Figure 31**, when compared to studies on the survivability of adult *An. Gambiae,* illustrates the magnitude of the difference that RH has on different life stages. For instance, when colonies are maintained for this investigation at 65-75% RH, the adults live for weeks.

Sex differences

The results (**Figure 28**) from the desiccation experiment show that females tended to have a higher success rate of eclosion than male *An. gambiae* pupae.

Sexual dimorphism with *An. gambiae* is well researched, especially in the area of genomics (Baker et al., 2011). However, there are only a handful of studies of the role sexual dimorphism plays in *An. gambiae* with regards to the impact on CHC profiles during early development stages (Caputo et al., 2005a; Carlson & Service, 1979). The Caputo study only goes as far as using CHCs for forensic entomology, using CHCs to correctly identify the sex of *An. gambiae* (2005). Whereas Carlson & Service only mention unsaturated hydrocarbons in males and females *An. gambiae* as being very similar, with the main focus of the paper on CHC differentiation between species (1979). However, post-eclosion CHC plasticity is well studied, such as for adults (Rajpurohit et al., 2021).

Sexual dimorphism occurs within the CHC profiles of *An. gambiae* (Caputo et al., 2005b; Thailayil et al., 2018). This study reiterates this idea to a greater extent. Our results show a complete absence of dimethylalkanes in male pupa shells when desiccated, whereas dimethylalkanes were shown to be present in female pupa shells (**Figure 23**). According to the Caputo et al (2005), study there were "significant" CHC sex differences between adult *An. gambiae*. However, the studied concluded that they "did not find any single specific CHC that could differentiate males from females", hence, all dimethylalkanes present in female adults were also present in males, however in different quantities. This indicates a stark difference between our pupal GC-MS data and the *An. gambiae* adult data from Caputo et al (2005) with regards to dimethylalkanes.

Only one other study has investigated CHCs within mosquito pupae (Shaalan et al., 2019), focussing on *Ades caspius* and *Culex pipiens*. The study only looked at changes in n-alkanes (2019). Hence, as of writing this thesis there is no data on non-alkane CHCs such as alkenes, methylalkanes and dimethylalkanes in *An. gambiae* pupae for comparison, nor papers on the relationship between eclosion success and CHC composition.

Could sex differences in CHCs explain the difference seen in hatching success between males and females? It is known that different combinations of CHCs alter the physical properties of the cuticle (Menzel et al., 2019). Therefore, it is possible for the viscosity of the lipid cuticle layer to impact the ease with which adults eclose from their shells. An increase in abundance of dimethylalkanes, which increases lipid viscosity could perhaps in part explain why females have more success eclosing than males.
According to **Figure 23** females have a higher abundance of branched dimethylalkanes whereas dimethylalkanes seem to be completely absent in males. It is impossible to confirm if there is a relationship between CHC composition and eclosion success from the data at present within this study. The data confirms that there is a difference in eclosion success and CHC composition between sexes. However, this does present an interesting hypothesis for future investigations to determine if viscosity of the cuticle is a mechanism partially determining eclosion success. Furthermore, sexual dimorphism impacts a large variety of characteristics such as wing shape and size. In Culicidae, females tend to have slightly larger wings, which could equally impact eclosion success by affecting the difficulty of escaping from the pupa shell (Virginio et al., 2015).

It should also be stated that for the male pupae for the desiccation experiment there were some runs with an above average level of contamination, making analysis of compounds slightly more difficult, even for those with a wealth of experience in analysing GC-MS spectra. Hence, this study would benefit from repeat collections and analysis of male and female pupae. This would yield a higher degree of validity that these changes in CHC compositions are true and the resulting difference in CHCs between sexes is not simply due to contamination. As mentioned in the results one of the most common eclosion outcomes was "half-hatching", wherethe adult *An. gambiae* would become trapped in their exuvia, shown in **Figure 9**. However, there is very little literature concerning this phenomenon and more importantly what induces it (other than desiccation). Research by Khorshidi *et al*., noted this phenomenon in *Liriomyza sativae* (2017).The paper found that when cyromazine (an insect growth regulator used as an insecticide) was added to the water the adult fly would get trapped in its pupal exuvia. Similarly research on *Aedes aeqypti,* again on an insect growth regulator (Lufenuron), found that in the presence of this chemical around 20% of L2 instar either could not moult and remained trapped inside the new exuviae or possessed bulged abdomen, while others showed ruptured exoskeletons (Panmei et al., 2020). However, nearly all papers that mention this phenomenon in mosquitoes were about insect growth regulators at the larval stage. Indicating an area for future study on what other climactic conditions induce this half-hatching phenomenon.

CHC vs desiccation

One of the main aims of the study was to investigate the pupal response to desiccation. The primary focus was to understand if there is a chemical response in terms of CHC profile change to desiccation. CHC changes in response to both desiccation and changes in temperature has been well documented in the literature in *Drosophila melanogaster* (Rajpurohit et al., 2021; Stinziano et al., 2015). *An. Coluzzi* (a sub-type of *An. gambiae*) adults' CHC profiles have been known to alter depending on the season, with an increased abundance of CHCs during dry seasons, as a form of desiccation tolerance (Arcaz et al., 2016). *An. gambiae* CHCs response to both insecticides and desiccation as adults has also been noted within the literature (Balabanidou et al., 2019). The vast majority of published research at present focuses entirely on post-eclosion CHC changes (such as in adults), with only some research confirming CHC changes are related to the age of the pupa case for a variety of species such as *Calliphora vicina, Lucilia sericata* and *Chrysomya megacephala*; however this is mainly in the realm of forensic entomology (Moore et al., 2017; Nasir et al., 2019). **Experiment 3 & 4** aimed to see if this plasticity occurs in reaction to environmental changes during the pupal stage.

Experiment 3 for the most part was the precursor experiment to **Experiment 4** and hence many short comings in **Experiment 3** were remedied for **Experiment 4,** the biggest of which was being able to record the exact time samples spent dessicating in their pupal form. Hence, many things that could be said for **Experiment 3** are allocated to the discussion section below for **Experiment 4** instead. Although there are a few important observations from **Experiment 3.** Like **Experiment 4, Experiment 3** reinforces the idea that the time in which the pupae spend desiccating has a minimal impact on their CHC profiles. Further to this both **Experiment 3 & 4** show minimal changes in the CHC composition. Both comprise primarily of alkanes and methylalkanes, however there seems to be a greater abundance of dimethylalkanes for females in **Experiment 4** than **Experiment 3.** It is unknown why this is the case. Perhaps this is caused by slight differences in experimental procedures between the experiments or contamination in **Experiment 3**, making the dimethylalkanes (which are the most difficult compound to identify correctly) even harder to identify.

Experiment 4, **Figure 21** (PCA desiccation time) shows that there is no discernible relationship between the length of time the pupae spent desiccating and changes in their CHC profiles. Although other types of chemical plasticity in response to environmental change has been noted in pupae such as colour plasticity of the exuvia (Mayekar & Kodandaramaiah, 2017). This indicates that although chemical changes do occur during the pupal stage in response to the environment perhaps CHCs are excluded from this environmental related plasticity. More research is needed to not only confirm the absence of CHC plasticity in response to environmental changes in pupae but also the reasons behind this, especially why CHC plasticity is common in adults but, as this study indicates, less so in pupae.

There also may be limitations within **Experiment 4** which make it difficult to determine whether desiccation has an observable impact on *An. gambiae* CHC profiles. It is observed in the literature that for adult *An. gambiae* the more extreme the temperature and humidity conditions are, the greater the effect on CHC abundance and composition (Wagoner et al., 2014). Furthermore, the longer the adult is exposed to these conditions, the greater the impact on the CHC profile (Wagoner et al., 2014). For example, one study on aphids used a desiccating period of 24 h to induce changes in CHCs (far above the maximum desiccation time for our study which is 8.5 h) (Yang et al., 2022). To see the maximum change in the pupae CHC profile the pupae will have to be exposed to the

maximum temperature and humidity stress for the longest period of time. However, in some cases CHC profiles are known to dramatically change between alive and dead samples such as in *Chrysomya megacephala*, although this does not seem to be the case for *An. gambiae* from the results in **Figure 17** (Guang Hui Zhu et al., 2013). However, a premature death of the pupae caused by these extreme conditions may halt developmental changes in its CHC composition. If the stresses are too much and lead to fatalities of the pupae, such as at 83.5% RH (**Figure 31**), it will be difficult to distinguish if the changes are a result of environmental stresses or the chemical changes that occur during and after death (despite the magnitude of these changes being seemingly minimal) (Ping, 2020).
However, maximising stress to a level which can be reflected in the CHC profiles may be more challenging than previously thought. It is widely agreed in the literature that the immature development stages (egg, larva and pupa) are the most vulnerable to both predation and environmental stresses with immature developmental time being highly correlated to mortality rate (Nielsen & Axelsen, 1988; Ning et al., 2017). *Danaus plexippus* pupae and larvae, for example, have a higher sensitivity to both "extreme" temperatures (above 33 °C for this taxa) and fluctuations in temperature compared to adult *D. plexippus* (York & Oberhauser, 2002). Therefore, it could be possible that the temperature or humidity changes that would induce an observable change in the CHC profile of *An. gambiae* pupae are too great and causes the pupae to die before the change can be observed. This proposes areas for future investigations where *An. gambiae* pupae are exposed to maximum survivable desiccation stresses (highest temperature, lowest humidity and longest time desiccating) to see if there is a CHC profile difference between that population and those that are maintained in water.

The PCA analysis of the pupa shells from the desiccation **Experiment 3 & 4** although, show no obvious difference between times spent desiccating and CHC composition. The desiccation experiment shows a complete absence of alkenes when compared to pupae in the age experiment and salinity experiment. This can be seen more easily in **Figure 33**, a master figure comparing CHCcompositions between all experiments.

Figure 33 shows more clearly the contrast between the CHC compositions of pupae. **Figure 33** shows that pupae that were allowed to develop in water (saline and DI) (**Experiment 1** & **2**) and pupae that were desiccated (removed from water) (**Experiment 3** & **4**) have notably different profiles. Most notably the presence of alkenes from experiments where the pupae were left in water and the absence in pupae that were removed from water. As explained above this makes sense due to alkenes' role in both increasing CHC profile volatility and reducing desiccation resistance.

It may seem obvious to suggest that because alkenes are known to disrupt lipid packing thereby decreasing waterproofing, it follows they would be missing from the desiccated pupae, especially when compared to pupae from the age experiment, which were left in optimal conditions (**Figure 33,** master graph). However, one important caveat to mention is the 4 different experiments used slightly different methodologies, the most important of which is that for the age and salinity experiments pupae were directly frozen before hatching and CHCs extracted, whereas in the desiccation experiment only pupae shells were frozen and CHCs extracted. Hence this discrepancy between alkenes could be a reaction to environmental stresses or simply the difference in CHC extraction between pupae and pupae shells. This presents another area for a future investigation of non-desiccated pupae shells with desiccated pupae shells to see if these differences in alkene abundance still persist.

Further analysis of the composition of the CHC profiles with respect to time spent desiccating (**Figure 19 & 21**) shows that there is no evident relationship between the time spent desiccating and changes in the ratio of alkanes, methyl-branched alkanes and dimethylalkanes. Many of the reasons why no changes are seen are mentioned above, such as the length of time desiccating being too short to induce profile change. However, it does beg the question for a future investigation, as to why there are no CHC changes within experiments between samples, with regards to time spent desiccating but changes with regards to desiccating and non-desiccating pupae.

However, it is important to note that the relationship between CHCs and desiccation resistance is not always as clear. Other physiological factors should also be included in further studies to gain a more holistic view of how *An. gambiae* resists desiccation. For example, on the Sahelian belt mosquitos are subject to strong daily fluctuations in temperature and relative humidity. There is a difference in desiccation resistance and water balance of adult female mosquitos *An. gambiae* and *An. arabiensis* (Gray & Bradley, 2005). One of the biggest factors impacting desiccation tolerance is having a higher body water content prior to desiccation (Gray & Bradley, 2005). Interestingly no changes in rate of water loss during desiccation or water content at death were noted (Gray & Bradley, 2005). This is interesting, as it suggests that CHCs mayhave less impact on desiccation tolerance than other studies would suggest. Metabolic rate and respiratory patterns also showed no statistically significant difference on desiccation tolerance between these species (Gray & Bradley, 2005)

Concluding thoughts

This study shows sexual dimorphism occurs with regards to eclosion success in *An. gambiae* pupae. Female pupae are likely to have a lower mortality rate and higher success rate of eclosion when compared to males under desiccation conditions. Males are more likely to become trapped in their shells during the eclosion process ("half-hatching"), under desiccation conditions. CHC analysis of female and male pupae show dimethylalkanes are present in female pupae shells but not in male shells. Branched hydrocarbons such as dimethylalkanes decrease the melting point of the lipid layer making it more "liquid like" (Menzel et al., 2019). This in part may explain the differences seen with eclosion success rates between sexes. However, more research is needed to determine the mechanisms at play. This study confirms that pupal CHCs in *An. gambiae* change depending on the age and sex of the pupae. However, changes in salinity and how long the pupae are desiccating for has a negligible impact on the CHC composition. This highlights the reduced plasticity of pupal CHCs to react to environmental stresses when compared to the adults (Wagoner et al., 2014b), possibly suggesting a greater vulnerability in the pupal life stage to environmental stresses and perhaps insecticides (insecticides were not directly tested in this study but it can be inferred due to strong links between reduced CHC plasticity and vulnerability to insecticides (Adams et al., 2021). Hence, targeting *An. gambiae* pupae may prove to be more effective in the ongoing fight to reduce vector transmission. However, more research is required, such as if the reduced plasticity is a result of biological factors or reduced time for the pupae to react to environmental stresses due to the short time frame of the pupal life stage.

This study quantifies the CHC differences with *An. gambiae* pupae within different climatic scenarios, such as drought. This study shows a discrepancy in eclosion success rates between male and female pupae under desiccation conditions and aims to provide a theory behind the discrepancy using CHCs as a framework.

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