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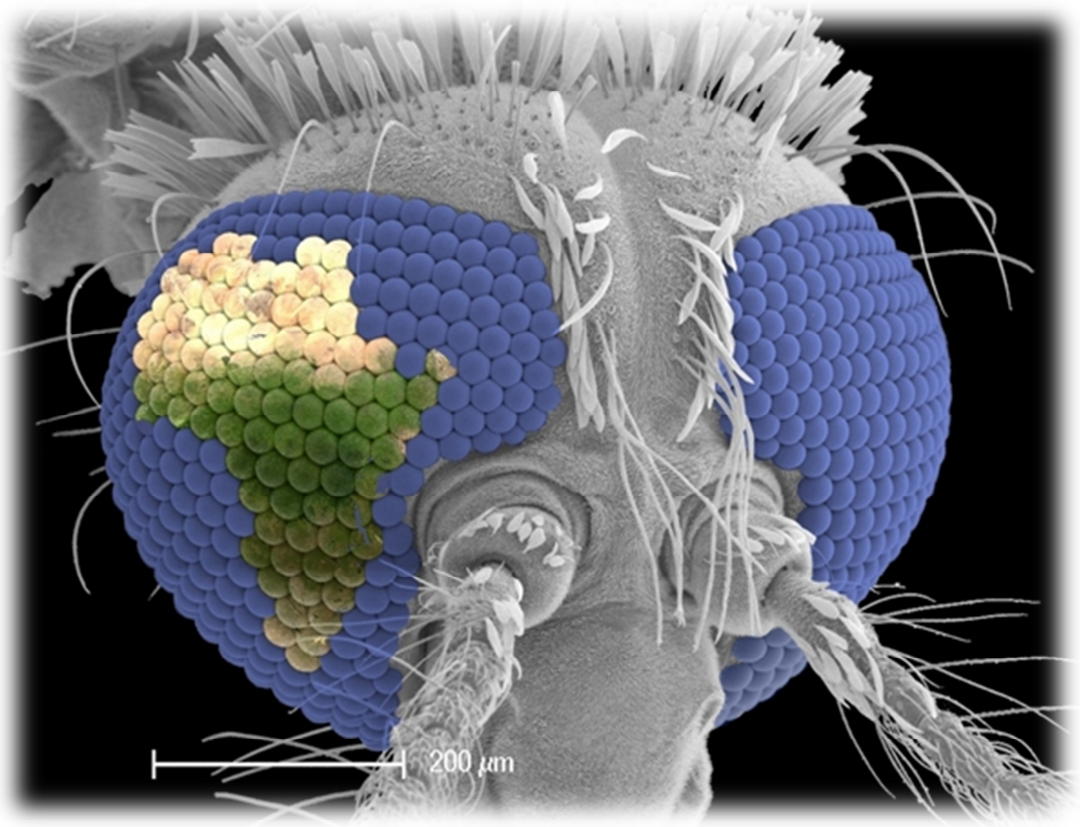
Respiratory physiology and anthropophilic behavioural traits of the main malaria vector *Anopheles gambiae* Giles (Diptera: Culicidae)

Ph.D. dissertation submitted to the Faculty of Sciences of the University of Neuchâtel

by

Jérôme Frei



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Like me, most scientists are happy to be higher primates preadapted for no good reason to making fabulously complicated twigs with which to fish out delicious and entertaining nuggets from the termite mound of consensual reality. Some nugget collections form patterns that jibe with accepted theory, itself a product of induction based on observation.

John R. B. Lighton 2007

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Abstract

Keywords: *Anopheles gambiae* Giles, malaria, mosquito, flow-through respirometry, resting metabolic rate, gas exchange pattern, temperature, body size, discontinuous gas exchange cycle, carbon dioxide, olfactometer, anthropophily, axillary odour, axilla bacteria, lactic acid, (R)/(S)-3-hydroxy-3-methylhexanoic acid, (R)/(S)-3-methyl-3-sulfanylhexan-1-ol, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Corynebacterium jeikeium.*

Anopheles gambiae Giles (*An. gambiae*) is the main vector of malaria. To fulfil metabolic demand *An. gambiae* is particularly dependent on blood feeding which is an important driving force of its strong vectorial capacity. It is therefore important to better understand how its metabolism scales with body size and, as an ectotherm, evaluate the thermal sensitivity of its metabolism. Meanwhile, understanding how respiratory gases are exchanged, which gas exchange pattern (GEP) is employed by *An. gambiae* is also of broader importance as little is known about the respiratory physiology of smaller insects. For this purpose a flow-through respirometer system was pushed to its limit in terms of both its precision and temporal resolution in order to fully resolve the GEP of resting female *An. gambiae*. The underlying components constituting the GEP could be identified and quantified, including: the standard CO_2 production rate of resting *An. gambiae* ($s\dot{V}CO_2$) as a proxy for resting metabolic rate, the inter-burst CO_2 emission rate ($i\dot{V}CO_2$) and duration (P_{int}), and CO_2 burst frequency (F), duration (P_b), amplitude (A) and volume (V_b). Over the temperatures and body size ranges tested, $s\dot{V}CO_2$ varied over a 10-fold range (20 to 32°C, 0.9 to 2.3 mg, 0.6 to 6 µl CO_2/hour). Multiple regression analysis demonstrates a positive and almost isometric scaling with living body mass and an apparent Q_{10} of 2.13, i.e. for a 10°C increment $s\dot{V}CO_2$ is more than doubled. Comparison of two age groups reveals lower and more controlled $s\dot{V}CO_2$ by older (6 days) *An. gambiae,* suggesting the existence of an optimum age for metabolic control. Considering that a higher metabolic demand imposed by the environment may enhance foraging and contact to hosts, these relationships provide an important foundation for bottom-up modelling for diseases transmitted by mosquitoes.

At rest, the GEP of *An. gambiae* can be characterised by the cyclic repetition of CO_2 bursts intercalated with inter-burst periods with a lower CO_2 emission rate. Individuals presenting particularly low $s\dot{V}CO_2$ values exchange respiratory gases not only cyclically but also discontinuously (gas exchange is negligible during inter-burst periods). Disturbed *An. gambiae* presented a continuous GEP. It is concluded that *An. gambiae* uses all three GEPs

described so far in the literature. With increasing temperature it is shown that F strongly modulates the gas exchange rate by increasing faster than $s\dot{V}CO_2$. This discrepancy is almost compensated by lower V_b values at higher temperature suggesting a decrease in the haemolymph buffering capacity for CO_2 with increasing temperature. F is independent of body mass whereas V_b scales out of proportion with body size suggesting a relatively larger tracheal volume in bigger mosquitoes. Considering mosquito size and the wide panel of behaviours undertaken during adult life, it is suggested that the ability to employ various GEPs and modulating its components might be an advantage to adapt the respiratory gas exchange pattern used (discontinuous and cyclic to only cyclic and eventually to continuous) to metabolic demand depending on the situation encountered in adult life.

Another major contributor to the vectorial capacity of *An. gambiae* is its odour-mediated preference to bite humans over other vertebrate host and certain human types over others. The second part of the thesis focuses on the identification of potential infochemicals that may explain this odour-mediated anthropophilic and discriminative host seeking behaviour of *An. gambiae*. For this purpose an experimental paradigm integrating both a sound behavioural evaluation concepts and an appropriate testing environment is used. An improved dual-choice olfactometer that tests the host seeking behaviour of *An. gambiae* in the presence of continuous well-controlled intermittent CO_2 stimulation is presented in both arm is developed. This background stimulation simulates the presence of two potential hosts whilst acting as a releaser of odour perception. Olfactometer tests with lactic acid, a human eccrine signature, added on one arm in the presence of CO_2 pulses as sensitizer, confirm earlier findings that this infochemical not necessarily augments anemochemotactic upwind flight of the insect vector but influences the mosquito's discriminative behaviour. After confirming the validity of the experimental paradigm it is used to evaluate the responses of *An. gambiae* to odours from human male and female axillary sweat incubated with 3 human axilla bacteria species. *Staphylococcus epidermidis* was selected for its low odour-producing pattern, *Corynebacterium jeikeium* for its strong Nα-acylglutamine aminoacylase activity liberating carboxylic acids including (R)/(S)-3-hydroxy-3-methylhexanoic acid (HMHA), and *Staphylococcus haemolyticus* for its capacity to liberate sulfur-containing compounds including (R)/(S)-3-methyl-3-sulfanylhexan-1-ol (MSH). It is demonstrated that *An. gambiae* is able to discriminate for the olfactometer arm conveying odour generated by incubating any of the three bacteria species with either male or female sweat. Whereas *An. gambiae* cannot discriminate between male and female sterile sweat samples in the olfactometer, the mosquito consistently shows a preference for male sweat over female sweat incubated with the same

bacterium, independent of the species used as inoculum. Axillary sweat incubated with *C. jeikeium* rendered *An. gambiae* particularly responsive and this substrate elicited the strongest preference for male over female sweat. HMHA and MSH are suspected to be unique to human odour. It is shown that when tested on their own, neither HMHA nor MSH elicited a clear discriminating response but may affect human host approach by *An. gambiae* or enhance activation followed by sustained upwind flight over longer distances.

Résumé

Mots clés: *Anopheles gambiae* Giles, malaria, moustique, respirométrie à flux continu, taux métabolique au repos, schéma respiratoire, température, taille corporelle, échange gazeux cyclique et discontinu, dioxyde de carbone, olfactomètre, anthropophilie, odeur axillaire, bactéries des aisselles, acide lactique, (R)/(S)-3-hydroxy-3-méthylhexanoïque acide, (R)/(S)-3-méthyl-3-sulfanylhexane-1-ol, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Corynebacterium jeikeium.*

La vie adulte des moustiques est marquée par la répétition de séquences comportementales incluant: la recherche d'un partenaire sexuel, la recherche de ressources (repas sanguin sur un hôte ou sucre d'origine végétale) et la recherche d'un site de ponte ; ces séquences comportementales étant intercalées de périodes plus ou moins longues de repos. L'entreprise de l'un ou l'autre de ces comportements typiques est influencée par des facteurs aussi bien internes (comme par exemple le bagage génétique ou l'état physiologique) qu'externes (comme les conditions environnementales ou les signaux émis par l'hôte). Dans ce contexte, le principal vecteur de la malaria, *Anopheles gambiae* Giles (*An. gambiae*), se distingue d'autres moustiques en plusieurs points : il commence sa vie adulte avec peu de réserves métaboliques, il concentre ses repas sanguins et doit souvent même les multiplier, afin de pouvoir pondre. Ce moustique est endophage, endophile et démontre un comportement anthropophile (préférence à piquer des êtres humains à par rapport à d'autres vertébrés) dicté principalement par la signature olfactive de l'homme. Dans cette thèse, je me suis donc intéressé à la physiologie respiratoire et à l'identification de signaux chimiques potentiels typiques à l'homme qui régulent la vie adulte de ce moustique.

Comprendre comment ce moustique échange les gaz respiratoires avec son environnement ou quel schéma respiratoire (SR) il utilise est fondamental, car on en sait très peu sur la physiologie respiratoire des insectes de petite taille. En tant que petit ectotherme et sachant que ce moustique est particulièrement dépendant de l'hématophagie pour répondre à ses besoins métaboliques de base, une meilleure compréhension des variations de son SR en fonction de sa taille et de la température est également importante. Pour ce faire, un système de respirométrie à flux continu a été poussé vers ses limites en termes de précision et de résolution temporelle, afin de résoudre le SR utilisé par *An. gambiae*. Au repos, le SR de *An. gambiae* est caractérisé par la répétition cyclique d'événements excrétoires de CO_2 marqués, suivis de périodes d'émission plus basses. Les composantes sous-jacentes constituant le SR ont pu être identifiées et quantifiées. Ces dernières incluent : le taux métabolique au repos représenté par le taux de production standard de CO_2 au repos ($s\dot{V}CO_2$), la durée (P_{int}) et le taux d'émission de CO_2 ($i\dot{V}CO_2$) entre les événements excrétoires de CO_2 marqués, ainsi que la fréquence (F), le volume (V_b), l'amplitude (A) et la durée (P_b) de ces derniers. Sur l'intervalle de taille et de température expérimentés, $s\dot{V}CO_2$ varie d'un facteur de 10 (20 à 32°C, 0.9 à 2.3 mg, 0.6 à 6 µl CO_2/heure). Une analyse par régressions multiples démontre une relation positive et isométrique de

$s\dot{V}CO_2$ avec la taille et un Q_{10} apparent de 2.13 (pour une augmentation de 10°C, $s\dot{V}CO_2$ est plus que doublé). Sachant qu'un taux métabolique plus élevé imposé par l'environnement pourrait provoquer une recherche de ressources nutritionnelles accrue, voire un contact plus fréquent avec l'hôte, cette relation est un fondement important pour toute tentative de modélisation bottom-up des maladies transmises par les moustiques. La comparaison entre deux groupes d'âge distincts démontre un $s\dot{V}CO_2$ plus bas et mieux maîtrisé chez les *An. gambiae* plus âgés (6 jours), suggérant ainsi un âge optimal pour le contrôle du métabolisme. Les individus présentant un $s\dot{V}CO_2$ particulièrement bas, en plus de respirer de manière cyclique, respirent aussi de manière discontinue (échanges de gaz proche de 0 durant la période de basse émission). En activité, *An. gambiae* utilise un SR continu. Il est conclu que ce petit insecte est capable d'utiliser les 3 SR décrits à ce jour dans la littérature chez les insectes (SR continu, cyclique et cyclique/discontinu). Lors d'une augmentation de température, il est démontré que F module fortement le taux d'échanges gazeux en augmentant plus vite que le taux métabolique au repos. Ce découplage est compensé par une diminution de V_b, suggérant ainsi une diminution de la capacité tampon pour le CO_2 dans l'hémolymphe lorsque la température augmente. F varie indépendamment de la masse corporelle, alors que V_b augmente de manière disproportionnée avec cette dernière, suggérant ainsi un volume trachéal relativement plus élevé chez les individus de plus grande taille. Si l'on tient compte de leur taille et de la variété de comportements que les moustiques sont capables d'entreprendre durant leur vie adulte, il est suggéré que la capacité d'utiliser divers SR peut être un avantage, afin d'adapter le schéma respiratoire à la demande métabolique imposée selon les circonstances rencontrées durant la vie adulte.

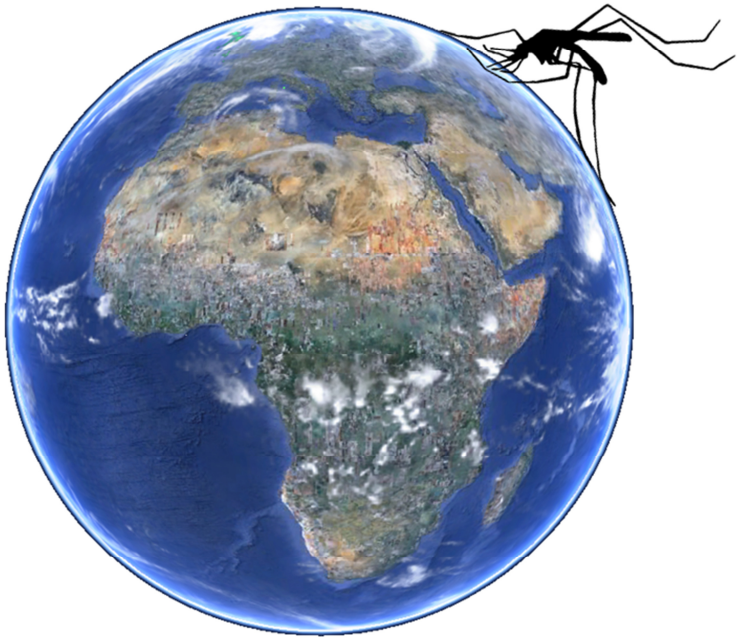
An. gambiae, en plus d'être anthropophile, montre même une préférence pour certains types d'humains. Il est connu que le mécanisme derrière ce comportement sélectif est médié par l'odeur émise par l'hôte. La seconde partie de cette thèse s'est donc concentrée sur l'identification de signaux chimiques émis par l'hôte potentiellement impliqués dans ce comportement sélectif. Pour ce faire, un paradigme expérimental intégrant une bonne évaluation du comportement dans des conditions expérimentales optimales a été développé et utilisé. Ce dernier comprend : i) une population test de moustiques adultes spécialement élevée et prête à rechercher un hôte, ii) un olfactomètre amélioré à deux ports permettant de mesurer l'intensité de la réponse chimioanémotaxique du moustique et sa capacité discriminative en redirigeant son vol vers l'une des deux odeurs sources présentées, iii) la présence inhérente de fluctuations de CO_2 contrôlées afin de simuler la présence de deux hôtes potentiels et augmenter la sensitivité des moustiques et iv) des conditions de vol optimales (température, humidité et vitesse du vent).

Les fluctuations de CO_2 ont pu être mesurées et reproduites de manière consistante dans les deux ports de l'olfactomètre. Cette stimulation a augmenté la réceptivité des moustiques à répondre à des odeurs. Des expériences conduites avec l'ajout d'acide lactique (une signature olfactive humaine d'origine eccrine) dans un des ports de l'olfactomètre confirment que ce composé volatile n'intensifie pas la

réponse chimioanémotaxique de *An. gambiae* mais influence son comportement discriminant. Après avoir validé le paradigme expérimental avec l'acide lactique, ce dernier a été utilisé pour évaluer la réponse du vecteur à des odeurs provenant de l'incubation de sueur mâle et femelle avec 3 espèces de bactéries peuplant l'aisselle humaine. *Staphylococcus epidermidis* a été choisi car il produit une odeur moins intense, alors que *Corynebacterium jeikeium* et *Staphylococcus haemolyticus* ont été choisi pour leurs contributions biologiques importantes dans la production d'une odeur axillaire intense. Ces deux dernières bactéries, par le biais d'une activité enzymatique spécifique et prononcée, libèrent respectivement des acides carboxyliques dont notamment le (R)/(S)-3-hydroxy-3-méthylhexanoïque acide (HMHA) et des composés volatiles soufrés tel que le (R)/(S)-3-méthyl-3-sulfanylhexane-1-ol (MSH). Les expériences avec l'olfactomètre démontrent que les trois bactéries, de par leur action sur la sueur mâle ou femelle, libèrent des composés volatiles influençant le comportement aussi bien chimioanémotaxique que discriminant de *An. gambiae*. Si le vecteur n'a pas pu distinguer la sueur mâle de la sueur femelle stérile, l'action de chaque bactérie a rendu la sueur mâle plus attractive de manière consistante. L'action de *Corynebacterium jeikeium* a généré la réponse chimionanémotaxique la plus intense et a engendré la préférence la plus forte pour la sueur mâle. Les composés volatiles HMHA et MSH sont suspectés d'être propres à l'odeur humaine mais aucun des deux composés volatiles n'a généré de préférence telle que celle observée pour l'acide lactique. Il n'est cependant pas exclu que ces deux signaux chimiques influencent le comportement du moustique au moment où il approche l'hôte ou encore engendre une activation suivie d'un vol soutenu en direction de l'hôte à des distances plus éloignées.

Table of Contents

1 General introduction



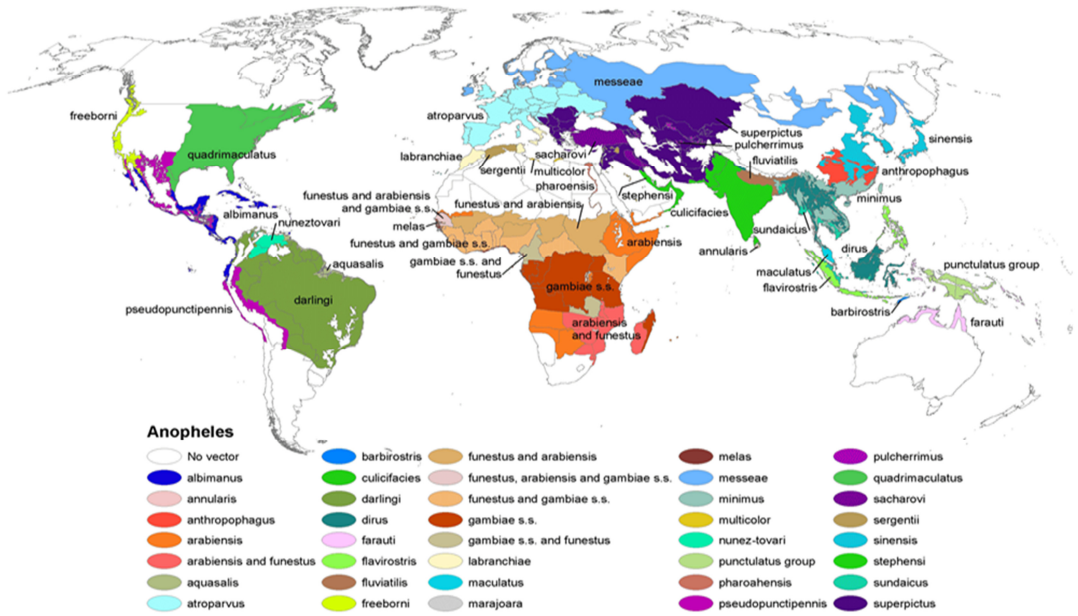
Despite tremendous investments and progress to control malaria its burden on human population prosperity remains important. Close to half the earth's population is under the threat of malaria infection and the estimated number of reported cases ranges from 148 to 304 million per year of which 235 to 639 thousand lead to death. Malaria primarily affects the Sub-Saharan portion of the African continent and it is an important contributing force to poverty in that continent (Brooker et al., 2000; Sachs and Malaney, 2002; WHO, 2016). Malaria infection is caused by eukaryotic protists of the genus *Plasmodium* and transmitted within human population by mosquitoes of the genus *Anopheles*. To combat the parasite artemisinin-based combination therapy is used against *Plasmodium*. Vector control methods such as long-lasting insecticidal treated bed nets and indoor residual spraying of insecticide have also proved to be efficient. Traps equipped with lures furnish adequate tools to monitor vector populations and hence use available resources more efficiently to contain malaria outbursts. Vector control improvement requires a better understanding of the physiological and behavioural fundamentals of malaria mosquitoes

regulating their intimate interaction with human beings. This thesis is a contribution to knowledge on the physiology and chemical ecology of the principal African malaria vector *Anopheles gambiae* Giles. By paying particular attention to *Anopheles gambiae* Giles this thesis is introduced with some important fundamentals of its biology. The important behaviour depicted by adult mosquitoes, including those that position *Anopheles gambiae* Giles as the principal malaria vector, are described in further details. Finally the two main themes and objectives of the thesis are introduced and outlined.

1.1 *Anophelinae* among other mosquito species

In total, there are 41 genera of mosquitoes regrouping ~3500 species. Mosquitoes are *Diptera* that belong to the *Culicidae* family. Within this family they are classified into three subfamilies, namely the *Anophelinae*, the *Culicinae* and the *Toxorhynchitinae*. The *Culicinae* subfamily is the largest and the most diversified and is therefore divided into tribes (the *Culcini*, the *Aedini*, the *Sabethini* and the *Mansoniin*, etc). The *Anophelinae* subfamily regroups three genera: *Anopheles* (Meigen), *Bironella* (Theobald) and *Chagasia* (Cruz). The *Anopheles* genus is constituted of ~ 430 species from which ~ 100 are known for their ability to transmit malaria however only 30-40 effectively do so in nature. The *Anopheles* studied species in this thesis is *Anopheles gambiae* (Giles 1902) henceforth abbreviated *An. gambiae* throughout the thesis. With *An. funestus* and *An. arabiensis* it belongs to the most efficient malaria vectors. *An. gambiae* is classified in the *Anopheles gambiae* complex which regroups 8 species which are morphologically undistinguishable. These include *An. merus* Dontiz, *An. melas* Theobald, *An. arabiensis* Patton, *An. quadriannulatus* Theobald, *An. bwambae* White, *An. amharicus* Hunt, Coetzee & Wilkerson, *An. coluzzi* Coetzee & Wilkerson and *An. gambiae* Giles, the latter two being previously known as the 'M' and 'S' form of the complex (Besansky et al., 1994; Fanello et al., 2002, Coetzee et al., 2013). *An. gambiae* as well as *An. arabiensis* and *An. funestus* (member of the *An. funestus* complex) are only found in Africa. *An. gambiae* and *An. arabiensis* have a panmictic distribution across tropical Africa. *An. funestus* has a similar distribution than *An. gambiae* but its population further extends into Southern Africa. In general, the three species can be considered as sympatric and their distribution perfectly matches where malaria incidence on human populations is highest (Figure 1.1A and B).

A



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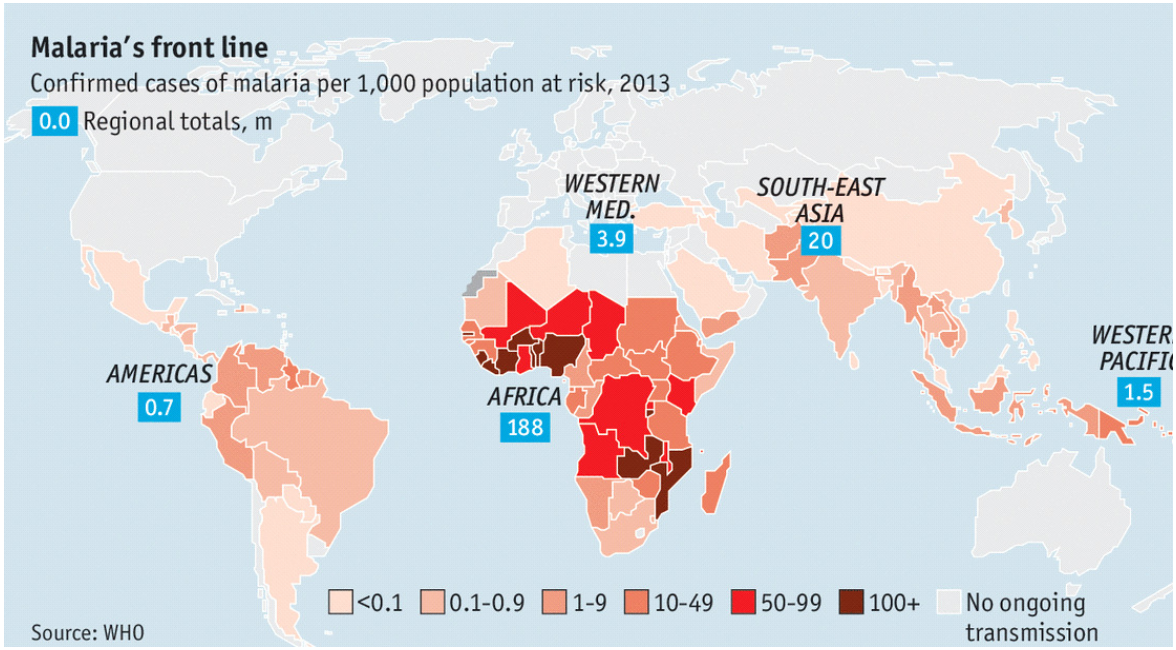


Figure 1.1 *Anophelinae* distribution and worldwide incidence of malaria. Map **A** the distribution of the potential malaria vectors (*Anopheles*) across the world (Kiszewski and Teklehaimanot, 2004). Map **B** represents the estimated incidence of malaria per 1000 population in 2013. Note the correlation of the most affected area in Africa (>50 cases per thousand population) and the geographic distribution of *An. gambiae*, *An. arabiensis* and *An. funestus*.

1.2 The life cycle of mosquitoes

Mosquito undergo 'complete metamorphosis' distinguished by four stages: eggs, larvae (4 instars), pupae and imagoes or adults, the three first stages being aquatic (Fig. 1.2). Typically an adult female gently lands on the water surface of a shallow pool of water and lays from a few tens to several hundred eggs either singly or in batches. Larval eclosion follows, depending on aquatic conditions, more or less rapidly and grows through three moults. Within a few days the 4^{th} instar larva develops into a pupa which already encloses a pharate adult insect form. Air intake at the water surface releases the insect from the pupal encapsulation and after wing unfolding the adult mosquito leaves the aquatic habitat to pursue its life span in the few meters of atmosphere above land. The adult is a typical dipteran characterized by only one pair of wings and a pair of halters. Like all Diptera, mosquitoes feed on fluids, but are equipped with exceptionally well-adapted mouthparts to probe plant juice (nectar, rotten fruits or honeydew) and perforate the hard skin of higher vertebrates to gain access to an already well processed resource: blood. For subsistence and to meet the high metabolic demand required for flight, both the male and female mosquito regularly feed on nectar, honeydew and plant sap. In addition, females feed on blood in order to constitute yolk protein in the fat body for egg production except for species of the *Toxorhynchitinae* subfamily in which females produce eggs entirely from materials of plant origin. The role of adult males mainly resides in female insemination and mating with the vast majority of females that occurs before seeking a blood meal on a vertebrate host. Males form swarms constituted of up to thousands of individuals near oviposition sites. Females entering the swarm are rapidly seized by males and copulate with them. Once inseminated, females are rendered refractory to further copulation and store the spermatozoids in their spermatheca. After mating an adult female mosquito seeks a vertebrate host and takes up a blood meal. Right afterwards the female mosquito flies to a refuge to digest its blood meal and permit oocytes maturation. Then, the adult female seeks a suitable oviposition site where it lays its eggs. Adult females lay eggs several times within their life span but every gonotrophic cycle requires at least one blood meal or more depending on the species (for more details see Clements, 1992 and 1999).

1.3 Notable behavioural traits of adult mosquitoes

The successive repetition of host seeking - blood meal take up - oviposition behavioural sequences (gonotrophic cycle) are determinant in the reproductive and survival success of mosquitoes (Fig. 1.2). An *An. gambiae* colony can be easily maintained in the laboratory by reproducing the cycle artificially (see appendix 9.1.1). Engagement in this typical behavioural sequence has is driven by internal (genetic and physiological status) and external cues (external stimuli coming from the surrounding environment) (Takken and Knols, 1999). The physiological status of the insect depends on factors such as age, size, nutritional state, circadian rhythm and gonotrophic stage. A good example of where the physiological status of the mosquito influences its behaviour is during blood feeding. After a blood meal and following its digestion the mosquito will seek a suitable oviposition site. However, if the first blood meal did not allow accumulation of sufficient protein to permit oocyte maturation and egg laying the female will engage in a second blood meal. This behaviour is termed multiple feeding and clearly shows how the physiological status or metabolic demand influences behaviour (Xue and Edman, 1991). As a matter of course, environmental conditions highly influence mosquito behaviour. Habitat and climatic factors such as temperature and humidity varying on a daily and seasonal basis constitute conditions to which the mosquito as an ectotherm is forced to adapt its behaviour. For example, to digest its blood meal a mosquito may seek a cool and humid refuge to limit metabolic expense and water loss (Kessler and Guerin, 2008). Depending on its physiological status and environmental conditions, the sensory apparatus of the insect will be more or less responsive to external stimuli to find/select a suitable conspecific to mate, a host to take a blood meal, find a sugar source and oviposition site. Mosquito flight behaviour is activated and guided to its end target by external stimuli such as chemical, visual as well as physical cues. Visual cues such as contrasting contours have been noted to be used by diurnal mosquitoes to find a host whereas colours seem to be less important (Allan et al., 1987; Muir et al., 1992). Although to a limited extent, night active mosquitoes such as *An. gambiae* have also been shown to respond to vertical and ground patterns but only under conditions similar to full-moon luminosity (Rubio-Palis, 1992; Gibson, 1995). A host targeted for a blood meal releases notable amounts of heat and moisture different from ambient levels in their vicinity. Those physical cues play an important role in eliciting a landing response close to the host (Khan et al., 1968). *Anopheles* males possess elaborate flagella on the antenna which are sensitive to the flight tone of conspecific females. Flying females that enter a swarm are sensed by males which rapidly seize them to

mate (Charlwood and Jones, 1980). By taking into consideration the extensive research performed to date on the sensory cues that direct mosquitoes towards their resources (i. e. mate, sugar source, host or oviposition site) olfactory cues seem to be the most important (Takken, 1991; Takken and Knols, 1999). Antenna and maxillary palps bear hundreds of sensilla that contain receptor cells sensitive to semiochemicals (see appendix 9.1.2 and Pitts and Zwiebel 2006 for a detailed review). Mating behaviour for some species (notably *Aedes* and *Mansonia*) implicates olfactory stimuli in that males use the odour of the vertebrate host to locate and intercept female that take a blood meal on the same host (McIver et al., 1980; Jaenson, 1985). Whether or not females from the *Anopheles* genus use olfactory cues (i. e. pheromones) provided by swarms to locate them remains unclear. Plant nectar is an important sugar source for the mosquito to sustain flight, in particular immediately after emergence. Both male and female mosquitoes use this resource for survival and use the host plant volatiles as olfactory cues (Foster, 1995; Foster and Takken, 2004). Depending on the species, mosquitoes exploit the blood of a large panel of higher vertebrates (mammals, birds, reptiles and amphibians). Olfactory cues play a major role in host seeking behaviour. At long distances where visual and physical cues are not available, wind carries infochemicals that elicit upwind flight (anemotaxis) and directs the mosquito towards its blood host (Takken, 1991; Takken and Knols, 1999). Finally, oviposition site location behaviours have also been reported as being mediated by olfactory cues in many species (Bentley and Day, 1989). Undoubtedly olfactory cues play a major role in regulating many aspects of adult mosquito behaviour.

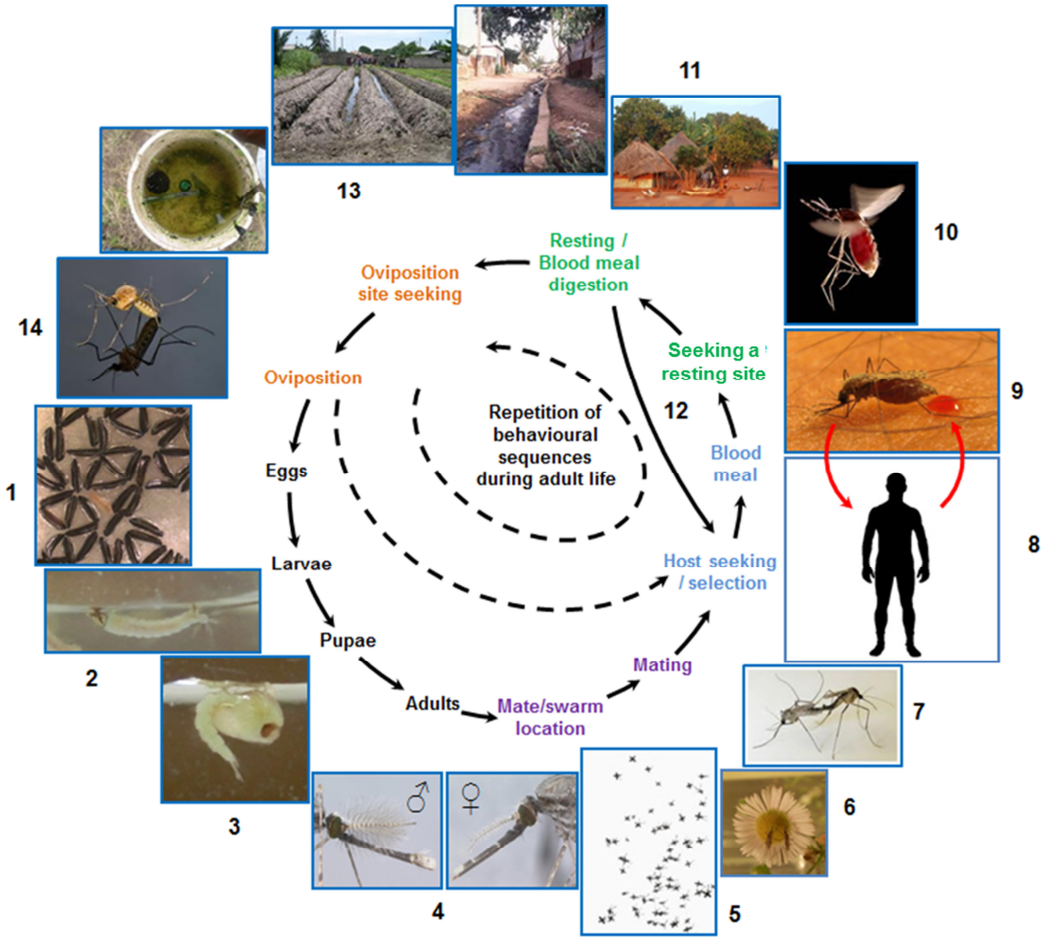


Figure 1.2 life cycles of *An. gambiae* and notable particularities distinguishing it from other mosquito species. Eggs laid singly as opposed to in batches (**1**). Horizontal position of the larva to feed and breathe at the water-air interface as larvae of this species do not possess a syphon (**2**). Pupae resting at the water air interface (**3**). *An. gambiae* has maxillary palps as long as the proboscis and males and females can be distinguished by the structure of their antennae (**4**). Swarm of males (**5**). Two females feeding on a flower (**6**). Copulation (**7**). Preference for human hosts (=anthropophily) and feeding inside human habitations (=endophagy) (**8** and **9**). Preference for resting site to digest blood meal inside human habitations (=endophily) (**10** and **11**). When the female was interrupted or did not ingest sufficient nutrients to produce mature eggs during the blood meal it may seek another blood meal (= multiple feeding) before oviposition (**12**). *An. gambiae* mainly exploits shallow water pools related to human activities as oviposition sites (**13** and **14**). The two red arrows represent the malaria parasite transmission cycle described in Box 1 below. After Clements (1992 and 1999).

1.4 Behavioural particularities of female *An. gambiae*

The life cycle of *An. gambiae* is marked by physiological and behavioural particularities that distinguish it from other mosquito species and position it as the most important vector of malaria by contributing to its vectorial capacity.

1.4.1 Feeding habits: multiple, concentrated, large and long blood meals

Compared to other mosquito species notably Culicinae, Anophelinae and particularly *An. gambiae* seem to have a different physiological strategy to acquire nutrients for energy and egg maturation through blood feeding. Some Culicinae species, notably *Aedes aegypti*, already during the larval stage cumulate quite a lot of reserves (Briegel 1990a). In contrast, teneral *An. gambiae* are 'skinny' (term used by Briegel, 2003 with reference to their low lipid and protein content) and have a small body size compared to other mosquitoes. To cope with this disadvantage *An. gambiae* completes teneral metabolic reserves by investing a larger portion of blood meal nutrients for basal needs and less in yolk synthesis for egg maturation. The first blood meals may be used to compensate for low teneral reserves whereas nutrients from subsequent blood meals are eventually allocated to oogenesis (Briegel, 2003; Fernandes and Briegel, 2005). In parallel to acquire sufficient reserves for egg maturation, *An. gambiae* has adapted different strategies. It is able to feed successfully on blood from hosts rapidly (~ ½ day) after emergence (Briegel and Hörler, 1993; Hörler and Briegel, 1995). During blood ingestion, it concentrates protein by simultaneous water removal (Fig. 1.2, image 9). This way *An. gambiae* ingests up to 10-12 µl of blood (up to 4 times its own weight) and requires an extended feeding time (Briegel and Rezzonico, 1985). In contrast, *Aedes aegypti*, even though a much larger mosquito only ingests up to 2-5 µl of blood (Briegel 2003). The ability to filter blood more efficiently is also accompanied by a strong tendency in *An. gambiae* to undertake multiple feeding at short intervals (Briegel and Hörler, 1993; Hörler and Briegel, 1995). Sugar feeding can also contribute to metabolic demand of female *An. gambiae* in a domestic environment (Foster 1995; Gary and Foster 2004). However it has been shown that *An. gambiae* can replace sugar with increased blood feeding without suppressing reproductive fitness even if life span is reduced (Briegel and Hörler 1993; Gary and Foster 2001; Straif and Beier 1996). Strong flight performance can be maintained with blood feeding alone by *An. gambiae* (Kaufmann and Briegel 2004). Sugar feeding, seems to not occur during but between gonotrophic cycle if blood donor are not available and under environmental constraints (Gary

and Foster 2006). In an endophilic and endophagic context metabolic demand seems to be particularly associated with blood feeding behaviour by female *An. gambiae* (Beier 1996).

1.4.2 Propensity for human habitat

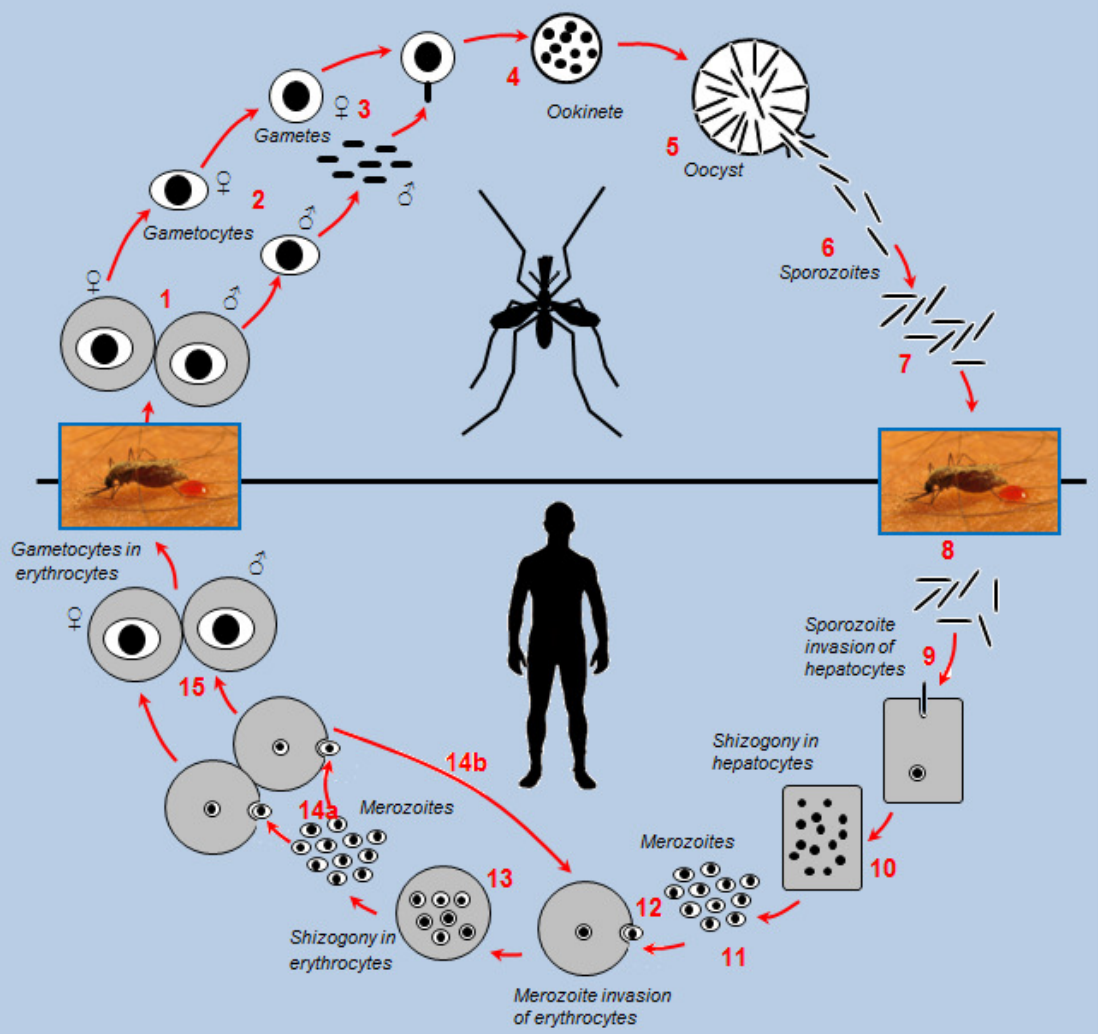
Another particularity of *An. gambiae* is its synanthropism (an animal that lives near and benefits from an association with humans and the man-made habitats that humans create around themselves). *An. gambiae* spends a significant period of its adult life in the vicinity of human habitations which ensures a continuous contact to human beings. It is endophagic in that it prefers to feed indoor in human dwellings and endophilic in that it prefers to rest indoor. The temperate and humidity characteristics of human habitations provide the vector ideal conditions to lower metabolic demand and save energy. After feeding on a human host indoor, nearby secluded indoor sites also provide ideal resting sites in order to quietly digest the freshly-taken blood meal (Chandler et al., 1975; Githeko et al., 1996). Meanwhile *An. gambiae* usually lays eggs on clear, unpolluted and shallow rain-filled water pools generated by human related activities. Hoof prints, puddles and discarded containers filled with water provide excellent oviposition sites. Irrigation in agriculture is also a source of water that may be utilised by the vector to oviposit (Muirhead-Thomson, 1951; Clements, 1999). The proximity to humans is not only marked spatially but also temporally. *An. gambiae* mating, blood feeding, resting and blood meal digestion followed by oviposition are regulated by an endogenous circadian rhythm that is reset daily when luminosity changes from photophase to the scotophase. The species is crepuscular and nocturnal with behaviours such as mating and oviposition occurring at twilight whereas blood feeding is maximal during the night between 22h00 and 4h00 (Jones and Gubbins, 1978). During the day, when humans are active, the mosquito rests and digests its blood meal. Blood feeding at night is probably an optimal strategy as it is at that time that human hosts are asleep and vulnerable to mosquito bites.

1.4.3 Odour-mediated preference to bite humans

An. gambiae is not only synanthropic; it is also demarked from other mosquito species by its anthropophilic character. This ectoparasite has a strong preference for humans over other vertebrate hosts (Garrett-Jones et al., 1980). There is evidence that selective human host-seeking behaviour of *An. gambiae* relies in the difference of odour emitted by humans compared to other vertebrate hosts (Costantini et al., 1993; Mboera et al., 1997; Costantini et al., 1998; Dekker and Takken, 1998; Dekker et al., 2001 and 2002) and certain human types compared to others (Knols et al., 1995; Dekker et al., 2002; Mukabana et al., 2002). By contrast, it has been shown that other *Anopheles* species tend to have a less selective or even a

rather zoophilic host-seeking behaviour. For example, and to contrast with *Anopheles gambiae*, *Anopheles quadriannulatus* seems to be truly zoophilic. This particularity strongly contributes to its high vectorial capacity compared to other mosquito species. To ensure development and reproductive success, the malaria parasite needs to be transmitted twice: the infection bite by a mosquito and further transmission by second bite of the same mosquito, Box 1. The probability of this occurring is enhanced when the targeted host pool remains restricted. In this regard, it must be noted that the apparent high vectorial capacity is also associated to the susceptibility of the vector to *Plasmodium falciparum,* the parasite that causes the most severe form of malaria, by comparison to *P. vivax*, *P. ovale* and *P. malariae* that cause less sever forms of malaria.

Box 1 Life cycle of the malaria parasite within the mosquito and human hosts



The sexual reproduction of *Plasmodium falciparum* takes place when a female *An. gambiae* ingests a blood meal containing erythrocytes infected with male or female gametocytes of the parasite (**1**). The gametocytes differentiate into male and female gametes (**2-3**) which after fertilization form a motile zygote called the ookinete (**4**). The ookinete migrates through the digestive tract wall of the mosquito, forms an oocyst (**5**) on the midgut's edge and divides into several thousand sporozoites (**6-7**) which are then able to reach the salivary glands of the mosquito through its haemolymph. Through a subsequent mosquito bite (e.g. the next gonotrophic cycle), these sporozoites can be transmitted and may infect a healthy human (**8**). If the infection is successful, sporozoites rapidly reach the liver through the circulatory system and penetrate hepatocytes (**9**). After asexual reproduction (exoerythrocytic shizogony, **10**) hepatocytes burst and release merozoites in the circulatory system (**11**). Merozoites invade erythrocytes (**12**) where they grow into trophozoites and multiply (another round of shizogony) causing erythrocytic burst which releases newly formed merozoites (**13**). These merozoites are in turn able to re-invade other erythrocytes (**14a and b**). This process occurs cyclically with erythrocytic burst happening every ~ 48 hours. The erythrocytic burst corresponds to the cyclical peaks of fever human beings are submitted to when they present malaria symptoms. Meanwhile, after invasion of erythrocytes, some merozoites may develop into gametocytes (**15**) and reinitiate a life cycle. Adapted from Peters, 1987; Ghosh et al., 2000; Combes, 2001.

1.5 Focus and outline of the thesis

As stated by Takken and Knols (1999), for their survival and reproductive success, mosquitoes depend on a series of characteristic behaviours such as mating, foraging, and oviposition which are governed by internal and external cues. In other words, engagement in one of these consecutive behaviours, next to being genetically determined or species specific depends on the physiological conditions in which a mosquito is developing and to which external stimuli it is confronted. A better understanding of these drivers is essential if one wants to improve mosquito control. In this thesis, both internal as well as external cues are addressed.

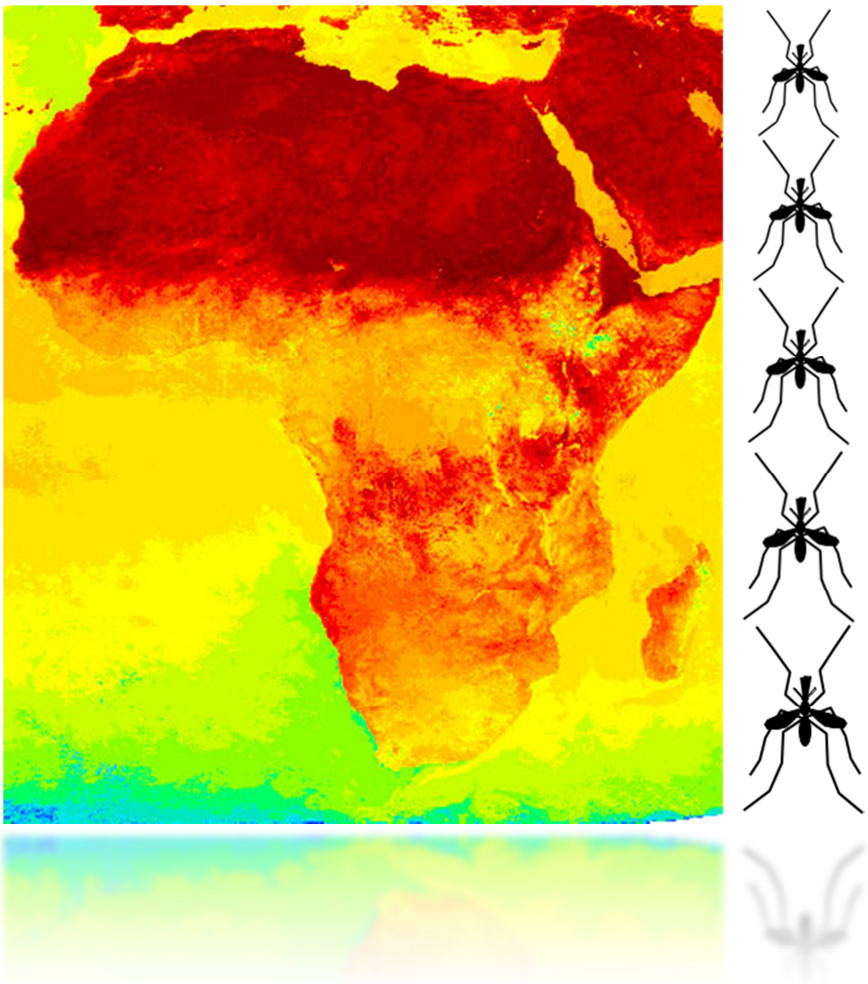
In a first stage (Chapters 2 and 3); I focused my interest on a major internal cue that govern the adult life of *An. gambiae* namely its respiratory physiology. Respiratory physiology includes two processes that are interdependent but that must be distinguished in order to avoid confusion. Cellular respiration or metabolism refers to the rate at which oxidative phosphorylation produces energy (mainly ATP) and waste products; whereas gas transport or gas exchange refers to the way respiratory gases (CO_2 and O_2) are transported from and to metabolic active tissues. Like most insects and as tiny ectotherms, adult mosquitoes have a metabolic rate that rapidly responds to changes in their activity and ambient temperature, and scales positively with body mass. The multiple feeding strategy of *An. gambiae* to blood feed in order to fulfil basal metabolic demand underlines the close relationship between its metabolism and host seeking behaviour. As stated earlier (see 1.4), *An. gambiae* uses a larger proportion of blood meals to fulfil basal metabolic demands than other mosquitoes. Metabolic variation is therefore intimately linked with host-seeking behaviour. Although important, relatively few studies document both metabolic rate and respiratory gas exchange pattern variation as a function of temperature and body size in blood feeding arthropod vectors of disease. This is rather unfortunate considering their impact on human health. More fundamentally, gas exchange patterns in smaller insects in the size range of *An. gambiae* remains poorly investigated. I therefore investigated the influence of body size and temperature, among other factors, on the metabolic rate (Chapter 2) and the gas exchange pattern of *An. gambiae* (Chapter 3). This is achieved with a flow through respirometry system specially adapted to unravel gas exchange in such a small insect.

The second part of the thesis (Chapters 4 and 5) focuses on external cues influencing the behaviour of the main vector of malaria. Knowing that *An. gambiae*, as a night active

mosquito, mainly uses olfactory cues to locate its host, that it has a strong preference for humans over other vertebrate hosts and is even able to distinguish certain human types over others (see 1.4), I focused my interest on this selective host-seeking trait with the aim of identifying human-specific kairomones. I accordingly tested the response of the vector to human odour effluvia produced by incubating a unique and representative human sweat pool with 3 bacteria species involved in the generation of the typical human axillary malodour. Furthermore, I investigated the response of *An. gambiae* to two volatile carboxylic acids recently identified as major characteristic constituents of human axillary malodour and that seem to vary significantly between human types. In order to test the differential response of *An. gambiae* a dual choice olfactometer was used.

Each chapter of the thesis is introduced separately and followed with a detailed discussion. A chapter can therefore be read individually and the thesis is ended with a set of concluding remarks (**Chapter 6**) that pick up on the highlights of particular findings and discussion points.

2 Mass scaling and thermal sensitivity of resting metabolic rate in *An. gambiae*



2.1 Introduction

Metabolic rate (MR) is a biological rate of fundamental importance as it sets the rate at which a living organism may uptake resources from its environment and how fast it may be able to transform and allocate them to survival, growth and reproduction to promote survival of the species (Brown et al., 2004). Due to their small size and as ectotherms insects' MR is particularly responsive to changes in their activity and ambient temperature (Chown and Nicolson, 2004). When actively flying, insects maintain the highest mass-specific MR known in the animal kingdom (Suarez, 1998; Suarez et al., 2000). Compared to when at rest, the MR of a flying insect can increase up to 100 fold (Joos et al., 1997). 10 °C increases in temperature can lead to more than fourfold increase in MR (Nespolo et al., 2003). Like for all living organism, insects have a MR that scales positively with the ¾ power of body mass and exponentially with temperature. This relation has important biological implications and is still debated (Kleiber, 1932; Gillooly et al., 2001; Chown et al., 2007). MR can be determined by either the rate of CO_2 production, or the rate of O_2 consumption. In insects MR is commonly investigated with the technique of flow-through respirometry (FTR) (Lighton, 2008). In this method, CO_2- and H_2O-free air is directed, at a known and precise flow rate, into a respiratory chamber containing the insect. This air then flows into a gas analyser where partial pressure of gases (usually CO_2 for best accuracy) is measured. In order to fulfil basal metabolic demand *An. gambiae* engages in blood feeding, sugar feeding and eventually when reserves are sufficient it may take the opportunity to lay egg to pursue its life cycle. Understanding the parameters that influence its MR is therefore of crucial importance as this internal physiological cue has a major influence on the behaviours undertaken that may be relevant for the fitness of the vector but also of wider importance in terms of malaria transmission rate. When it comes to mosquitoes that transmit important diseases, laboratory investigations using accurate FTR to measure MR of these important vectors of diseases have only been reported on few occasions. The first study investigated the effect of size, age, activity and blood feeding on the MR of *Culex tarsalis* (Gray and Bradley, 2003). The same authors investigated the effect of malaria infection in *Aedes aegypti* on the MR: during blood digestion infected mosquitoes had a lower MR suggesting that midgut invasion and sporogony were not increasing MR (Gray and Bradley, 2006b). The only available study on *An. gambiae* with FTR in the laboratory compared the MR between *An. arabiensis* and *An. gambiae* under desiccation stress. *An. arabiensis* lives in drier habitats and is more resistant to desiccation stress. Consequently, the aim of the study was to see if this species showed a notable

difference in MR as an adaptation to control respiratory water loss. However no notable differences were found between the two *Anopheles* species and resistance to desiccation was attributed to higher water content at emergence (Gray and Bradley, 2005). These studies have all shown that mosquitoes seem to exchange gases in a cyclical manner (periodic CO_2 outbursts). However, one year later, using the winter mosquito *Culiseta inornata* as a model, Gray and Bradley showed that the observed gas exchange pattern (GEP) is highly affected by measurement conditions notably the flow rate that affects the temporal resolution of an FTR-system (Gray and Bradley, 2006a). More recently the first field investigations complemented our knowledge on MR variation in wild caught *An. gambiae sensu lato* by taking into account various factors such as temperature, wing length (as a proxy for body size), sex, gonotrophic/feeding status and sex (Huestis et al., 2011). However, in this latter research MR was measured using the constant-volume technique which does not allow temporal resolution of the GEP used by *An. gambiae* (Lighton, 2008).

To date no accurate investigation on how MR and GEP parameters at rest are modulated by temperature and body size has been made for *An. gambiae* especially by clearly knowing the contribution of size variation in individual mosquitoes and using the FTR technique. In this thesis chapter, the relationship between temperature, body mass and resting MR (RMR) is clearly defined for *An. gambiae*. For this purpose, RMR of individual *An. gambiae* covering a very wide body size range (known parameters: dry mass, water content, wing length) on a temperature gradient from 20 to 32 °C is measured. The results are compared with previous findings and discussed in an eco-epidemiological context (consequences of RMR variation). RMR measurements are performed with the highest flow rate possible in order to establish the data base to resolve the parameters that constitute GEP of *An. gambiae*. An in-depth analysis of the parameters constituting the GEP and how it evolves with increasing size of individual mosquitoes and with temperature is treated in the following chapter.

2.2 Material and Methods

2.2.1 Mosquitoes

The colony of *An. gambiae* Giles (Diptera, Culicidae) 16cSS strain, derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa and classified by Mnzava and Curtis (1989) as the 'S' form, was maintained in a climate chamber (28°C, 80% relative humidity) under a 12:12 light:dark cycle with 2 h simulated sunrise and sunset (see Appendix 9.1.1). The mosquito culture was maintained by feeding females on a Guinea pig (Cavia porcellus L.) once a week and eggs were recovered on wet filter paper. The larvae were reared in trays (290 x 210 x 55 mm) containing 200 ml distilled water (4 mm deep) and fed with pulverized Tetramin® fish food on a daily basis. In order to produce mosquitoes of different sizes larvae were grown at 4 different densities (75, 225, 375 and 525 larvae per tray) but fed with the same regime (day 1: 18 mg, day 2-9: 36 mg fish food). Higher/lower densities lead to smaller/bigger mosquitoes (Timmermann and Briegel, 1999). For a given density the day of maximum pupation was constant. Most of the larvae grown at densities of 75, 225, 375 and 525 larvae per tray pupated on days 6, 7, 8 and 9, respectively. The yield, expressed as the percentage of larvae reaching the pupal stage, varied marginally for each density (75: 86.55±1.75, 225: 88.22±0.94, 375: 86.62±0.97 and 525: 86.69±0.12, mean ± standard error, n=6), indicating that mortality between densities was fairly constant. Pupae of each density were placed in four cages (200 x 200 x 260 mm) separately and all emerged the following day. In these cages mosquitoes were maintained by giving them access *ad libitum* to sucrose 10% and distilled water on cotton wool. This permitted targeted respirometry experiments according to size with three and six day old female *An. gambiae*. These two age categories were chosen because they coincide with two key steps in the adult life stage. Three day-old mosquitoes have just reached full maturation and six day-old mosquitoes have just reached optimal age for host seeking (Jones and Gubbins, 1978).

2.2.2 Wet body mass, water content, dry mass and wing length of mosquitoes

Mosquitoes were weighed with a microbalance (Mettler Toledo MX5; accuracy ±0.001 mg). Mosquitoes were weighed prior and after experimental trials. Immediately after an experiment the mosquito was placed inside a 5 ml glass vial and stored in the freezer (-20°C). Subsequently mosquitoes were dried in an oven at 70°C for 48 hours to permit measurement of their dry mass. To evaluate wing length, the distance from the alula to the distal end was measured (Fig. 2.1). For this a microscope equipped with an objective incorporating a

graduation was used. Before measurement, the wings were laid flat on a glass slide and covered with a thin layer of 90% glycerol.

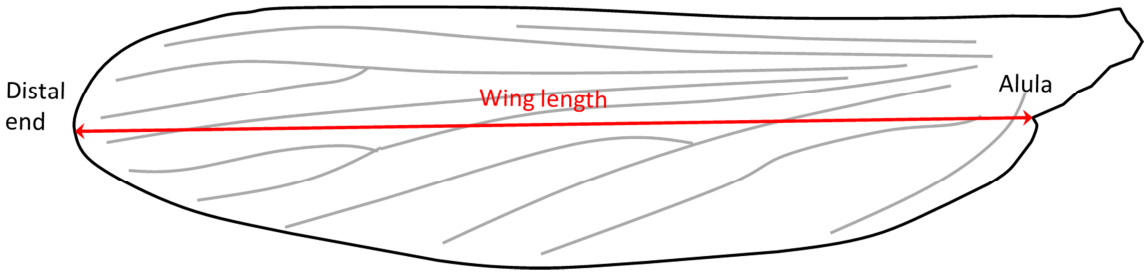


Figure 2.1 Wing length measurements.

Wing length (WL), wet body mass prior (M_{wetp}, in mg) and after respirometry (M_{weta}, in mg) as well as dry mass (M_{dry}, in mg) was determined for each individual *An. gambiae*. Using these measures, the water content ($W_{content}$, in mg water per mg dry mass) prior and after respirometry ($W_{content.p}$ and $W_{content.a}$) as well as water loss (W_{loss}, in mg) and the mean living body mass (M_{alive}, in mg) during respirometry were determined for each individual *An. gambiae*:

$$W_{content.p} = \frac{M_{wetp} - M_{dry}}{M_{dry}} \qquad W_{content.a} = \frac{M_{weta} - M_{dry}}{M_{dry}}$$

$$M_{alive} = \frac{M_{wetp} + M_{weta}}{2} \qquad W_{loss} = M_{wetp} - M_{weta}$$

Linear regressions were used to evaluate the relationship between M_{dry}, M_{wetp} or WL and larval density as well as the relationship between M_{dry} or M_{wetp} and WL. Analysis of variance (ANOVA) was used to infer on differences in body mass (M_{wetp} , M_{weta} and M_{dry}), $W_{content.p}$, $W_{content.a}$ and W_{loss} between 3 or 6 days-old mosquitoes and experimental condition groups (20→32°C or 32→20°C ramps, see 2.2.3 below).

2.2.3 Flow-through respirometry

The MR of *An. gambiae* was estimated by measuring the CO_2 production rate ($\dot{V}CO_2$) using FTR. Unless otherwise stated, all hardware and software devices constituting the FTR circuitry were acquired from Sable Systems International (http://www.sablesys.com/, Las Vegas, Nevada, USA). The devices include 1) a LI-7000 (LI-COR, Lincoln, Nebraska, USA) infra-red CO_2 gas analyser calibrated on a regular basis with a reference gas bottle with a

known CO_2 concentration (high quality CO_2, 987 ppm, 2% error; Carbagas, Switzerland), 2) an air pump (SS3) with a flow rate manual regulator, 3) a multivalve switcher (8 channels) to switch automatically between mosquito chambers (RM8 multiplexer), 4) a 0-500 ml min^{-1} mass flow valve (Sierra Instruments, Wetzikon, Switzerland) controlled by a mass flow controller (MFC-2) for accurate regulation to the desired air-flow rate, 5) a thermistor probe to measure temperature accurately and 6) a Peltier element temperature-controlled cabinet (PTC-1; ±0.1°C). For control, data acquisition and monitoring purposes all devices were connected to a user interface (UI-2) which in turn was connected to a computer where Expedata© was used as software. For individual mosquito chambers, 2 ml medical plastic syringes were cut to obtain a volume of 0.5 ml. Syringes were fitted to tubing using rubber O-rings to ensure gas tightness. The entrance of the mosquito chamber was equipped with a resistance (fine-mesh nylon flow screen) to ensure a turbulent flow and gas mixture. To ensure a CO_2 concentration at zero and prevent adverse effects of water vapour on CO_2 concentration in the air, an air-scrubber contained within a glass cylinder was used to extract water vapour and CO_2 from the airflow. The glass cylinder constituting the air-scrubber was filled with a layer of soda lime followed by a layer of silica gel. The silica gel was placed downstream of the soda lime in the air-scrubber as the chemical reaction fixing CO_2 to the soda lime produces water vapour. The two scrubber substrates were regularly replaced or recycled (for silica gel) as soon as their indicator colours changed (white to purple for soda lime and blue to pink for silica gel). For most accurate respirometer ascarite (for CO_2 scrubbing) and drierite (desiccant) should be used but for reasons of expense soda lime and silica gel were used. Following preliminary trials it turned out that these chemicals led to sufficient accuracy. The FTR system used is schematised in Figure 2.2. Using the SS3 pump, compressed air was pulled from a carboy (10 L empty glass bottle opened to ambient air) through the air-scrubber and fed through the mass flow valve set at 250 ml min^{-1}. The carboy served to buffer eventual ambient air pressure fluctuations that could alter the precision of the FTR system. Once the flow rate was set at 250ml min^{-1}, the air flow passed into the PTC-1 cabinet through a copper tubing coil to ensure cabinet temperature uptake. Then the air was fed into the RM8-multiplexer which was outside the temperature cabinet but placed as close as possible to the door which had holes for tubing connections to the individual mosquito chambers. The tubing between the RM8 multiplexer and the PTC-1 temperature cabinet was kept as short as possible whereas tubing within the cabinet to the mosquito chamber was longer. Temperature measurements confirmed that the airflow had the temperature set for the PTC-1 cabinet. Finally, the air was sent to the LI-7000 to accurately measure the CO_2

concentration in ppm at a sampling rate of 5 Hz. Only one mosquito was measured at a time. Consequently two chambers were used to measure CO_2 concentration and the flow alternated between one empty serving as the baseline (for 2 minutes) and the other containing the mosquito (for 5 minutes). An extract from a typical record is presented in Figure 2.3. The chambers not being measured were continuously flushed with an additional airflow (flush, see Fig. 2.2) to maintain similar air conditions in all mosquito chambers even when they were not monitored. This procedure is of particular importance to avoid hypercapnia in the test chamber when it is not being monitored. This flow was also air-scrubbed and also went through a copper tubing coil within the PTC-1 cabinet. Measurements confirmed that there was no temperature difference between the additional flow and the flow subjected to CO_2 measurement. Accordingly, the thermistor probe was placed in a third chamber which allowed continuous temperature measurement during respirometry. Recordings lasted for 2.5 hours during which heating or cooling temperature ramps where programmed with the PTC-1. Mosquitoes were submitted to increasing temperature ramps with five constant temperature plateaus at 20, 23, 26, 29 and 32°C lasting each approximately 20 minutes. The 3°C temperature increases took approximately 5 to 6 minutes. Since measurements performed at each temperature were made with a mosquito that spent increasing time in the test chamber, an equivalent number of mosquitoes were submitted to temperature ramps where the temperature decreased. Prior to start of experiments each mosquito was left to acclimatize in the PTC-1chamber at the initial temperature for 30 minutes (either 20 or 32°C depending on heating up or cooling down experiments). All recordings were made within a 2-month period.

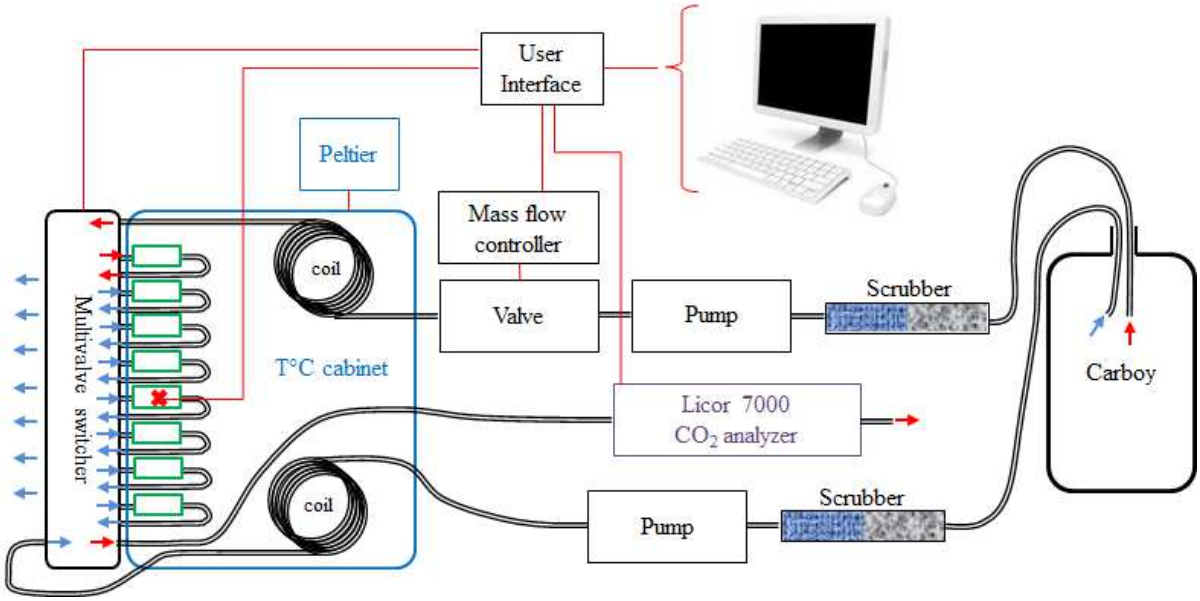


Figure 2.2 Scheme of the FTR circuitry. Double black lines depict the tubing network and red lines a simplified scheme of the cabling network. In green the mosquito chambers connected to the multivalve switcher (RM8-multiplexer). The blue frame depicts the insulated PTC-1 cabinet where temperature is accurately controlled by the Peltier element. Note the copper tubing coils to bring the air-flow to the temperature regulated by the Peltier element. The infrared CO_2 gas analyser (LI-7000) is indicated in purple. Red arrows indicate the direction of the air-flow used for measurements and blue arrows the direction of the flush used in order to avoid hypercapnia in chambers not being monitored. The red cross indicates the position of the thermistor probe used to measure temperature in an additional mosquito chamber. The computer equipped with Expedata© software allows control of all the devices and the acquisition of the data via the user interface.

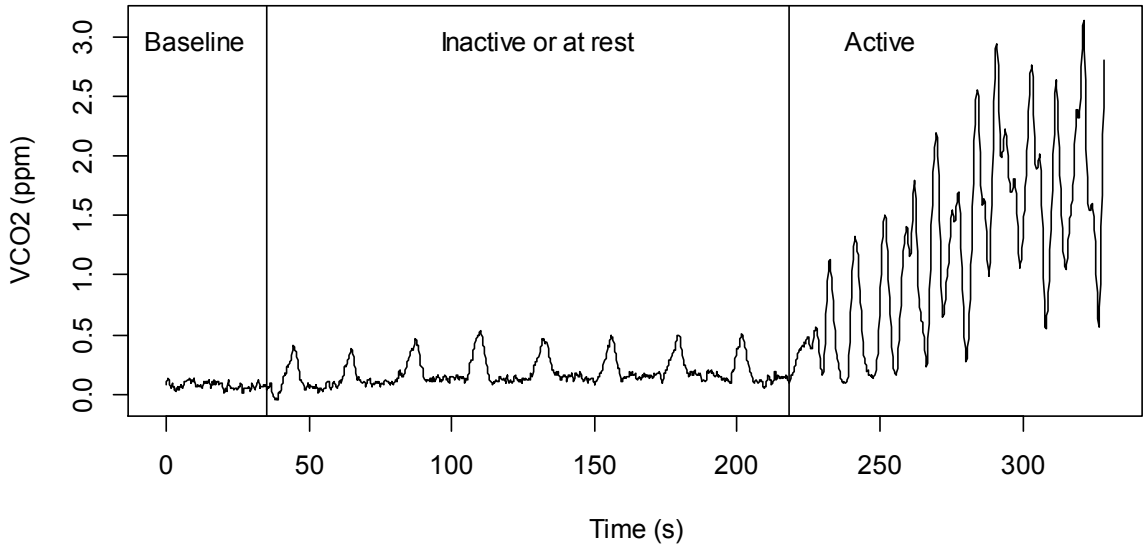


Figure 2.3 GEP employed by a female *An. gambiae* at rest and when active. Activity of the mosquito was induced by slightly shaking the mosquito chamber. At rest, $\dot{V}CO_2$ is lowest and characterized by a periodic expiration of discrete CO_2 bursts (middle panel). When active the regular nature of the GEP is disrupted and $\dot{V}CO_2$ increases strongly (right panel). The baseline level is provided on the left (CO_2 concentration measurement in an empty chamber).

2.2.4 Data correction, transformation, selection and evaluation

Using Expedata software, data were at first lag corrected (duration of gas transport from the animal chamber to the gas analyser), then using the baseline level of CO_2 measured in the empty chamber the data were drift corrected. Finally, using the recorded flow rate and the CO_2 concentration in ppm the data were transformed into µl CO_2 per hour. RMR (measured as $\dot{V}CO_2$ at rest and henceforth abbreviated as $s\dot{V}CO_2$) was identified by taking into account the recording sections when $\dot{V}CO_2$ was lowest and when mosquitoes exchange CO_2 in periodic, discrete bursts (Gray and Bradley, 2003 and 2005). Recording sections meeting these criteria were readily recognisable. Preliminary observations indicated that any slight movement by the mosquito in the test chamber generated a high increase in $\dot{V}CO_2$ production and a clear interruption of the regular nature of the GEP of *An. gambiae* (Fig. 2.3). For temperature the mean obtained from the thermistor probe over the selected record section was used (Fig. 2.4). In addition, during the 2.5 hour recordings only data located within the stable temperature plateaus were selected for further analysis, i.e. at 20, 23, 26, 29 and 32°C ± 0.1°C. In order to be valid as a measure the recording period also needed to last at least for 120 seconds (2

minutes). With baseline measurement interruptions more than one period could qualify as valid within a temperature plateau (usually not more than 3 to 4).

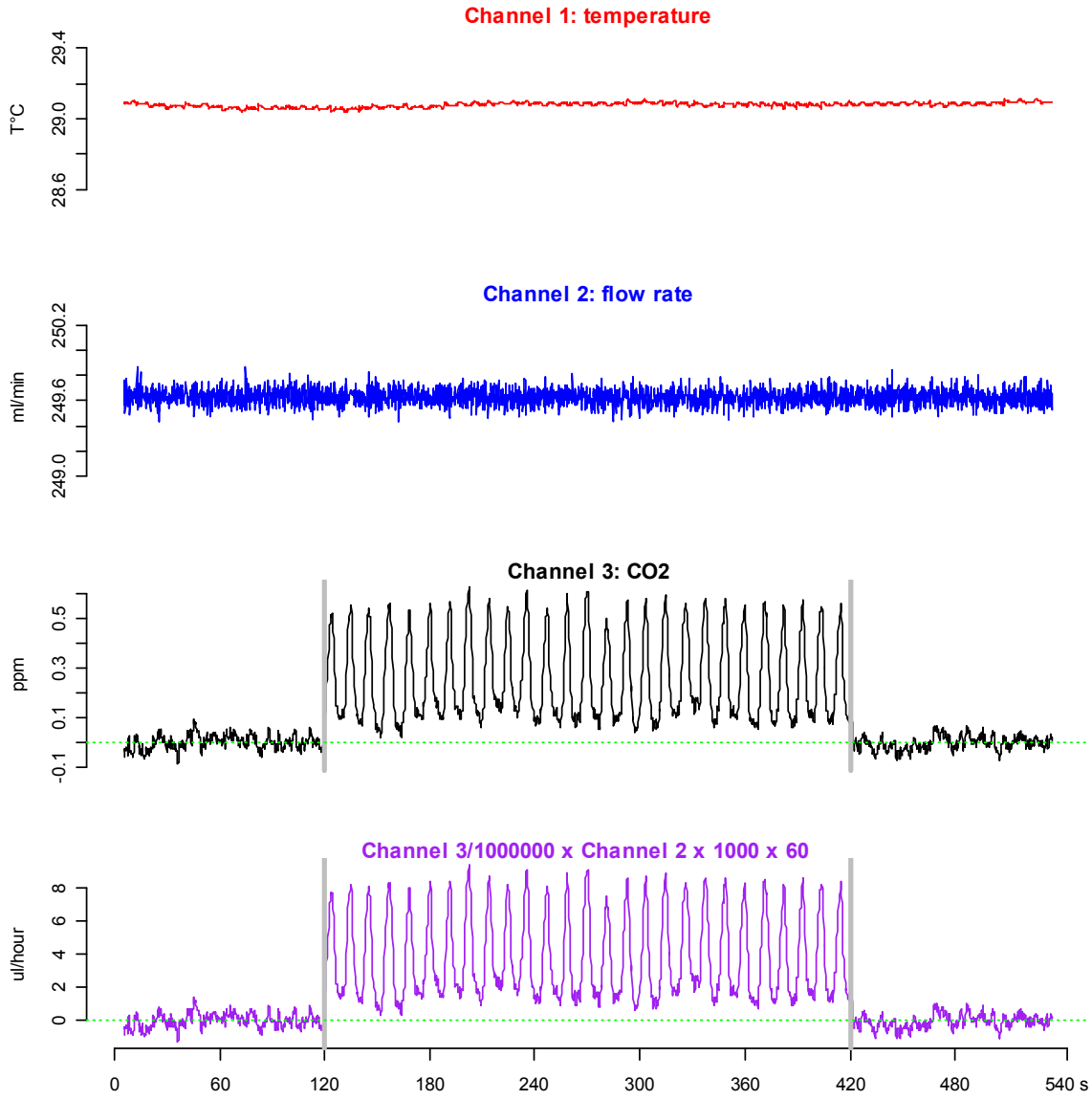


Figure 2.4 Example of recordings with the flow-through respirometry. Channel 1 (red) represents the output of the thermistor probe (T°C), Channel 2 (blue) the output of the mass flow controller (ml min^{-1}) and Channel 3 (black) the output of the LI-7000 (ppm CO_2) linked to a chamber with a mosquito. To convert parts per million to µl $hour^{-1}$ Channel 3 output in ppm CO_2 is divided by 10^6, multiplied by Channel 2 output in ml min^{-1} leading to expression of ml CO_2 min^{-1}. This value is then multiplied by 1000 to obtain µl min^{-1} and finally multiplying by 60 leads to expressing CO_2 production in µl $hour^{-1}$ (the trace in purple). Grey lines indicate transitions between measurements made between the empty chamber (baseline level) and the chamber with the mosquito (insect measurement). Note the stability of temperature and flow rate within a narrow range. Also note the stability of the baseline around 0 ppm (dashed green lines).

In order to describe and make inferences on the relationship between RMR, body size and temperature the following equation was used, as described in Lighton (1988b):

$$\mathrm{Log}_{10}(s\dot{V}CO_2) = a + b \times \log_{10} Body\ size + c \times Temperature$$

where body size is either represented by the living body mass (M_{alive}), dry mass (M_{dry}) or wing length (WL), *a* is the intercept, *b* the body size estimate and *c* the temperature estimate. For inference, ordinary multiple regression analysis in R using linear model function was used (R Core Team 2013). The response variable of the multiple regression was $s\dot{V}CO_2$ and the explanatory variables were either continuous (M_{alive} or M_{dry} or WL and temperature) or categorical for age with two levels. Variables were checked for normality using a Shapiro – Wilks test, and, where necessary, distributions were normalized by Log_{10} transformation (Zar, 1999). In order to assess the importance of model parameters, the maximum model was computed first. Subsequently, stepwise elimination of parameters to end up with the best model fit was performed. Non-significant interaction terms were removed first followed by non-significant single terms. Between each simplification, an ANOVA between both models was performed in order to confirm that removing was justified (non-significant ANOVA indicates that both models do not differ and justifies removing of terms). In all statistical evaluations the significance threshold were set at $\alpha = 0.05$ ($P>0.05$: NS (value), $P<0.05$*, $P<0.01$** and $P<0.001$***). Values complementing means in the text and error bars in figures represent standard errors or 95% confidence intervals when complemented with a CI_{95} level.

2.3 Results

2.3.1 Size range covered of the studied *An. gambiae* population

Varying larval density allowed the production of adult *An. gambiae* covering a wide size range. Table 2.1 summarizes sizes parameters according to larval density and age of mosquitoes. The biggest mosquito was more than twice the size of the smallest. Three day-old mosquitoes had a dry mass ranging from 0.279 to 0.695 mg, a wet mass ranging from 0.972 to 2.084 mg and wing lengths ranging from 2.50 to 3.25 mm. Although slightly higher, values were similar for 6 day-old mosquitoes with dry masses ranging from 0.315 to 0.811 mg, wet masses from 1.031 to 2.305 mg and wing lengths from 2.47 to 3.42 mm.

Table 2.1 Size range covered in the studied population of female *An. gambiae*.

Age (days)	Density (larvae/tray)	M_{dry} (mg)	M_{wetb} (mg)	WL (mm)
3	75	0.523 - 0.695	1.700 - 2.084	3.015 - 3.240
	225	0.411 - 0.603	1.201 - 1.821	2.835 - 3.038
	375	0.336 - 0.454	1.060 - 1.469	2.723 - 2.902
	525	0.279 - 0.387	0.972 - 1.278	2.470 - 2.745
6	75	0.525 - 0.811	1.639 - 2.305	3.127 - 3.420
	225	0.422 - 0.614	1.232 - 1.765	2.768 - 3.195
	375	0.392 - 0.532	1.299 - 1.542	2.745 - 3.240
	525	0.315 - 0.345	1.031 - 1.150	2.475 - 2.700
3	Overall	0.279 - 0.695	0.972 - 2.084	2.470 - 3.240
6	Overall	0.315 - 0.811	1.031 - 2.305	2.475 - 3.420
Overall	Overall	0.279 - 0.811	0.972 – 2.305	2.470 - 3.420

This table indicates the size range for the populations of mosquitoes from which metabolic data were used.

2.3.2 Relationships between larval density, body mass and wing length

Data from three day-old (closest to emergence) mosquitoes revealed larval density to be inversely proportional with mosquito Log_{10} body mass (Fig. 2.5 and Table 2.2). To understand the relationship between wing length and body mass, Log_{10} dry body and wet body mass was regressed against Log_{10} wing length by including age as a categorical variable (ANCOVA). However, the categorical variable age was not significant when included in the regression (P=0.72 and 0.75 when using M_{wetp} and M_{dry} as response variables, respectively). Wing length was best correlated with dry body mass and the regression analysis revealed dry body mass to increase proportionally with almost the cubic (2.899±0.207) value of wing

length (Fig. 2.6 and Table 2.3). Regressing Log_{10} cubic wing length of three day-old mosquitoes with larval density in which they were grown (Table 2.2) consolidated this outcome in that the obtained regression coefficients were very similar to those obtained with the body mass.

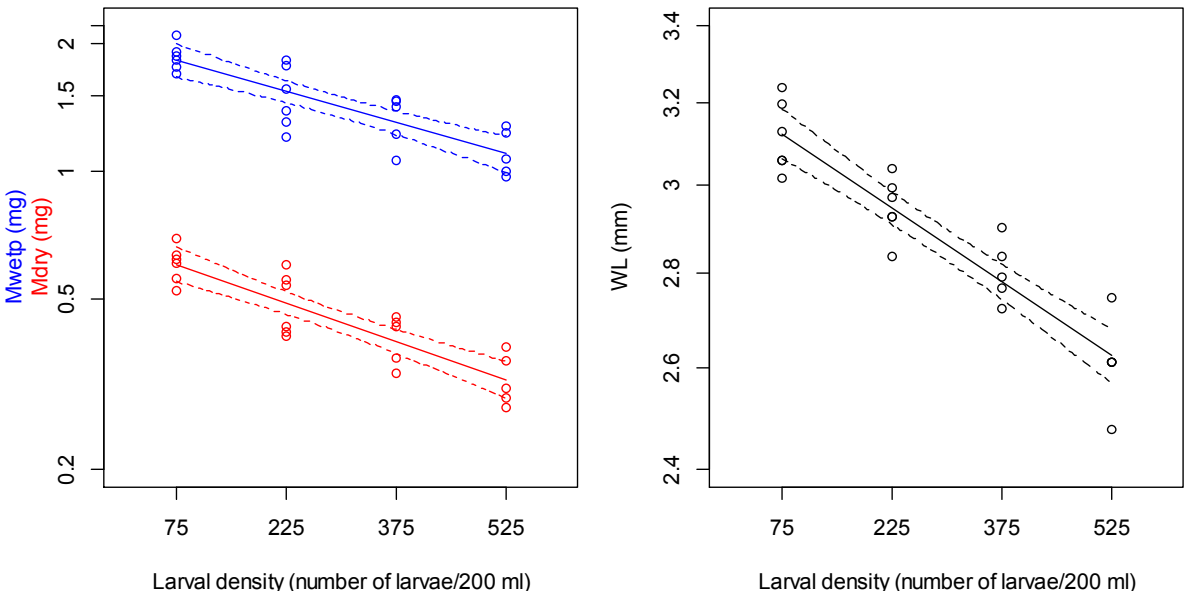


Figure 2.5 Relationship between larval density with Log_{10} body mass (M_{dry} in red and M_{wetp} in blue) in the left panel, and with Log_{10} wing length in the right panel. The Y-axis is in a logarithmic scale on both panels. Linear relationships and 95% confidence limits are traced from the regression coefficients described in table 2.2.

Table 2.2 Summary of linear regression applied to Log_{10} body mass and Log_{10} wing length against larval density.

Response variable	Explanatory variables	Estimates ± SE	r^2	*t*-value	*P*
Log_{10} M_{dry}	Intercept	$-1.74\times10^{-1} \pm 2.38\times10^{-2}$	0.77	-7.30	4.6×10^{-7} ***
	Density	$-6.01\times10^{-4} \pm 7.18\times10^{-5}$		-8.36	5.8×10^{-8} ***
		F-statistics: $F_{1, 20}$*: 69.89,* $p = 5.86 \times 10^{-8}$			
Log_{10} M_{wetb}	Intercept	$2.97\times10^{-1} \pm 2.31\times10^{-2}$	0.70	12.83	4.1×10^{-11} ***
	Density	$-4.88\times10^{-4} \pm 6.98\times10^{-5}$		-7.00	8.6×10^{-7} ***
		F-statistics: $F_{1, 20}$*: 48.93,* $p = 8.69 \times 10^{-7}$			
Log_{10} $(WL)^3$	Intercept	$1.521 \pm 1.60 \times10^{-2}$	0.85	100.8	$< 2\times10^{-16}$ ***
	Density	$-5.05 \times10^{-4} \pm 4.55\times10^{-5}$		-11.1	5.3×10^{-10} ***
		F-statistics: $F_{1, 20}$*: 123.1,* $p = 5.36 \times 10^{-10}$			

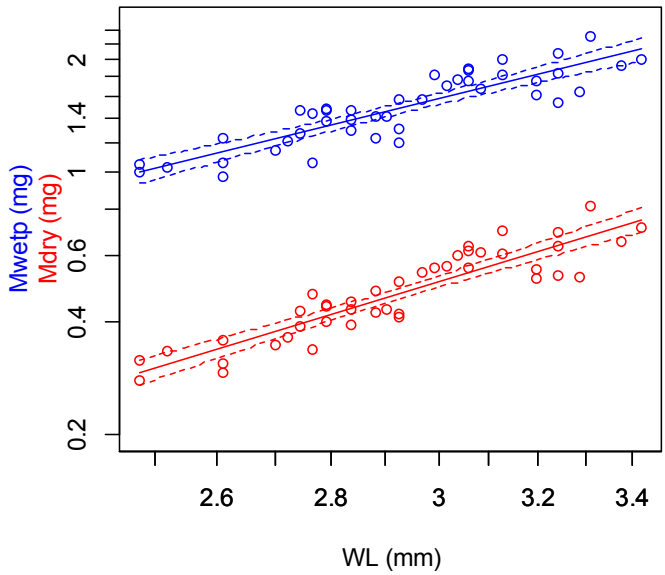


Figure 2.6 Relationship between log_{10} wing length and log_{10} M_{dry} (red) and Log_{10} M_{wetp} (blue). Axes are in logarithmic scale. Linear relationships and CI_{95} limits are traced from the regression coefficients described in Table 2.3.

Table 2.3 Summary of linear regression applied to Log_{10} body mass against Log_{10} wing length.

Response variable	Explanatory variables	Estimates ± SE	r^2	*t*-value	*P*
Log_{10} M_{wetb}	Intercept	-0.914 ± 0.091	0.77	-10.06	1.2×10^{-12} ***
	Log_{10} WL	2.329 ± 0.195		11.97	5.8×10^{-15} ***
		F-statistics: $F_{1, 41}$*: 143.2,* $p = 5.88 \times 10^{-15}$			
Log_{10} M_{dry}	Intercept	-1.674 ± 0.097	0.82	-17.32	$< 2\times10^{-16}$ ***
	Log_{10} WL	2.899 ± 0.207		14.01	$< 2\times10^{-16}$ ***
		F-statistics: $F_{1, 41}$*: 196.3,* $p < 2.2 \times 10^{-16}$			

2.3.3 Body mass, water content and water loss before and after respirometry

The *An. gambiae* populations used to perform respirometry trials for one of the other experimental conditions (heating or cooling) and age category did not differ in their M_{wetb}. Using their M_{dry} measured after respirometry, the water content prior to respirometry ($W_{content.p}$) was calculated and revealed to be similar in the mosquitoes used to perform experiments under heating or cooling conditions. Three day-old mosquitoes had a water content prior to respirometry ($W_{content.p}$) slightly higher than six day-old ones but this difference was insignificant. For all the mosquitoes that underwent respriometry their dry body mass (M_{dry}), wet body mass (M_{weta}) and water content after respirometry ($W_{content.a}$) were measured. It was found that experimental condition did not influence two of these parameters in that three and six day-old mosquitoes had a similar dry mass and wet mass after respirometry. However, their water content after respirometry differed significantly depending on age with 6 day-old mosquitoes losing significantly more water than 3 day-old ones. Descriptive and inferential statistics (ANOVA) of these parameters are summarized in Table 2.4 (means and p-values for each group) and plotted in boxplots in Figure 2.7.

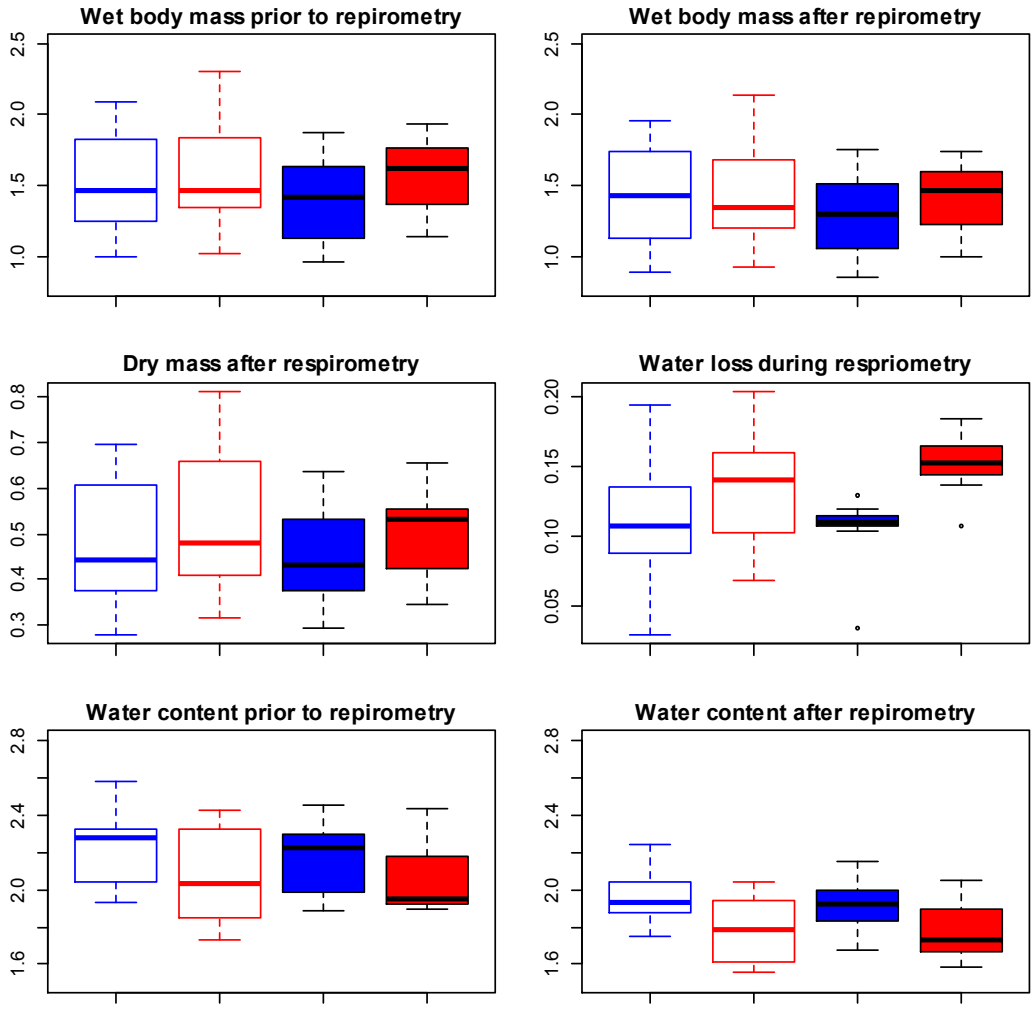


Figure 2.7 Boxplots representing the effect of age (3 day-old in blue and 6 day-old in red) and experimental condition (heating: empty boxplots and cooling: filled boxplots) on M_{wetb} (mg), M_{weta} (mg), M_{dry} (mg), W_{loss} (mg H_2O), $W_{content.p}$ and $W_{content.a}$ (mg H_2O/mg dry mass).

Table 2.4 *An. gambiae* body mass, water content, prior and after respirometry trial as well as water loss depending on experimental condition (heating and cooling) and age (3 and 6 days).

	Heating		Cooling		P value of ANOVA on	
	3	6	3	6	Age	Experimental condition
M_{drv} (mg)	0.485±0.041	0.518±0.046	0.448±0.033	0.513±0.035	0.21	NS (0.59)
M_{wetb} (mg)	1.537±0.107	1.559±0.111	1.406±0.091	1.567±0.090	0.37	NS (0.53)
M_{weta} (mg)	1.429±0.109	1.424±0.107	1.299±0.091	1.416±0.087	0.58	NS (0.48)
W_{loss} (mg H_2O)	0.107±0.015	0.135±0.012	0.106±0.007	0.151±0.007	**	NS (0.52)
$W_{content.p}$	2.217±0.062	2.061±0.069	2.162±0.058	2.076±0.067	0.06	NS (0.75)
$W_{content.a}$	1.973±0.046	1.785±0.051	1.910±0.041	1.771±0.053	**	NS (0.41)

$W_{content}$ in mg H_2O per mg dry mass.

2.3.4 Overview of the data selected for resting metabolic rate evaluation

In total, measurements were made from 43 mosquitoes in the respirometer (Table 2.5). From these 43 mosquitoes and following the data selection criteria, most of the recordings could be evaluated at each temperature. In almost all (Table 2.5, 205/215 = 95%) tested condition (individual × T°C × age), a record period could be extracted to analyse mass scaling and thermal sensitivity of $s\dot{V}CO_2$. The mean of valid periods for further analysis under each test conditions exceeded the set minimum of 120 seconds (Table 2.5, values in brackets). It must be noted that the mean duration of recordings tended to decrease towards the end of the 2.5 hours of respirometry. It was therefore justified to perform experiments by both decreasing and increasing temperature in order to compensate for this bias.

Table 2.5 Overview of the data used following the data/selection procedure.

Increasing/ Decreasing T°C	Age (days)	N	Number of mosquitoes evaluated at T°C					
			20	23	26	29	32	All T°C
20→32°C	3	11	11 (613)	11 (398)	10 (392)	11 (403)	10 (321)	**53/55 (425)**
	6	12	12 (594)	11 (415)	12 (402)	12 (355)	11 (390)	**58/60 (431)**
	3 and 6	23	23 (603)	22 (406)	22 (397)	23 (379)	21 (355)	**111/115 (428)**
32→20°C	3	11	10 (349)	11 (551)	11 (504)	11 (478)	11 (514)	**54/55 (479)**
	6	9	8 (451)	9 (416)	7 (432)	8 (378)	8 (572)	**40/45 (450)**
	3 and 6	20	18 (400)	20 (484)	18 (468)	19 (428)	19 (543)	**94/100 (465)**
	Total	**43**	**41 (501)**	**42 (445)**	**40 (432)**	**42 (403)**	**40 (449)**	**205/215 (446)**

N: the number of mosquitoes from which data were selected under each experimental condition. At each temperature the number of mosquitoes which could be evaluated following selection criteria is provided and in brackets the mean recording period in seconds (under some conditions more than one recording period could be used).

2.3.5 Body size scaling and thermal sensitivity of resting metabolic rate

Unsurprisingly $s\dot{V}CO_2$ scaled positively with body size and temperature but $s\dot{V}CO_2$ was significantly lower in 6 day-old *An. gambiae* (Fig. 2.8). Independent of using M_{alive} or M_{dry} or WL as body size explanatory variables, the temperature estimate was 0.033 (CI_{95}: 0.030-0.036) which corresponds to an apparent Q_{10} of $10^{10*0.033} = 2.13$ (CI_{95}: 1.99-2.29) meaning that $s\dot{V}CO_2$ is multiplied by 2.13 for a 10°C increment (Cossins and Bowler 1987, Chown and

Nicolson 2004). The estimate for M_{alive} was 0.98 (CI_{95} = 0.85-1.11). When using M_{dry} or WL to predict $s\dot{V}CO_2$, their respective estimates differed form M_{alive} (0.86, CI_{95}: 0.76-0.98 and 2.71, CI_{95}:2.35-3.08, respectively).

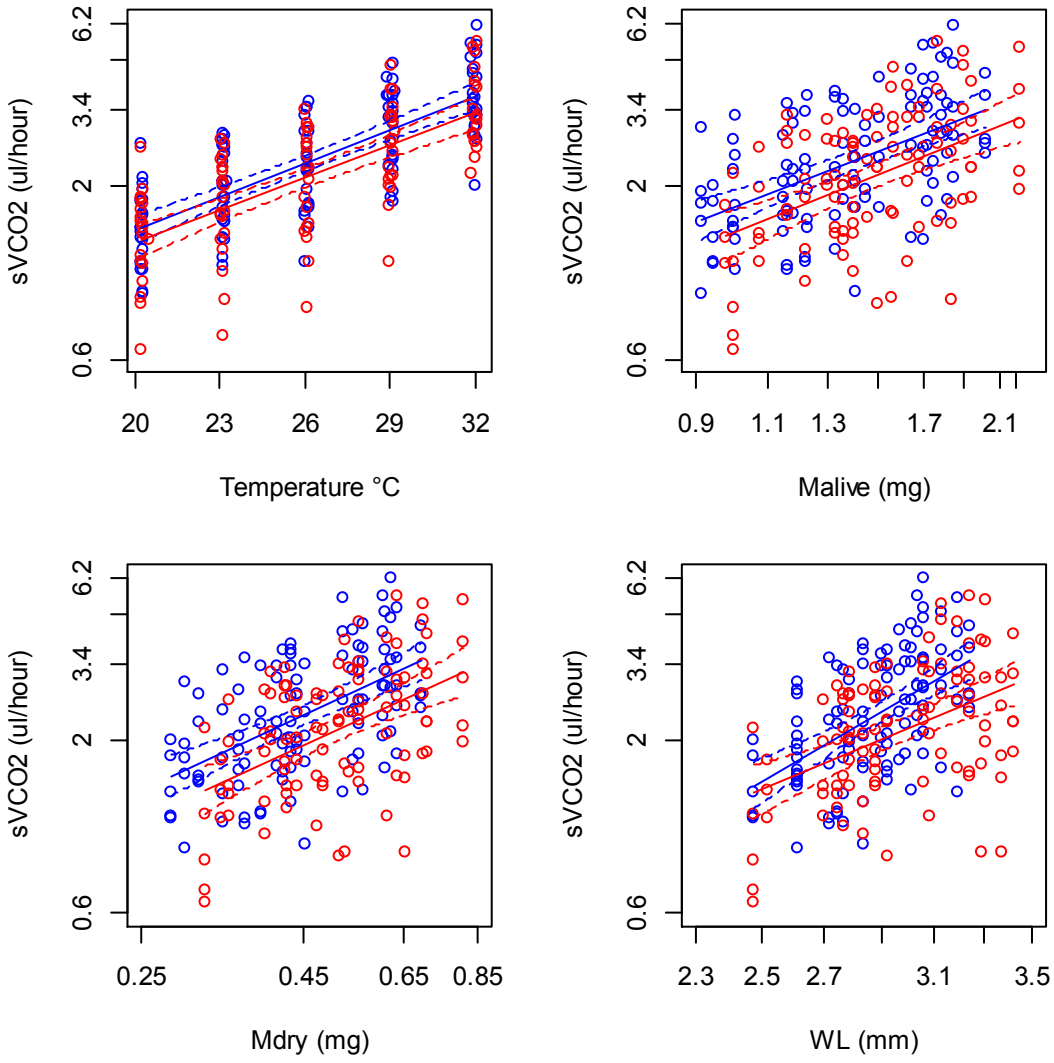


Figure 2.8 Relationships of $s\dot{V}CO_2$ plotted against temperature and mass (M_{alive} and M_{dry}) and size (WL) in female *An. gambiae*. Solid lines represent regression plots for each age (blue: 3 days and red: 6 days) and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on outputs from equations 1 to 3 (see text).

From multiple regression analyses $s\dot{V}CO_2$ of individual *An. gambiae* can be predicted with good accuracy (r^2 close to 80%) using the following equations incorporating experimental temperature with M_{alive} or M_{dry} or WL and age (for 6 day-old *An. gambiae* the value in brackets needs to be added to the intercept):

$$\log_{10} s\dot{V}CO_2 = -0.622\ (-0.071) + 0.980\ \times \log_{10} M_{alive}\ + 0.033 \times T \quad (1)$$

$$\log_{10} s\dot{V}CO_2 = -0.187\ (-0.087) + 0.867\ \times \log_{10} M_{dry}\ + 0.033 \times T \quad (2)$$

$$\log_{10} s\dot{V}CO_2 = -1.824\ (-0.083)\ + 2.716\ \times \log_{10} WL\ + 0.033 \times T \quad (3)$$

2.4 Discussion

2.4.1 Predicting resting metabolic rate of *An. gambiae*

An. gambiae is a small insect that exchanges gases at a very low rate. Because CO_2 gas analysers are more accurate compared to O_2 gas analysers (Lighton, 2008), RMR measurements are here represented by the mean production of CO_2 at rest ($s\dot{V}CO_2$). Unsurprisingly, RMR was found to scale positively with body size and temperature. The lowest measured RMR, 0.64 µl CO_2/hour came from a ~1 mg mosquito measured at 20°C and the highest at 6.15 µl CO_2/hour from a mosquito with twice as much weight and at 32°C. This means a 10-fold increase in RMR over the applied temperature range which is quite impressive. Six day-old mosquitoes had a lower RMR. This relationship was established on an *An. gambiae* population covering a wide size range by varying larval density and at five different temperatures over a 12 °C increment. The wide range of the investigation clearly contributed to the predictive power of equations 1 to 3 at around 80%, and a R^2 value that is considerably high for the size of the studied organism. Using FTR in the laboratory, Gray and Bradley (2005) found that a six day-old *An. gambiae* population with an average 0.5 mg dry mass produces on average ~1.6 – 2.1 µl/hour $s\dot{V}CO_2$ at 25°C. Feeding these data (T = 25°C and M_{dry} = ~0.5 mg) into equation 2, $s\dot{V}CO_2$ comes out at 1.94 µl/hour, meaning that our results corroborate those earlier findings. Direct measurement in the field yielded comparable results on *An. gambiae sensu lato* population (mainly composed of *An. arabiensis*) although age was not taken into consideration (Huestis et al., 2011). In the latter study, the effect of temperature and size (represented mainly with wing length) were also investigated. However, the authors did not provide a comprehensive equation of the type described here. Instead, a multivariate ANOVA was performed on the data in order to rank the importance of the different factors affecting MR. Moreover, individuals at rest were not clearly distinguished from active ones, a factor that may be important to infer proper relationships from the data. Prediction of $s\dot{V}CO_2$ based on living body mass (equ.1) is closest to reality. It must be noted, however, that predicting $s\dot{V}CO_2$ with dry mass provided a slightly better F-statistic. This might arise from the fact that dry mass can be measured with better accuracy. Further,

metabolically active tissues are probably best represented by the dry mass. $s\dot{V}CO_2$ prediction based on wing length, even if less accurate, remains very useful to predict $s\dot{V}CO_2$ for practical reasons since an accurate microbalance is not routinely available, especially in the field.

2.4.2 Body size scaling and thermal sensitivity of resting metabolic rate

Body mass was found to scale almost isometrically (=0.98) with RMR. The mass-scaling exponent of dry mass was 0.87 and 2.71 for wing length. The dry mass of the *An. gambiae* population ranged from 0.279 to 0.811 mg whereas the living body mass ranged from 0.91 to 2.22 mg. On a logarithmic scale these ranges correspond to 0.46 and 0.39 respectively, in other words, on a logarithmic scale the dry mass covers a wider interval than the living body mass and consequently will have a lower slope when plotted against $s\dot{V}CO_2$ (0.39/0.46 = 0.83 and $0.83 \times 0.980 = 0.82 \approx 0.87$). Given the relationship between wing length and dry mass ($M_{dry} \sim WL^{2.899}$, r^2=0.82) it was not surprising to find that wing length scaled with $s\dot{V}CO_2$ with an exponent of 2.71 ($\approx 2.899 \times 0.87$). The nutrient supply network model provides a theoretical background for MR to scale with (living body mass)$^{0.75}$ both across and between species on the basis of three key properties of a branching transport system: space filling networks, size-independent terminal branch units and optimization of energy expenditure by the network through natural selection (West et al., 1997, 1999). An alternative model suggests that the scaling of MR is a by-product of how cell size or number or a combination of both can explain living body mass variation (Kozlowski et al., 2003; Kozłowski and Konarzewski, 2004, 2005). This model proposes that living body mass variation is solely due to changes in cell number resulting in isometric scaling (exponent=1.00) whereas when mainly due to cell size variation to a scaling exponent approaching 0.67 (scaling relationship of surface area). This model predicts a scaling exponent of mass of 0.75 at the interspecies level, but values of mass between 0.67 and 1.0 within species. Chown et al. (2007) found a mass scaling exponent of 0.75 when investigating the relationship between living body mass and MR in 391 species of insects and a living body mass exponent of between 0.67 and 1.00 within ant species. Moreover, the ant species where body size variation mainly originated from cell number variation had a mass-scaling exponent approaching 1.00 (Chown et al., 2007). The variation in living body mass of the *An. gambiae* population investigated here was obtained by varying larval density for a constant food supply and the living body mass parameter was best correlated with wing length compared to dry mass and wet body mass (see Table 2.2, r^2 of 85% compared to 77 and 70%, respectively). This probably reflects body size variation due to the number of cells and explains a mass-scaling exponent of 0.98 which is very close to 1.00.

Size variation obtained by varying larval density has been shown to result in a linear relationship between WL^3 and protein, lipid and carbohydrate content at emergence (Briegel 1990b). Moreover, 6-day-old mosquitoes had a higher dry mass than 3-day-old mosquitoes but this difference was not significant (Table 2.4), meaning that they accumulated little reserves during their adult life which could have resulted in substantial cell size increases. Living body mass variation in mosquitoes can also be obtained by maintaining adults for a longer period and allowing them cumulate reserves over time. In this case the mass-scaling exponent would be expected to approach 0.67. In this context it is interesting to note that by doing so, Gray and Bradley (2003) obtained a mass-scaling exponent close to 0.67 by analysing the relationship between MR and living body mass in *Culex tarsalis* using FTR. As shown for ants (Chown et al., 2007), it seems that mass-scaling data on mosquitoes are rather consistent with the model proposed by Kozlowski et al. (2003) than the nutrient supply network model proposed by West et al. (1997, 1999). In terms of thermal sensitivity, RMR was found to more than double (Q_{10} = 2.13) for a 10°C increment. This finding is in agreement with Huestis et al. (2011) who found a Q_{10} of 2.07 from field measurements on *An. gambiae sensu lato*. The literature reports Q_{10} values usually ranging between 2.0 to 2.5 with extremes approaching 5 (Forlow and Macmahon, 1988; Lighton, 1988b; Cooper, 1993; Lighton and Wehner, 1993; Chown, 1997). Between individual Q_{10} variation was considerable (CI_{95}: 1.99-2.29) for the *An. gambiae* population studied here and did not seem to be correlated with wing length or wet body mass prior to respirometry. Compared to other findings, this intraspecific variation remains within a reasonable range but it cannot be excluded that it is associated with enzyme polymorphism (Nespolo et al., 2003).

2.4.3 Ecological and epidemiological implication of predicting resting metabolic rate

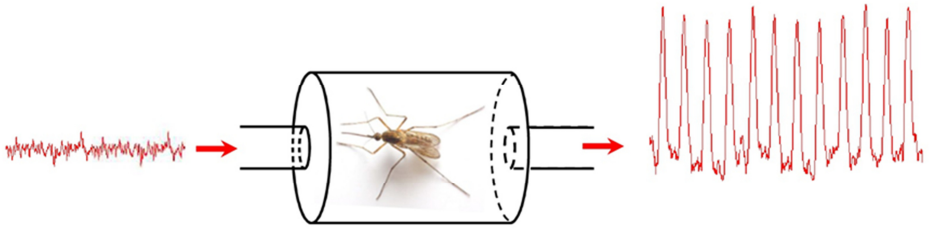
Being able to predict RMR on the basis of ambient temperature and body mass is quite trivial and has already been documented plenty of times for many organisms. Its importance should, however, not be underestimated when it comes to model malaria transmission rate and mosquito population dynamics. As an ectotherm and due to its tiny size, *An. gambiae* has an internal temperature that is almost aligned with the temperature of the surrounding environment. This latter therefore almost directly regulates *An. gambiae* MR (metabolic demand ~ environmental temperature). This is of particular importance for targeted behaviours such as mating, resource uptake and digestion (either blood or sugar) and oviposition, but *An. gambiae* spends most of its time resting (Jones and Gubbins, 1978). To respond to its metabolic demands *An. gambiae* is forced to undertake behaviours that will

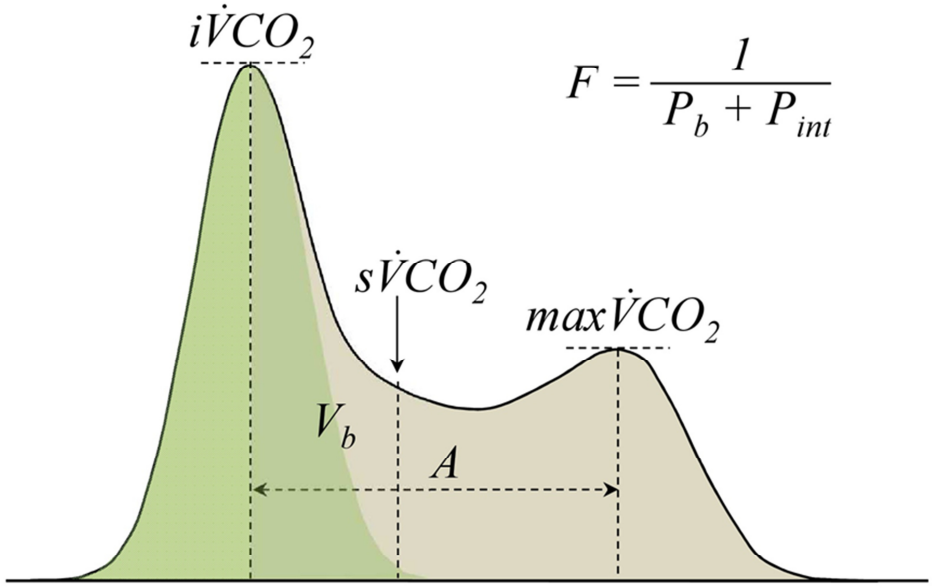
guarantee at least its own survival and propagation of the species to enhance its reproductive success. In case of an increase in temperature, as a temporary option, *An. gambiae* might seek a cool and humid refugium in order to maintain metabolic demand at a lower level and avoid depletion of reserves (Kessler and Guerin, 2008). It has been suggested that changes in behaviour and feeding activity can provide an effective mechanism for mosquitoes to reduce their MR and provide them with a means to survive aestivation (Huestis et al., 2011; Huestis et al., 2012; Huestis and Lehmann, 2014). However, under circumstances where a higher ambient temperature persists and when reproductive success is to be enhanced, sooner or later the mosquito will need to seek further resources either in the form of sugar from flowers or blood from a vertebrate host, the latter being obligatory for reproductive success. When the temperature increase does not lead to death it will likely lead to an increase in biting frequency and accelerated gonotrophic cycles. Since the development of the malaria parasite in the mosquito is also accelerated by higher temperatures it will eventually lead to an intensified malaria transmission rate. Such a scenario is realistic considering how enhanced vectorial capacity with temperature increases due to microclimatic changes as has been documented in the field for *An. gambiae* (Afrane et al., 2006; Afrane et al., 2008; Afrane et al., 2012). As suggested by Terblanche and Chown (2007) for trypanosomiases, if we assume that the malaria transmission rate is linearly linked to biting frequency then the malaria transmission rate could potentially increase by 146% due to a 5°C increment and by 213% across a 10°C increment as a result of the greater metabolic demand imposed on the vector by the environment. This relation is of particular importance in the context of human driven climate change. It has been underlined that the temperature change scenario looks quite different from predicted MR change scenario (Dillon et al., 2010). Due to climate change, it is predicted that temperature shifts will merely impact on the mid to high latitudes of the northern hemisphere whereas the physiological translation of such a scenario argues for a high metabolic impact in the tropics. Ectotherm biodiversity is mostly concentrated in the tropics and the non-linear effect of temperature on MR exacerbates small changes in the higher temperature range (Dillon et al., 2010). This scenario remains illustrative; one must not forget that the effect of temperature on mosquito population dynamics and malaria transmission rate is probably much more complex. Temperature affects the entire life cycle of *An. gambiae*, not only resting adults (Beck-Johnson et al., 2013).

2.4.4 Resting metabolic rate, water balance and age

As mentioned in the Introduction, it has been hypothesised that reducing RMR may be an adaptive strategy for mosquitoes to limit water loss (Gray and Bradley, 2005). However, these authors found no difference in RMR between *An. gambiae* Giles and *An. arabiensis*, the later inhabiting more arid conditions. According to their findings, desiccation resistance in *An. arabiensis* is merely due its ability to carry more water reserves. They also measured both $s\dot{V}CO_2$ and water loss rate at various ages with a gas analysers. Interestingly, they obtain similar results to those reported here: 6 day-old *An. gambiae* tended to have a lower RMR than 4 day-old *An. gambiae* and had a tendency to lose more water per hour (~ 60µg/hour) than younger individuals. Here similar results were found: 6-day-old mosquitoes lost approximately ~150 µg water within 2.5 hours (≈ 60µg/hour) and had a lower RMR than 3-day-old *An. gambiae*. In addition, 6-day-old individuals tended to have a lower water content than 3 day-old *An. gambiae* (Table 2.4). Teneral water reserves seem to play an important role in desiccation resistance in mosquitoes and RMR control seems to be optimal after a certain age. Following emergence and up to 2 to 4 day-old mosquitoes are still developing the functionalities of their organs and it is only after a certain age (~ 6 days) that RMR control stabilizes at its optimal but lowest level before aging alterations set in. There are three main pathways through which an insect can lose water: excretion, respiration and cuticular diffusion, whereas respiratory water loss usually represents a maximum of about 20% of total (Chown, 2002). A 6-day-old *An. gambiae* may have lost less water through respiration even though overall water loss was greater than for 3 day-old *An. gambiae*. However, the major source of water loss must have another origin. There is no reason to think that excretory events occurred more frequently in 6 day-old *An. gambiae*. A possible explanation may be that 6-day-old *An. gambiae* had a lower water content per unit dry mass. The accumulation of fat reserve with aging may limit overall water content. Glycogen reserves as an intracellular water storage possibility may also have evolved with aging. Finally, and not least important, the cuticle composition may have changed with age and it is well possible that the cuticle of 6-day-old *An. gambiae* is more permeable to water loss. On the sole basis of the present investigation, no inference can be made as to the adaptive value of reduced RMR to prevent water loss. However, the present findings are in line with Gray and Bradley (2005) who rejected the hypothesis that mosquito species that are more resistant to desiccation (*An. arabiensis*) reduce their RMR to limit water loss (see Chapter 3, for further analysis of this topic).

3 Gas exchange pattern of female *An. gambiae*





3.1 Introduction

In the previous chapter of this thesis the relation between RMR, temperature and body size was investigated. It basically reflects cellular respiration or the rate at which oxidative phosphorylation occurs and produces energy (mainly ATP) and waste products by individual *An. gambiae*. Respiratory physiology also includes the mechanisms underlying transport of respiratory gases (CO_2 and O_2), most frequently referred as the gas exchange pattern (GEP). As nicely remembered in Chown and Nicolson (2004), the statement of Miller in the early 80s still stands:

> *'The concept that insect respiration depends only on diffusion supplemented in larger species by ventilation is in need of an overhaul: the situation is much more complex.'*
>
> (Miller, 1981)

Although documentation remains scarce it is becoming clear that even smaller insects do not uptake or release respiratory gases simply by diffusion. At rest, an insect may employ a diversity of GEP. In order to characterize this diversity, subsequent descriptions qualified GEP from continuous, to cyclic and to cyclic-discontinuous (Gibbs and Johnson, 2004; Marais et al., 2005). The corridors or the tracheal network that transport respiratory gases from and to tissues is closed by a door called the spiracle in insects. Where spiracles are lacking or held open during activity the pattern is continuous. Cyclic GEP is periodic and lacks periods of no gas exchange with the atmosphere. Discontinuous and cyclic GEP (often referred as DGC: discontinuous gas exchange cycles) is distinguished from the others by the periodic occurrence of no gas exchange (discontinuity). Since it appears to be the most regulated and evolved manner of exchanging respiratory gases in insects it has by far enjoyed most attention in insect physiology research (Chown and Nicolson, 2004). The first observation of DGC originates from the beginning of the 1960s (Punt et al., 1957; Wilkins, 1960). Soon after, further investigation on diapausing saturniid pupae provided the first developed description of the pattern (Schneiderman, 1960; Levy and Schneiderman, 1966b, a; Schneiderman and Schechter, 1966). Classical DGC is characterized by the repetition of cycles composed of three consecutive phases. It starts with the closed-phase (C-phase) during which the spiracles all remain closed and gas exchange is negligible. During this period endotracheal O_2 is depleted by metabolic active of tissues and not fully replaced by CO_2 due to haemolymph buffering capacity resulting in a negative endotracheal pressure. The C-phase is followed by the flutter-phase (F-phase) during which spiracles open intermittently and gas

flux is low and largely inwardly convective due to negative endotracheal pressure. A DGC cycle ends with the open-phase (O-phase) during which gas exchanges rapidly (Box 3.1.). This description mostly originates from work on Lepidoptera, large insects where GEP is more easily measured. Depending on the insect species this 3-phase pattern may vary (Lighton, 1994, 1996; Chown and Nicolson, 2004; Chown et al., 2006).

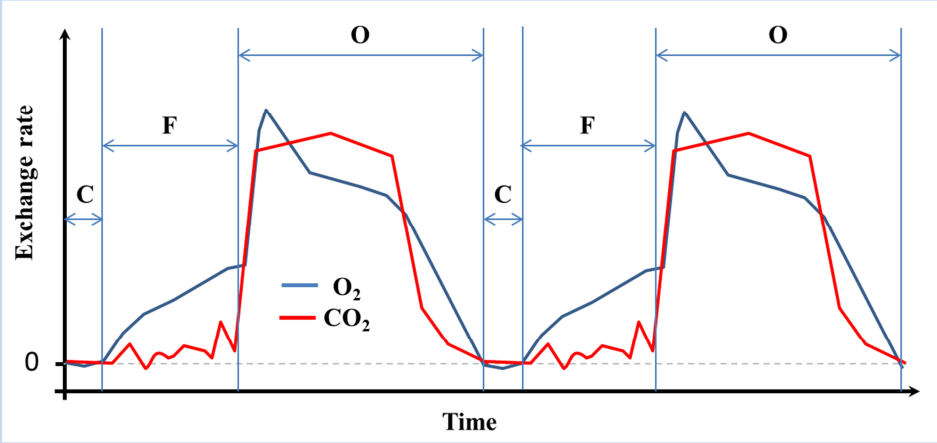
Box 3.1 Typical Discontinuous Gas exchange Cycle (DGC) of insects

DGC is characterized by constant repetition of three consecutive spiracular behaviours: **closed (C)** followed by the **flutter (F)** and ending with the **open phase (O)**.

C-phase: During this period input from the nervous system serves to contract spiracular muscles causing tight closure of spiracles. No gas exchange takes place with the surrounding atmosphere and tracheal O_2 is consumed by metabolic active of tissues that surround the tracheoles. Because of haemolymph buffering capacity the O_2 consumed is not replaced by CO_2 produced. This results in a negative endotracheal pressure.

F-phase: This phase is triggered by the nervous system when endotracheal PO_2 reaches a critical set point. During this phase the spiracles open slightly and close in rapid succession. The negative endotracheal pressure built up during the C-phase prevents any outward movement of gases (hypothetically including H_2O) during the F-phase. As a result, additional O_2 flows into the tracheal system to satisfy metabolic demand while little outward movement of any other nature takes place. The F-phase continues until the buffering capacity of the haemolymph is surpassed.

O-phase: Endotracheal PCO_2 continues to increase up to a higher critical set point where it activates total opening of the spiracles. During the O-phase gas exchange occurs normally with the atmosphere, mainly through diffusion.



DGC description and scheme adapted from (Lighton, 1988a; Chown and Nicolson, 2004; Chown et al., 2006)

So far, information on GEP exists on 18 orders and 118 species of insect. Cyclic and continuous GEP have been observed in all insect orders while DGC in less than 10 orders

(Marais et al., 2005; Contreras and Bradley, 2011). Why a limited number of insect orders have evolved DGC, as the apparent most regulated form of gas exchange, remains largely debated in terms of possible ultimate or proximal reasons. Several ultimate explanations have been proposed that are not mutually exclusive (reviewed in Chown et al. 2006). The 'hygric hypothesis' (Kestler, 1984), where the C- and F- phase are thought to limit respiratory water loss (during the C-phase there is no exchange and the F-phase is dominated by an inward convective flow), has dominated research literature for a couple of decades but has recently been seriously questioned (Hadley and Quinlan, 1993; Williams and Bradley, 1998; Chappell and Rogowitz, 2000; Chown and Holter, 2000; Gibbs and Johnson, 2004; Lighton and Turner, 2008; Contreras and Bradley, 2009; Contreras and Bradley, 2011). The more recent perspectives propose challenging alternative hypotheses: 'the chthonic hypothesis' of Lighton and Berrigan (1995) proposes that DGC evolved in order to enhance gas exchange in hypercapnic and hypoxic environments (e. g. underground), the 'oxidative damage hypothesis' of Hetz and Bradley (2005) suggests that DGC evolved in order to maintain endotracheal oxygen at a lower level and consequently prevent oxidative damage of tissues, and the 'strolling arthropod hypothesis' of Harrison et al. (2001), a more targeted explanation, proposes that maximizing spiracular closure prevents dust and parasite intrusion through the tracheal network.

Proximal or mechanistic explanations suggest that occurrence of DGC is MR dependent. Indeed, more and more studies show that a single species can employ all three GEP types and that DGC occurs at rest and at lower temperatures or when metabolic demand is lowest (Marais and Chown, 2003; Contreras and Bradley, 2009, 2010). The 'emergent properties hypothesis' of Chown and Holter (2000) suggest that DGC is the result of two competing/interacting sensory systems, one responding to endotracheal CO_2 and the other to O_2. This proximal explanation has since been confirmed in moth pupae: By manipulating endotracheal respiratory gas concentration Förster and Hetz (2010) revisited the way spiracles open and close. They noticed that when PO_2/PCO_2 is above/below a certain threshold the spiracles remain closed and open when both partial pressures are above their respective thresholds. They also demonstrate that the F-phase is explained by a time delay between sensing and entry of O_2 into the trachea resulting in small and fast open/close cycles until the endotracheal PCO_2 threshold is reached and the spiracles open fully (O-phase). To add an adaptive value to this explanation it has been suggested that DGC occurs when the central nervous system allocates control of partial pressure of respiratory gases to the segmental

ganglia in order to save energy, knowing that neural centres, even at rest, are particularly demanding metabolically (Matthews and White, 2010; Chown, 2011). This ultimate explanation or 'neural hypothesis' could explain why DGC has often been observed at lower temperature and when insects are at rest or when MR is particularly low. The role of the neural system in control of GEP by insects has recently been reviewed in Matthews (2017) where it is clearly stated that hypothesised hysteresis in chemoreception of respiratory gases still needs further research.

What do we know about the GEP of *An. gambiae*? First of all it is important to appreciate that there is only one technique available to resolve the GEP characteristics of an insect, namely FTR, and so far few studies have been performed on mosquitoes. As mentioned in chapter 2 of this thesis, FTR investigations have been performed on *Culex tarsalis* (Gray and Bradley, 2003), on *An. gambiae* and *An. arabiensis* (Gray and Bradley, 2005) and on *Aedes aegypti* (Gray and Bradley, 2006b). However, most of these investigations focused on quantifying MR not on the parameters characterizing GEP as such. Traces of GEP of *Culex tarsalis* and both anopheline species seem to indicate that mosquitoes employ cyclical gas exchange. The cited study on anopheline species estimated O-phase amplitude from the standard error of $s\dot{V}CO_2$ and DGC frequency was mentioned but poorly documented. One year later, using the winter mosquito *Culiseta inornata* as a model, the same researchers nicely demonstrated, that although GEP may appear cyclic, under appropriate measurement conditions (FTR: higher flow rate, minimal mosquito chamber volume and lower temperature) the pattern resolves to be close to a true DGC (Gray and Bradley, 2006a). *Culiseta inornata* weighs about 7 mg and these observations were made at 10°C at a flow rate of 200 ml/min indicating the winter mosquito to be the smallest insect species employing DGC observed so far. The reason why former investigations by the same authors did not document such GEP parameters is simply because a lower flow rate (50 ml/min) was used resulting in a very poor temporal resolution by the FTR system. These later findings suggest that highly regulated forms of breathing such as DGC are probably much more frequent used even by smaller insects or smaller mosquitoes like *An. gambiae*. For this reason, MR and GEP measurements described in this chapter of the thesis were performed with a relatively high flow rate of 250 ml/min. In this chapter the parameters constituting the GEP of *An. gambiae* are resolved and quantified by taking into account the influence of body size and temperature. The results are compared with other insects and treated in the context of earlier discussions concerning the origin of highly evolved form of gas exchange in insects.

3.2 Material and Methods

3.2.1 Parameters characterizing the gas exchange pattern of *An. gambiae*

The same data was used as in Chapter 2 (for methodological aspects such as FTR, female mosquito body size and data correction see sections 2.2.1 to 2.2.3). A quick look on Figure 2.3 shows the typical GEP employed by *An. gambiae* when at rest. This GEP can be characterized by calculating several parameters for each record (Figure 3.1):

- **$s\dot{V}CO_2$** (µl $hour^{-1}$): the mean CO_2 production rate at rest (representing the RMR and already calculated in Chapter 2)
- **$i\dot{V}CO_2$** (µl $hour^{-1}$): the mean inter-burst (including hypothetical C- and F-phase) CO_2 production rate
- **$b\dot{V}CO_2$** (µl $hour^{-1}$): the mean burst (O-phase) CO_2 production rate or contribution of burst volume to $s\dot{V}CO_2$
- **$max\dot{V}CO_2$** (µl $hour^{-1}$): the mean of maxima of CO_2 production reached during burst periods
- **A** (Δµl): the mean CO_2 burst amplitude
- **F** (mHz): the burst frequency
- **P_{int}** (s): the mean inter-burst period (including hypothetic C- and F-phase)
- **P_b** (s): the mean burst period representing the O-phase
- **V_b** (nl): the mean volume of a burst or the additional CO_2 excretion resulting from spiracle opening.

3.2.2 Evaluation of gas exchange parameters

F and $s\dot{V}CO_2$ were easily computed by using functions directly available in Expedata software (mean of the signal for $s\dot{V}CO_2$, already calculated in Chapter 2, and Fast Fourier transform for F). The other parameters necessitated alternative means of analysis of the raw data using R software (R Core Team 2013). The determination of the other variables was extracted from the Kernel density estimates (KDE) of the data constituting the records using *density function* in R. The bandwidth was chosen according to Silverman's rule of thumb (Silverman 1986). The KDE proved to be bimodal in most of the records. The KDE is very useful as its major mode ($Mode_1$) corresponds to $i\dot{V}CO_2$ (lowest CO_2 production rate) and the minor mode ($Mode_2$) to $max\dot{V}CO_2$ (Fig. 3.1). Both modes were determined by using 'quantmod' package in R using 'findPeaks function' for each record. In 8 cases out of 205,

the 'findPeaks function' delivered non plausible modes. In such records, where possible, both modes were manually determined (2 out of 8). Once both modes were known the other parameters characterizing the GEP of *An. gambiae* were determined using the following equations:

$$Mode_1 = i\dot{V}CO_2 \qquad Mode_2 = max\dot{V}CO_2$$

$$A = max\dot{V}CO_2 - i\dot{V}CO_2 \qquad V_b = \frac{s\dot{V}CO_2 - i\dot{V}CO_2}{F}$$

where $s\dot{V}CO_2$ and $i\dot{V}CO_2$ are in µl hour^{-1}, and F is expressed in hour^{-1}.

P_{int} and P_b are related to burst frequency since:

$$F = \frac{1}{P_{int} + P_b}$$

and can be defined as follow:

$$P_{int} = \frac{N_{int}}{(N_{int} + N_b) \times F} \qquad P_b = \frac{N_b}{(N_{int} + N_b) \times F}$$

where N_{int} and N_b are the number of data points of the record belonging to the inter-burst and burst period, respectively. Assuming that the data points of the inter-burst period are normally distributed around $Mode_1$, N_{int} corresponds to twice the number of data points below $Mode_1$ since a normal distribution is symmetric around the mean. N_b can be obtained by subtracting N_{int} from the total number of points in the record. To obtain P_{int} and P_b in seconds F must be in Hertz.

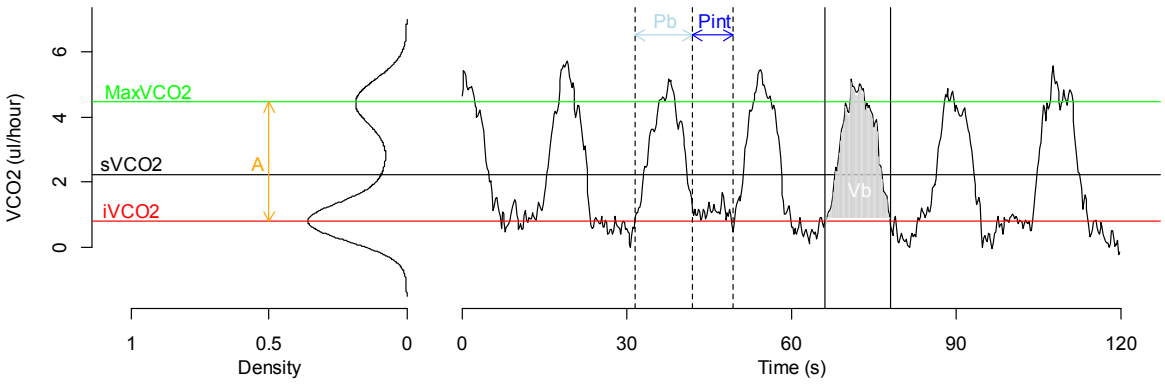


Figure 3.1 Parameters characterizing the GEP of *An. gambiae*: $s\dot{V}CO_2$, $max\dot{V}CO_2$, $i\dot{V}CO_2$, $b\dot{V}CO_2$, A, P_{int}, P_b and V_b. $b\dot{V}CO_2$ and F are not shown on the figure but $b\dot{V}CO_2 = s\dot{V}CO_2 - i\dot{V}CO_2$ and $F^{-1} = P_{int} + P_b$. On the right, a 2 minutes extract of a record and on the left its Kernel density estimate (KDE). Determination KDE of modes allows accurate determination of $i\dot{V}CO_2$ and $max\dot{V}CO_2$.

3.2.3 Statistics and inferences on discontinuity of the gas exchange pattern

In order to describe and make inferences on the relationship between GEP parameters, body size and temperature the following equation was used, as described in Chapter 2 (section 2.2.4):

$$\mathrm{Log}_{10}(GEP\ parameter) = a + b \times \log_{10} Body\ size + c \times Temperature$$

Ordinary multiple regression analysis was made in R using the linear model function (R Core Team 2013). The response variable of the multiple regression was any parameter characterizing the GEP and the explanatory variables were either continuous (M_{alive} or M_{dry} or WL and temperature) or categorical (age) with two levels. Variables were checked for normality using a Shapiro-Wilks test, and, where necessary, distributions were normalized by Log_{10} transformation (Zar, 1999). In order to assess the importance of model parameters, the maximum model first was computed. Subsequently stepwise elimination of parameters to find the best model fit was performed. Non-significant interaction terms were removed first followed by non-significant single terms. Between each simplification, an ANOVA between models was performed in order to confirm that removal was justified (non-significant ANOVA indicates that models do not differ and justifies removal of terms).

An empty mosquito chamber served as the baseline ($\dot{V}CO_2$ = 0 µl hour^{-1}). Taking into account all the baseline portions contained in the selected records, and after drift correction, the baseline had a mean of 0.005 µl hour^{-1} with a CI_{95} of -0.38 to 0.39 µl hour^{-1} (Fig. 3.2) and did not significantly differ from 0 (t-test: p=0.77). Moreover the baseline data points were normally distributed around the mean (Shapiro normality test: p=0.77). The mean baseline level and its CI_{95} limits were computed for each GEP record. In order to conclude on a 'true' DGC (a GEP where $\dot{V}CO_2$ falls back to zero periodically), i$\dot{V}CO_2$ needed to have a value falling within the CI_{95} limits of the baseline level.

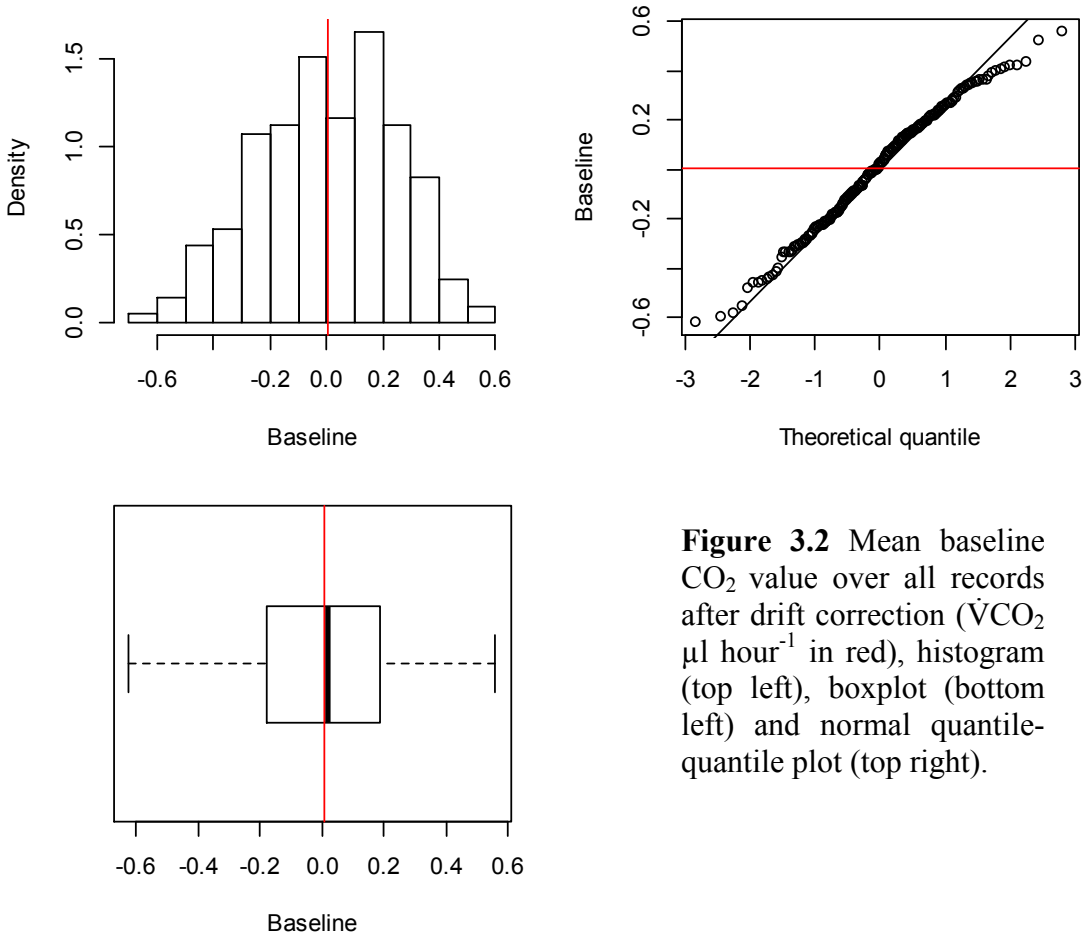


Figure 3.2 Mean baseline CO_2 value over all records after drift correction ($\dot{V}CO_2$ µl hour^{-1} in red), histogram (top left), boxplot (bottom left) and normal quantile-quantile plot (top right).

3.3 Results

3.3.1 Overview of parameters characterizing the gas exchange pattern of *An. gambiae*

Figures 3.3 to 3.8 represent the typical GEP at rest employed by 6 individual *An. gambiae* of different ages (3 or 6 day-old), of different body size (grown at different larval densities) and at each temperature (all records are provided in Appendix 8.2.1). Using mode determination on the basis of KDE, $i\dot{V}CO_2$ (=mode 1) and $max\dot{V}CO_2$ (=mode 2) could be determined in 199 of the 205 available records (Fig. 3.9). In the 6 remaining records only $s\dot{V}CO_2$ and F could be determined. A summary of all calculated parameters is provided in Table 3.1. The cyclical nature of the GEP was evident in all of the records. In 8 individuals, the cyclic nature of the GEP was accompanied by $i\dot{V}CO_2$ level almost undistinguishable from 0 µl hour^{-1} (within the CI_{95} of the baseline). Figure 3.4 shows an example of an *An. gambiae* with an $i\dot{V}CO_2$ approaching 0 µl hour^{-1} at 20°C.

Table 3.1 Summary of each parameter characterizing the GEP of female *An. gambiae* at rest for each age group and tested temperature.

Age	Body size*	T°C	$s\dot{V}CO_2$ (µl/hr)	$i\dot{V}CO_2$ (µl/hr)	A (Δµl/hr)	F (mHz)	P_{int} (s)	P_b (s)	V_b (nl)
		20	1.55±0.09	0.85±0.08	2.22±0.21	44.1±1.1	15.91±0.69	7.07±0.44	4.43±0.29
	1.43±0.07	23	2.03±0.12	1.27±0.09	2.45±0.27	58.8±1.2	11.19±0.48	5.92±0.46	3.83±0.35
3	0.47±0.03	26	2.35±0.15	1.28±0.09	2.81±0.27	78.3±1.6	7.39±0.44	5.45±0.36	3.89±0.30
	2.88±0.04	29	3.13±0.18	1.91±0.10	3.17±0.27	102.8±1.5	5.18±0.22	4.58±0.18	3.45±0.29
		32	3.89±0.23	2.37±0.13	3.37±0.25	134.5±2.9	3.84±0.17	3.66±0.18	3.13±0.24
		mean	**2.59±0.11**	**1.54±0.07**	**2.81±0.12**	**83.7±3.2**	**8.69±0.47**	**5.33±0.19**	**3.75±0.13**
		20	1.46±0.10	0.85±0.10	2.58±0.19	40.8±0.7	18.18±0.89	6.51±0.72	4.15±0.41
	1.49±0.07	23	1.84±0.13	1.05±0.11	2.69±0.22	52.8±1.0	12.58±0.48	6.48±0.46	4.36±0.39
6	0.52±0.03	26	2.17±0.17	1.24±0.13	2.81±0.20	70.6±1.4	8.99±0.33	5.31±0.29	3.89±0.33
	2.98±0.06	29	2.76±0.19	1.58±0.12	3.11±0.29	95.0±1.8	6.13±0.23	4.46±0.24	3.49±0.32
		32	3.54±0.23	2.18±0.15	3.56±0.33	124.2±2.1	4.41±0.09	3.68±0.11	3.07±0.32
		mean	**2.34±0.10**	**1.38±0.07**	**2.95±0.12**	**76.2±3.1**	**10.11±0.55**	**5.29±0.22**	**3.79±0.16**
3 and 6		**mean**	**2.47±0.07**	**1.46±0.07**	**2.88±0.08**	**80.1±2.3**	**9.38±0.36**	**5.31±0.14**	**3.77±0.10**

*Mean M_{alive} (mg), M_{dry} (mg) and WL (mm), respectively.

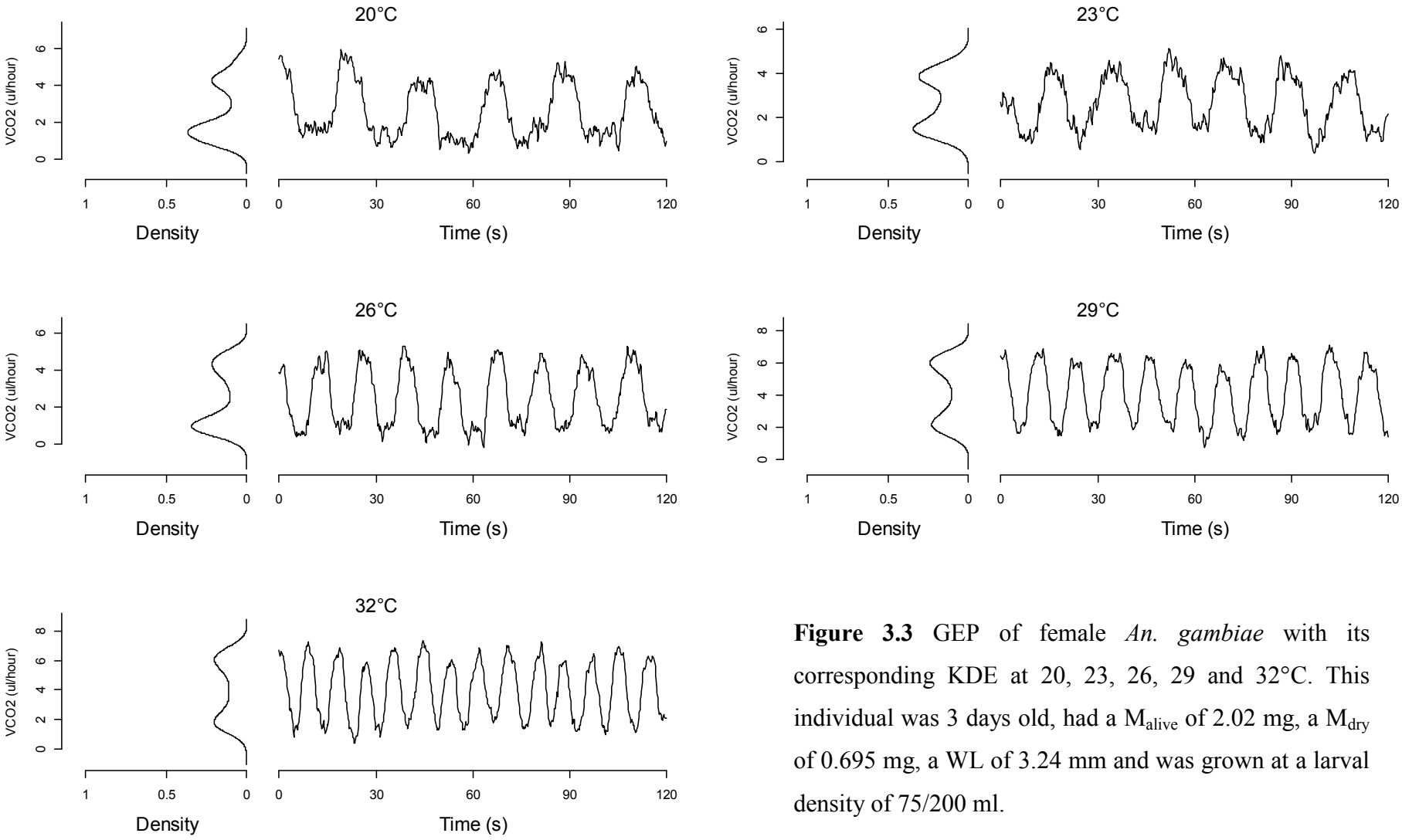


Figure 3.3 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 3 days old, had a M_{alive} of 2.02 mg, a M_{dry} of 0.695 mg, a WL of 3.24 mm and was grown at a larval density of 75/200 ml.

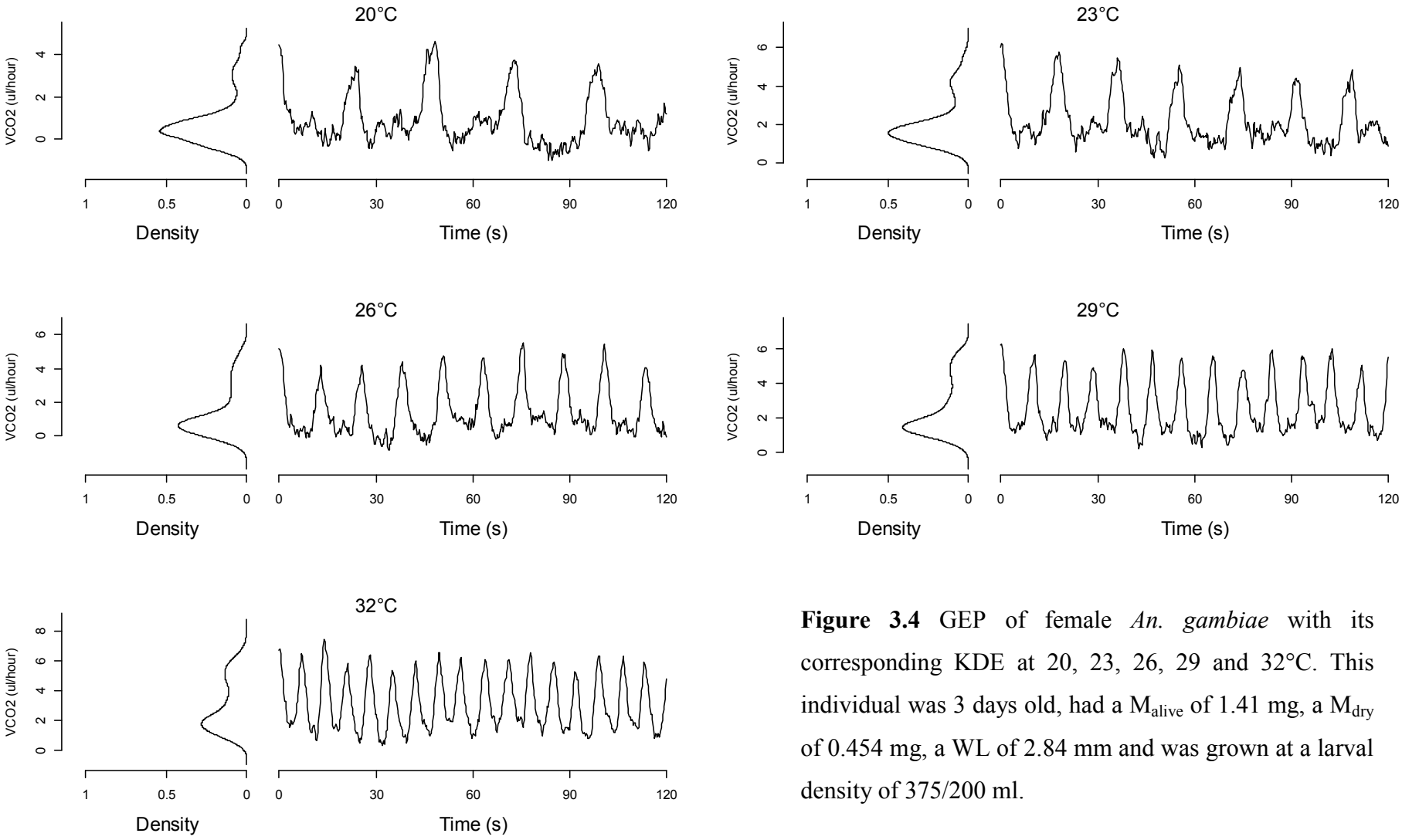
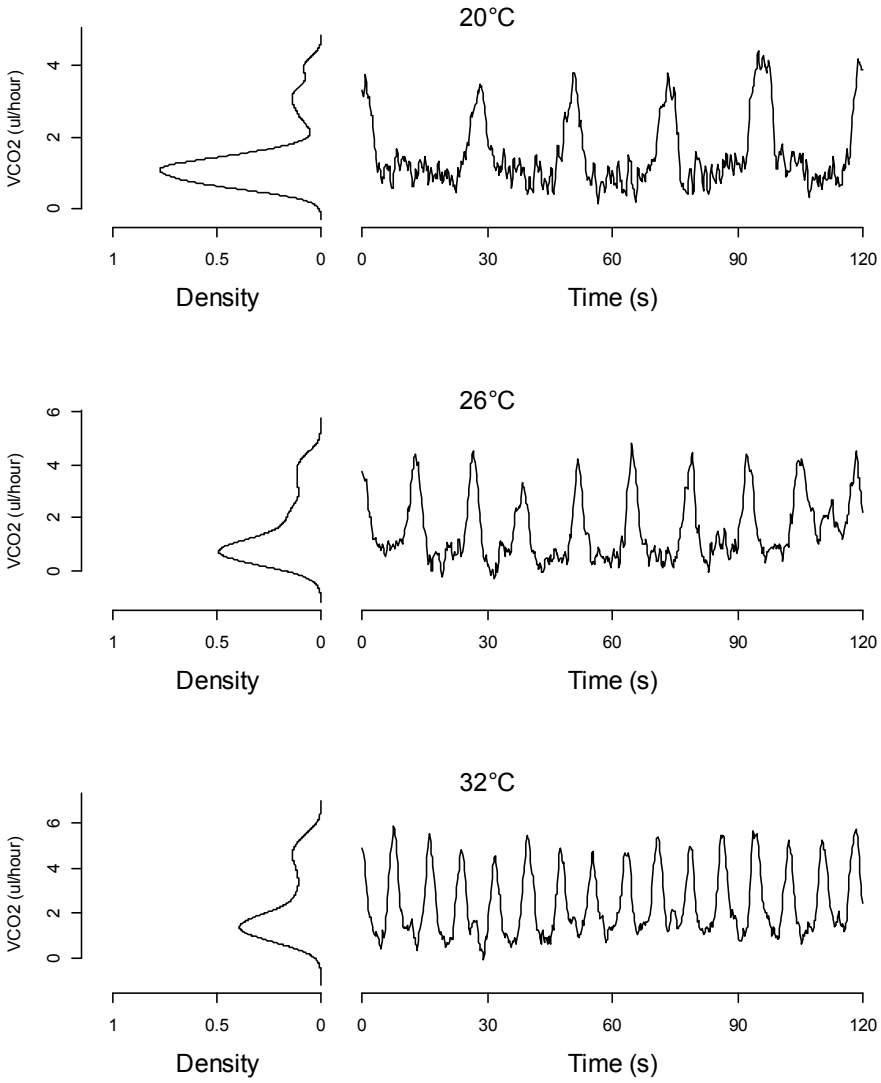


Figure 3.4 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 3 days old, had a M_{alive} of 1.41 mg, a M_{dry} of 0.454 mg, a WL of 2.84 mm and was grown at a larval density of 375/200 ml.



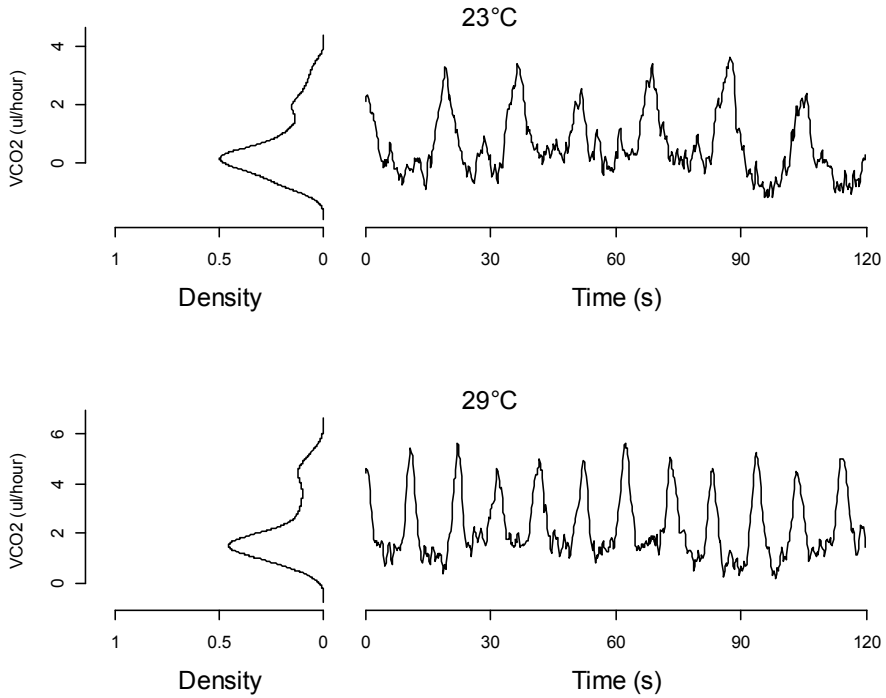


Figure 3.5 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 3 days old, had a M_{alive} of 1.00 mg, a M_{dry} of 0.309 mg, a WL of 2.61 mm and was grown at a larval density of 525/200 ml.

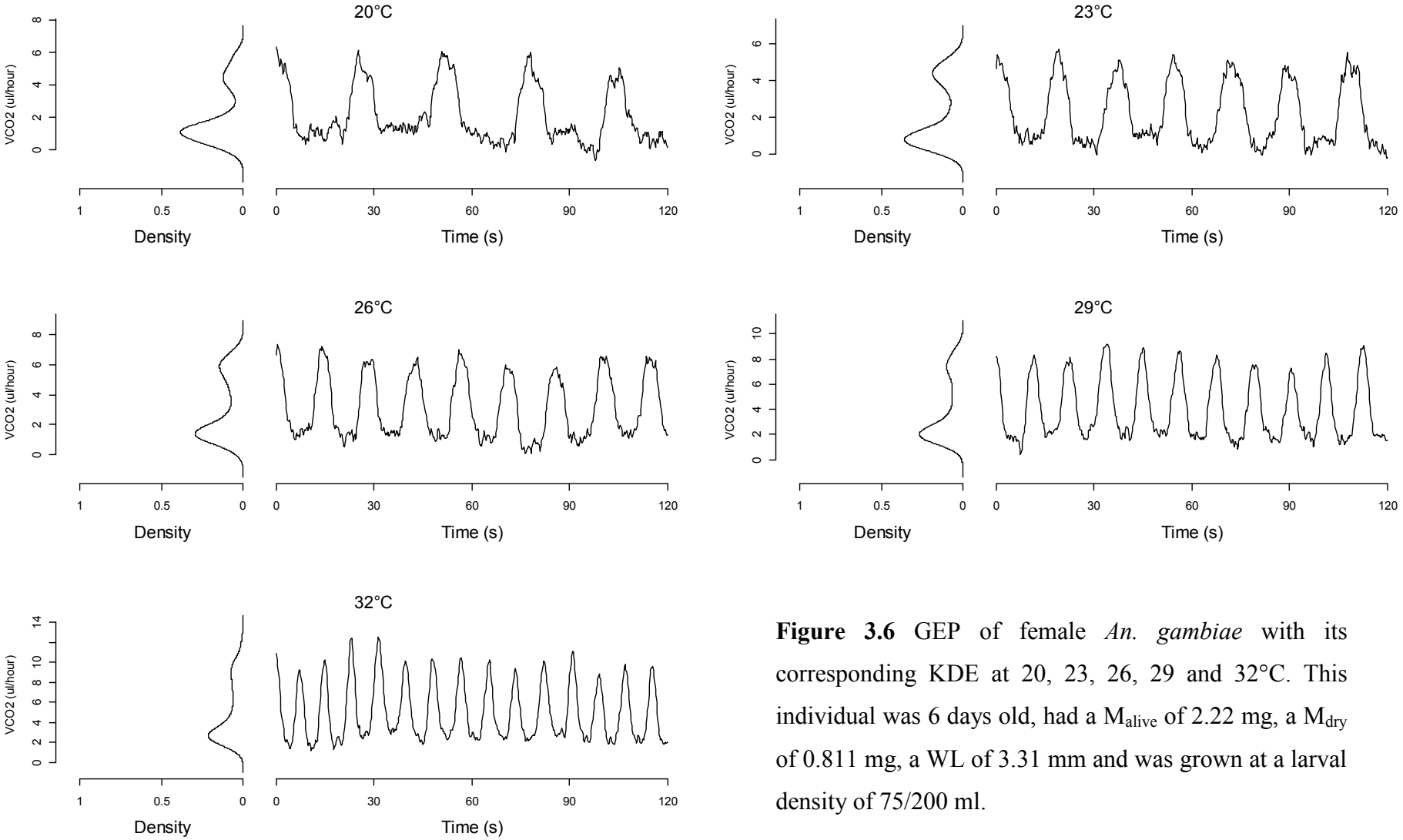


Figure 3.6 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 6 days old, had a M_{alive} of 2.22 mg, a M_{dry} of 0.811 mg, a WL of 3.31 mm and was grown at a larval density of 75/200 ml.

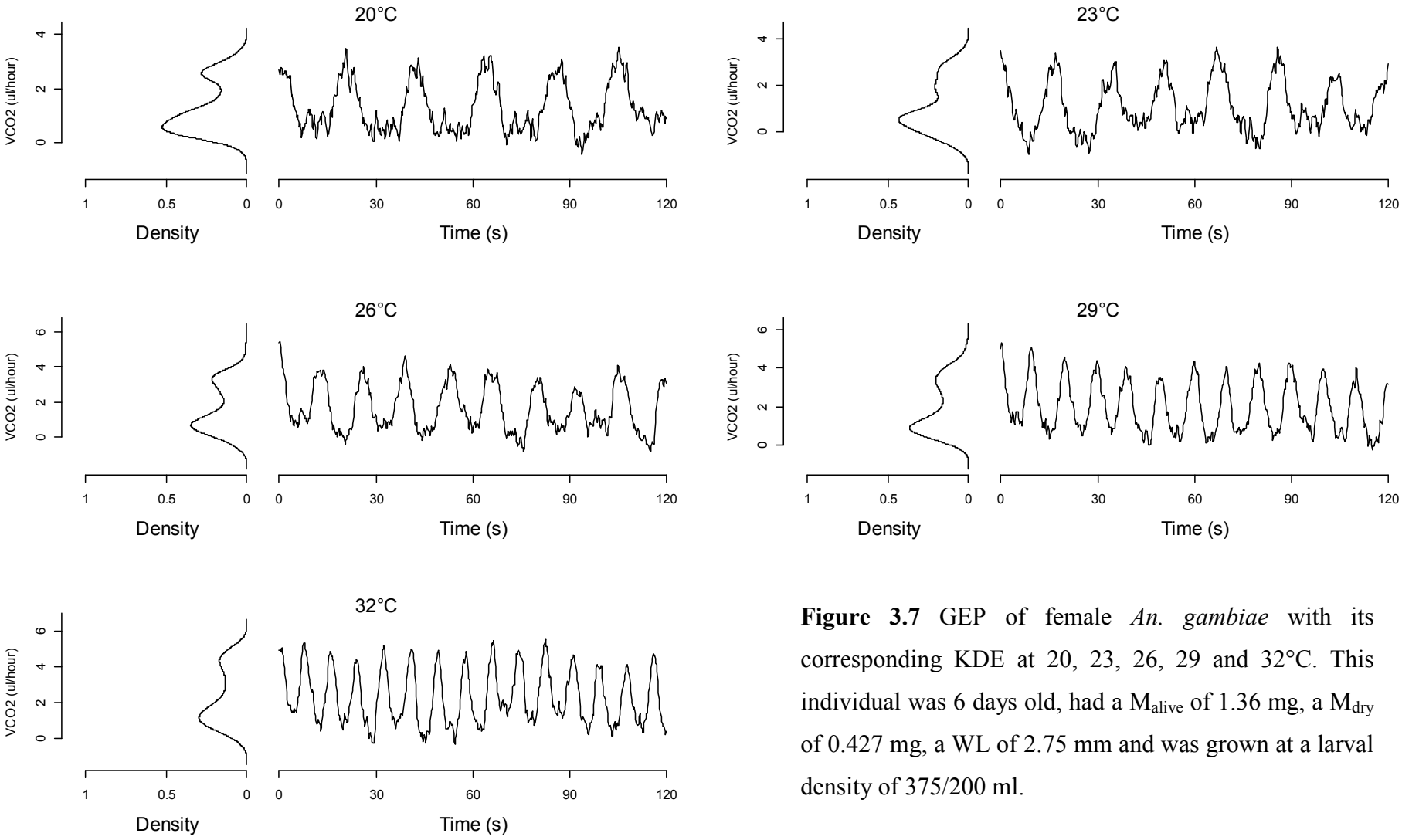


Figure 3.7 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 6 days old, had a M_{alive} of 1.36 mg, a M_{dry} of 0.427 mg, a WL of 2.75 mm and was grown at a larval density of 375/200 ml.

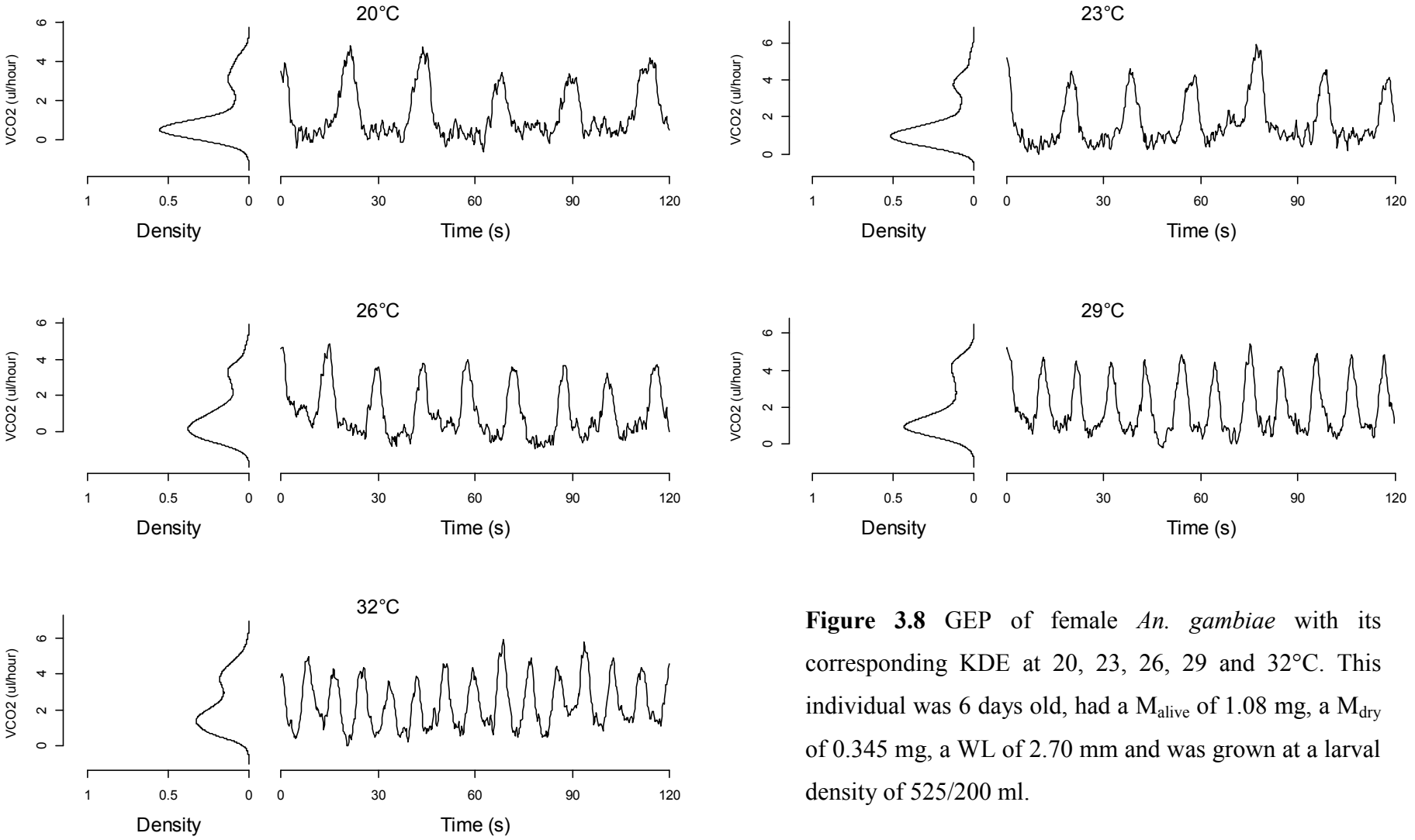


Figure 3.8 GEP of female *An. gambiae* with its corresponding KDE at 20, 23, 26, 29 and 32°C. This individual was 6 days old, had a M_{alive} of 1.08 mg, a M_{dry} of 0.345 mg, a WL of 2.70 mm and was grown at a larval density of 525/200 ml.

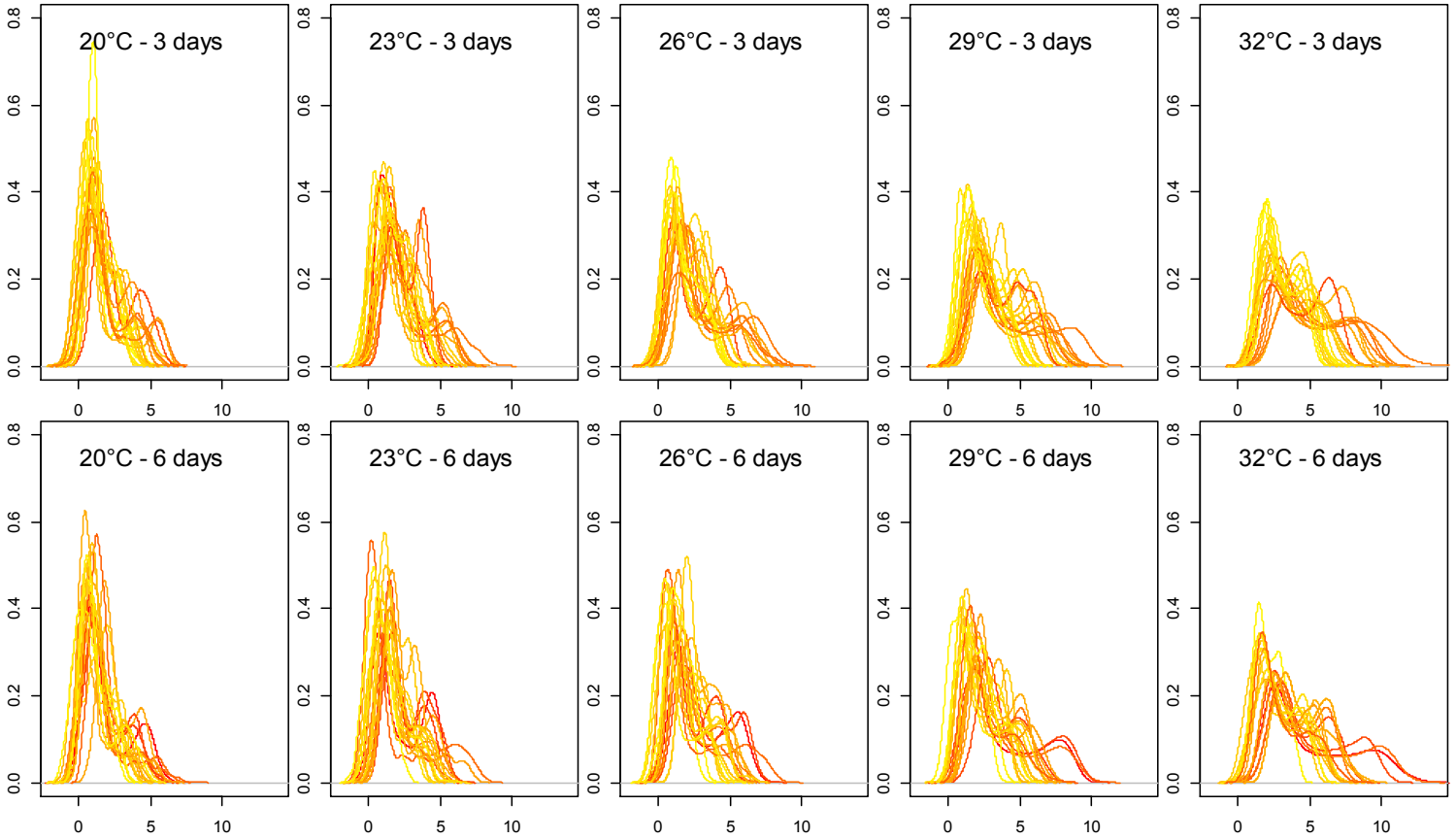


Figure 3.9 KDE for all records at each temperature and for both age groups; the colour gradient represents the size of the recorded female *An. gambiae* (the redder the bigger the mosquito). Both $Mode_1$ ($i\dot{V}CO_2$) and $Mode_2$ ($max\dot{V}CO_2$) are clearly distinguishable. Notable trends: the amplitude (A) or the distance between the modes increases with increasing temperature but particularly with increasing size; the relative importance between modes indicates the importance of the inter-burst duration (P_{int}) relative to burst duration (P_b) with P_{int} decreasing with both temperature and size increases, P_b decreasing with temperature increase but decreasing with size increase; the asymmetry between modes is particularly pronounced for 6 day-old female *An. gambiae*.

3.3.2 Body size scaling and thermal sensitivity of gas exchange pattern parameters

On the basis of the raw data summarized in Table 3.1, multiple regression analyses was used to establish body size scaling and thermal sensitivity of the different parameters characterizing the GEP employed by female *An. gambiae*. By using estimates calculated by the mean of multiple regressions, the relationship between each GEP parameters can be predicted using an equation of the form:

$$\log_{10}(GEP\ parameter) = a + b \times \log_{10} Body\ size + c \times Temperature$$

where the GEP parameter is either $s\dot{V}CO_2$, $i\dot{V}CO_2$, A, F, P_{int}, P_b or V_b. Body size is either represented by the living body mass (M_{alive}), dry mass (M_{dry}) or wing length (WL), *a* is the intercept, *b* the body size estimate and *c* the temperature estimate. After simplification of each multiple regression analysis it appeared that none of the interaction terms (2nd and 3rd order) remained significant. This means that a different body size scaling resulted solely in different *b* estimates, thermal sensitivity solely in different *c* estimates and differences between each age group solely in terms of intercepts *a*. All multiple regression analyses are shown in Table 3.2 for M_{alive}, in Table 3.3 for M_{dry} and in Table 3.4 for WL.

Table 3.2 Summary of multiple regression relationships of GEP parameters against temperature (T°C), M_{alive} (mg) and age group (3 and 6 day-old female *An. gambiae*).

Response variable*	Explanatory variables**	Estimates ± SE***	r^2	*t*-value	*p-values*
Log_{10} $s\dot{V}CO_2$	Intercept	-0.622 ± 0.041		-14.861	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	0.980 ± 0.065	0.78	15.099	$< 2\times10^{-16}$ ***
	Temperature	0.033 ± 0.002		21.491	$< 2\times10^{-16}$ ***
	Age	-0.071 ± 0.013		-5.545	9.1×10^{-11} ***
	F-statistics: $F_{3, 201}$*: 236.0,* $p < 2.2 \times 10^{-16}$				
Log_{10} $i\dot{V}CO_2$	Intercept	-1.021 ± 0.084		-12.151	$< 2\times10^{-16}$
	Log_{10} M_{alive}	0.862 ± 0.134	0.52	6.447	8.7×10^{-10} ***
	Temperature	0.039 ± 0.003		12.912	$< 2\times10^{-16}$ ***
	Age	-0.084 ± 0.026		-3.265	1.2×10^{-3} ***
	F-statistics: $F_{3, 195}$*: 71.63,* $p < 2.2 \times 10^{-16}$				
Log_{10} $b\dot{V}CO_2$	Intercept	-0.951 ± 0.053		-18.092	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	1.287 ± 0.084	0.71	15.318	$< 2\times10^{-16}$ ***
	Temperature	0.029 ± 0.002		15.007	$< 2\times10^{-16}$ ***
	Age	-0.054 ± 0.016		-3.357	9.5×10^{-4} ***
	F-statistics: $F_{3, 195}$*: 155,* $p < 2.2 \times 10^{-16}$				
Log_{10} A	Intercept	-0.215 ± 0.069		-3.116	2.1×10^{-3} **
	Log_{10} M_{alive}	1.416 ± 0.109	0.51	12.899	$< 2\times10^{-16}$ ***
	Temperature	0.016 ± 0.003		6.186	3.5×10^{-9} ***
	Age	0.006 ± 0.021		0.284	0.78 NS
	F-statistics: $F_{3, 195}$*: 69.31,* $p < 2.2 \times 10^{-16}$				
Log_{10} F	Intercept	0.8099 ± 0.017		45.603	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	-0.022 ± 0.027	0.95	-0.824	0.41 NS
	Temperature	0.041 ± 0.001		63.742	$< 2\times10^{-16}$ ***
	Age	-0.037 ± 0.005		-6.934	5.4×10^{-11} ***
	F-statistics: $F_{3, 201}$*: 1374,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_{int}	Intercept	2.266 ± 0.039		56.95	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	-0.194 ± 0.063	0.87	-3.09	2.3×10^{-3} **
	Temperature	-0.052± 0.001		-36.226	$< 2\times10^{-16}$ ***
	Age	0.073 ± 0.012		6.008	9.1×10^{-9} ***
	F-statistics: $F_{3, 195}$*: 450.7,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_b	Intercept	1.243 ± 0.053		23.62	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	0.427 ± 0.083	0.47	5.124	7.1×10^{-7} ***
	Temperature	-0.023 ± 0.002		-12.16	$< 2\times10^{-16}$ ***
	Age	-0.003 ± 0.016		-0.164	0.87 NS
	F-statistics: $F_{3, 195}$*: 58.47,* $p < 2.2 \times 10^{-16}$				
Log_{10} V_b	Intercept	0.678 ± 0.054		12.546	$< 2\times10^{-16}$ ***
	Log_{10} M_{alive}	1.315 ± 0.086	0.58	15.228	$< 2\times10^{-16}$ ***
	Temperature	-0.012 ± 0.002		-6.398	1.1×10^{-9} ***
	Age	-0.015 ± 0.017		-0.929	0.35 NS
	F-statistics: $F_{3, 195}$*: 90.9,* $p < 2.2 \times 10^{-16}$				

* GEP parameter, ** intercept (a) and estimates (b and c), *** $1.96 \times SE = CI_{95}$

Table 3.3 Summary of multiple regression relationships of GEP parameters against temperature (T°C), M_{dry} (mg) and age group (3 and 6 day-old female *An. gambiae*).

Response variable*	Explanatory variables**	Estimates ± SE***	r^2	*t*-value	*p-values*
Log_{10} $s\dot{V}CO_2$	Intercept	-0.187 ± 0.044		-4.237	3.4×10^{-5} ***
	Log_{10} M_{dry}	0.867 ± 0.055	0.79	15.933	$< 2\times10^{-16}$ ***
	Temperature	0.033 ± 0.001		22.190	$< 2\times10^{-16}$ ***
	Age	-0.087 ± 0.013		-6.867	8×10^{-11} ***
	F-statistics: $F_{3, 201}$*: 254.3,* $p < 2.2 \times 10^{-16}$				
Log_{10} $i\dot{V}CO_2$	Intercept	-0.635 ± 0.090		-7.093	2.3×10^{-11} ***
	Log_{10} M_{dry}	0.774 ± 0.114	0.53	6.799	1.2×10^{-10} ***
	Temperature	0.039 ± 0.003		13.071	$< 2\times10^{-16}$ ***
	Age	-0.099 ± 0.026		-3.816	1.8×10^{-4} ***
	F-statistics: $F_{3, 195}$*: 74.33,* $p < 2.2 \times 10^{-16}$				
Log_{10} $b\dot{V}CO_2$	Intercept	-0.386 ± 0.056		-6.935	$< 2\times10^{-16}$ ***
	Log_{10} M_{dry}	1.121 ± 0.071	0.71	15.845	$< 2\times10^{-16}$ ***
	Temperature	0.029 ± 0.002		15.360	$< 2\times10^{-16}$ ***
	Age	-0.073 ± 0.016		-4.563	9.0×10^{-6} ***
	F-statistics: $F_{3, 195}$*: 163.4,* $p < 2.2 \times 10^{-16}$				
Log_{10} A	Intercept	0.417 ± 0.072		5.817	2.4×10^{-8} ***
	Log_{10} M_{dry}	1.264 ± 0.091	0.54	13.864	$< 2\times10^{-16}$ ***
	Temperature	0.016 ± 0.002		6.469	7.7×10^{-10} ***
	Age	-0.017 ± 0.021		-0.843	0.40 NS
	F-statistics: $F_{3, 195}$*: 78.9,* $p < 2.2 \times 10^{-16}$				
Log_{10} F	Intercept	0.798 ± 0.019		41.526	$< 2\times10^{-16}$ ***
	Log_{10} M_{dry}	-0.024 ± 0.024	0.95	-1.015	0.31 NS
	Temperature	0.041 ± 0.001		63.796	$< 2\times10^{-16}$ ***
	Age	-0.037 ± 0.005		-6.75	1.5×10^{-10} ***
	F-statistics: $F_{3, 201}$*: 1376,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_{int}	Intercept	2.184 ± 0.042		51.108	$< 2\times10^{-16}$ ***
	Log_{10} M_{dry}	-0.157 ± 0.054	0.87	-2.901	4.1×10^{-3} **
	Temperature	-0.052 ± 0.001		-36.129	$< 2\times10^{-16}$ ***
	Age	0.075 ± 0.012		6.112	5.3×10^{-9} ***
	F-statistics: $F_{3, 195}$*: 447.8,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_b	Intercept	1.430 ± 0.056		25.372	$< 2\times10^{-16}$ ***
	Log_{10} M_{dry}	0.367 ± 0.072	0.47	5.134	6.8×10^{-7} ***
	Temperature	-0.023 ± 0.002		-12.152	$< 2\times10^{-16}$ ***
	Age	-0.008 ± 0.016		-0.548	0.58 NS
	F-statistics: $F_{3, 195}$*: 58.53,* $p < 2.2 \times 10^{-16}$				
Log_{10} V_b	Intercept	1.257 ± 0.057		22.099	$< 2\times10^{-16}$ ***
	Log_{10} M_{dry}	1.150 ± 0.072	0.60	15.897	$< 2\times10^{-16}$ ***
	Temperature	-0.012 ± 0.002		-6.487	7.2×10^{-10} ***
	Age	-0.035 ± 0.016		-2.135	0.06 NS
	F-statistics: $F_{3, 195}$*: 98.5,* $p < 2.2 \times 10^{-16}$				

* GEP parameter, ** intercept (a) and estimates (b and c), *** $1.96 \times SE = CI_{95}$

Table 3.4 Summary of multiple regression relationships of GEP parameters against temperature (T°C), WL (mm) and age group (3 and 6 day-old female *An. gambiae*).

Response variable*	Explanatory variables**	Estimates ± SE***	r^2	*t*-value	*p-values*
Log_{10} $s\dot{V}CO_2$	Intercept	-1.741 ± 0.095	0.77	-18.324	$< 2\times10^{-16}$ ***
	Log_{10} WL	2.716 ± 0.185		14.650	$< 2\times10^{-16}$ ***
	Temperature	0.033± 0.002		21.379	$< 2\times10^{-16}$ ***
	Age	-0.083 ± 0.013		-6.269	2.1×10^{-9} ***
	F-statistics: $F_{3, 201}$*: 226.6,* $p < 2.2 \times 10^{-16}$				
Log_{10} $i\dot{V}CO_2$	Intercept	-2.064 ± 0.188	0.53	-10.989	$< 2\times10^{-16}$ ***
	Log_{10} WL	2.522 ± 0.366		6.892	7.4×10^{-11} ***
	Temperature	0.039 ± 0.003		13.170	$< 2\times10^{-16}$ ***
	Age	-0.098 ± 0.026		-3.806	1.8×10^{-4} ***
	F-statistics: $F_{3, 195}$*: 75.06,* $p < 2.2 \times 10^{-16}$				
Log_{10} $b\dot{V}CO_2$	Intercept	-2.299 ± 0.128	0.66	-18.092	$< 2\times10^{-16}$ ***
	Log_{10} WL	3.314 ± 0.250		15.318	$< 2\times10^{-16}$ ***
	Temperature	0.029 ± 0.002		15.007	$< 2\times10^{-16}$ ***
	Age	-0.067 ± 0.018		-3.357	9.5×10^{-4} ***
	F-statistics: $F_{3, 195}$*: 125,* $p < 2.2 \times 10^{-16}$				
Log_{10} A	Intercept	-1.858 ± 0.153	0.52	-12.075	$< 2\times10^{-16}$ ***
	Log_{10} WL	3.992 ± 0.299		13.319	$< 2\times10^{-16}$ ***
	Temperature	0.016 ± 0.002		6.468	7.8×10^{-10} ***
	Age	-0.015 ± 0.021		-0.695	0.49 NS
	F-statistics: $F_{3, 195}$*: 73.4,* $p < 2.2 \times 10^{-16}$				
Log_{10} F	Intercept	0.887 ± 0.039	0.95	22.631	$< 2\times10^{-16}$ ***
	Log_{10} WL	-0.174 ± 0.076		-2.282	2.3×10^{-2} *
	Temperature	0.041 ± 0.001		64.429	$< 2\times10^{-16}$ ***
	Age	-0.036 ± 0.005		-6.603	3.5×10^{-10} ***
	F-statistics: $F_{3, 201}$*: 1406,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_{int}	Intercept	2.419 ± 0.090	0.87	26.673	$< 2\times10^{-16}$ ***
	Log_{10} WL	-0.392 ± 0.176		-2.227	2.7×10^{-2} *
	Temperature	-0.052 ± 0.001		-35.826	$< 2\times10^{-16}$ ***
	Age	0.073 ± 0.012		5.921	1.4×10^{-8} ***
	F-statistics: $F_{3, 195}$*: 439.1,* $p < 2.2 \times 10^{-16}$				
Log_{10} P_b	Intercept	0.752 ± 0.118	0.47	6.355	1.4×10^{-9} ***
	Log_{10} WL	1.197 ± 0.230		5.199	5×10^{-7} ***
	Temperature	-0.023 ± 0.002		-12.13	$< 2\times10^{-16}$ ***
	Age	-0.008 ± 0.016		-0.522	0.60 NS
	F-statistics: $F_{3, 195}$*: 58.9,* $p < 2.2 \times 10^{-16}$				
Log_{10} V_b	Intercept	-0.754 ± 0.127	0.55	-5.927	1.4×10^{-8} ***
	Log_{10} WL	3.504 ± 0.248		14.127	$< 2\times10^{-16}$ ***
	Temperature	-0.012 ± 0.002		-5.948	1.2×10^{-8} ***
	Age	-0.030 ± 0.017		-1.72	0.09 NS
	F-statistics: $F_{3, 195}$*: 79.09,* $p < 2.2 \times 10^{-16}$				

* GEP parameter, ** intercept (a) and estimates (b and c), *** $1.96 \times SE = CI_{95}$

3.3.3 Levels of CO_2 emission rates

Three levels of CO_2 production were distinguishable in the GEP employed by *An. gambiae*: MR at rest or the mean CO_2 production rate at rest ($s\dot{V}CO_2$), the inter-burst CO_2 production rate ($i\dot{V}CO_2$) and the maximal production rate ($max\dot{V}CO_2$) from which burst amplitude A could be determined ($A = max\dot{V}CO_2 - i\dot{V}CO_2$). The relation between $s\dot{V}CO_2$, temperature, body size and age groups was already established in Chapter 2 (see 2.3.5, Fig. 2.8 and equations 1 to 3). Similarly, inter-burst CO_2 production rate ($i\dot{V}CO_2$) also strongly depended on temperature and body mass, and differed between age groups (Fig. 3.10).

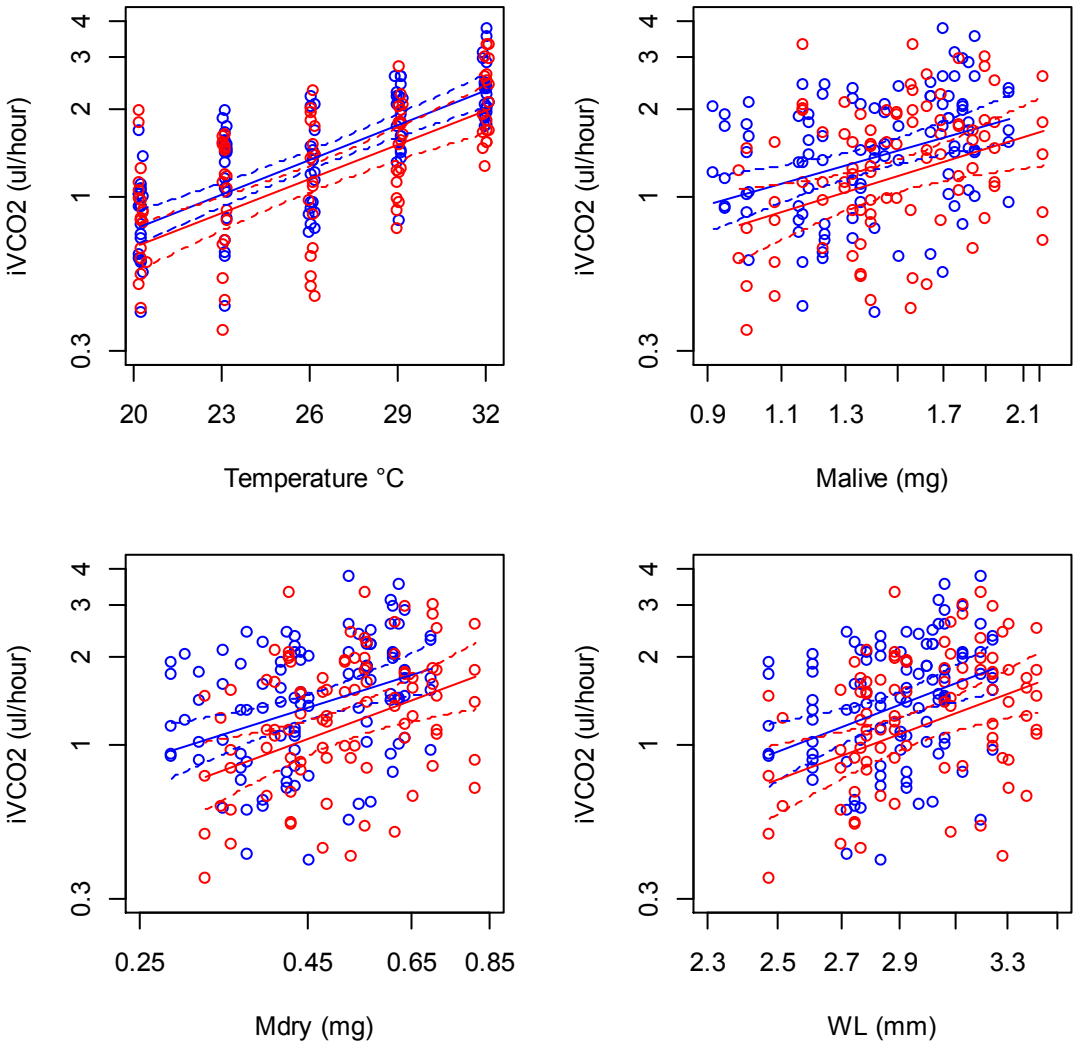


Figure 3.10 Relationships of $i\dot{V}CO_2$ with temperature and size (M_{alive} or M_{dry} or Wing length) in female *An. gambiae*. Solid lines represent regressions for each age (blue: 3 days and red: 6 days) and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4.

The body size estimates were slightly lower whereas the temperature estimates were slightly higher than observed for $s\dot{V}CO_2$. The temperature estimate was 0.039 (CI_{95}: 0.033-0.036) corresponding to an apparent Q_{10} of 2.47 (CI_{95}: 2.13-2.81). The body size estimates were 0.86 (CI_{95}: 0.59-1.12) for M_{alive}, 0.77 (CI_{95}: 0.55-0.99) for M_{dry} and 2.52 (CI_{95}: 1.80-3.24) for WL. Regression analysis revealed a lower r^2 (52 to 53 %) indicating that $i\dot{V}CO_2$ can be predicted with reasonable but lower accuracy than $s\dot{V}CO_2$. This lower accuracy is also reflected in the above indicated CI_{95}. According to Tables 3.2 to 3.4, $i\dot{V}CO_2$ can be predicted with the following equations (for 6 day-old female *An. gambiae* the value in brackets needs to be added to the intercept):

$$\log_{10} i\dot{V}CO_2 = -1.021\ (-0.084) + 0.862 \times \log_{10} M_{alive} + 0.039 \times T \quad (4)$$

$$\log_{10} i\dot{V}CO_2 = -0.635\ (-0.099) + 0.774 \times \log_{10} M_{dry} + 0.039 \times T \quad (5)$$

$$\log_{10} i\dot{V}CO_2 = -2.064\ (-0.098) + 2.522 \times \log_{10} WL + 0.039 \times T \quad (6)$$

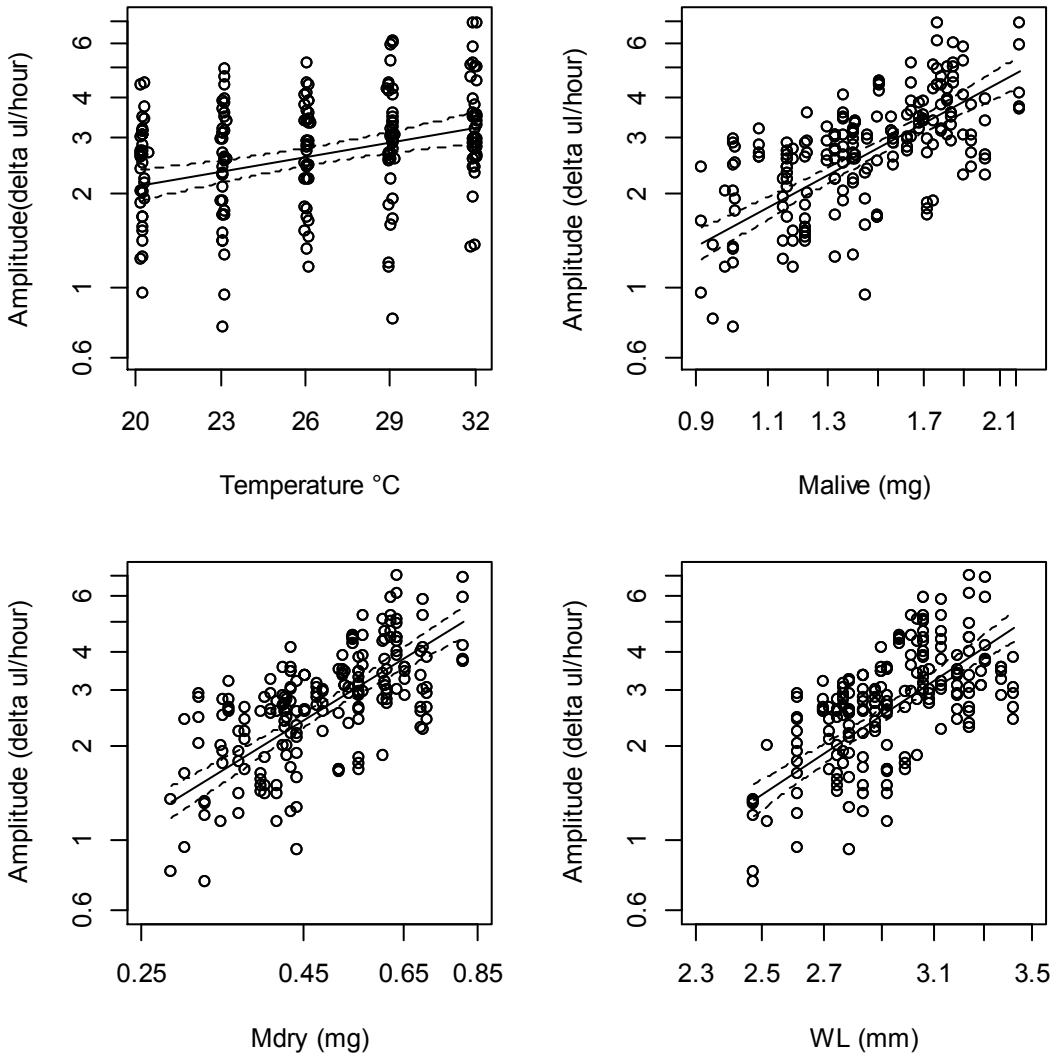


Figure 3.11 Relationships of burst amplitude (A) with temperature and body size (M_{alive} or M_{dry} or Wing length) in female *An. gambiae* (pooled data for the two age groups). Solid lines represent regressions and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4. Since

Burst amplitude A increased slightly with temperature but strongly with body size (Fig. 3.11). Multiple regression analysis revealed that age group did not influence burst amplitude (Tables 3.2 to 3.4, P=0.78, 0.40 and 0.49). The temperature estimate was 0.016 (CI_{95}: 0.033-0.036) corresponding to an apparent Q_{10} of 1.43 (CI_{95}: 2.28-2.61). Body size estimates were 1.42 (CI_{95}: 1.19-1.63) for M_{alive}, 1.26 (CI_{95}: 1.08-1.44) for M_{dry} and 3.99 (CI_{95}: 3.40-4.58) for WL. With reasonable accuracy (r^2 = 51-54 %) burst amplitude can be predicted using the following equations:

$$\log_{10} A = -0.215 + 1.416 \times \log_{10} M_{alive} + 0.016 \times T \quad (7)$$

$$\log_{10} A = -0.417 + 1.264 \times \log_{10} M_{dry} + 0.016 \times T \quad (8)$$

$$\log_{10} A = -1.858 + 3.992 \times \log_{10} WL + 0.016 \times T \quad (9)$$

3.3.4 Temporal characterization of the gas exchange pattern

The temporal breathing pattern of female *An. gambiae* was characterized by a periodic repetition of two events namely, a CO_2 expiration period (burst or spiracular opening during a period P_b) followed by a lower and constant CO_2 production rate period (inter-burst or spiracular closure during period P_{int}). This oscillating pattern occurred at a regular frequency F or $1/(P_{int}+P_b)$.

Burst frequency (F) was highly correlated with temperature and differed between age groups (Fig. 3.12, Tables 3.2 to 3.4). The temperature estimate was 0.041 (CI_{95}: 0.040-0.042) corresponding to an apparent Q_{10} of 2.57 (CI_{95}: 2.52-2.67). F varied independently of living and dry body mass (P = 0.41 and 0.31, respectively; Tables 3.2 and 3.4) but, with marginal significance, decreased slightly with increasing wing length (estimate: -0.175, CI_{95}: -0.33-0.02, P = 0.02, Table 3.4). Six day-old mosquitoes expired in individual bursts at a lower frequency than three day-old ones (Fig. 3.12 and Tables 3.2 to 3.4).

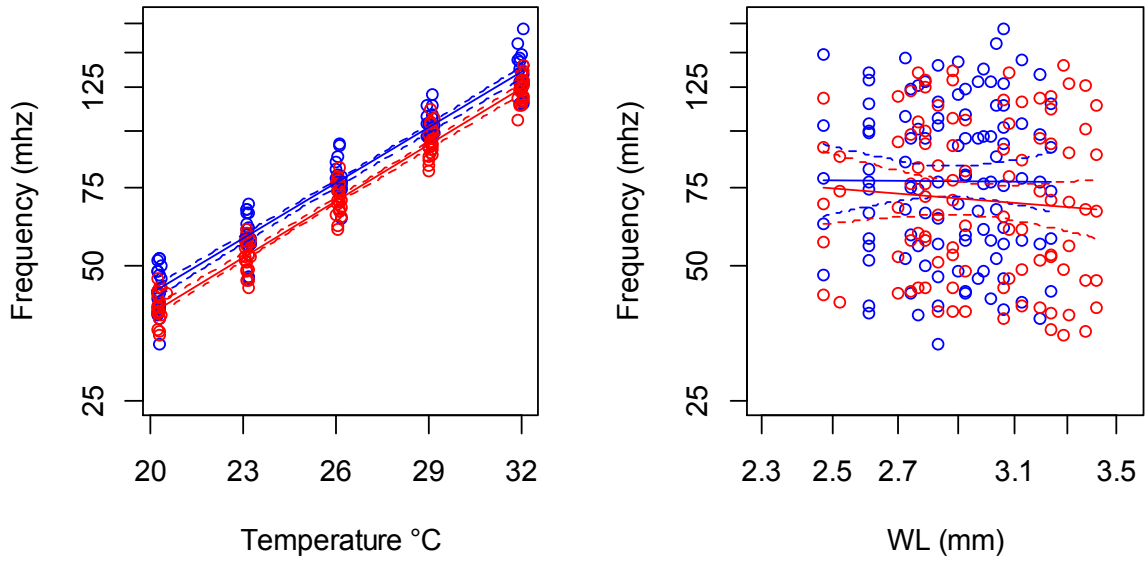


Figure 3.12 Relationships of burst frequency (F) with temperature and wing length in female *An. gambiae*. Solid lines represent regressions for each age (blue: 3 days and red: 6 days) and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4.

Burst frequency can be predicted with remarkable accuracy (r^2 = 95%) using the following equation (for 6 day-old *An. gambiae* the value in brackets needs to be added to the intercept):

$$\log_{10} F = 0.807(-0.037) + 0.041 \times T \qquad (10)$$

and when wing length is available:

$$\log_{10} F = 0.887(-0.036) - 0.175 \times \log_{10} WL + 0.041 \times T \qquad (11)$$

Compared with explaining burst frequency variation on the basis of temperature and age group alone, adding wing length increased the percentage of explained burst frequency variation (r^2) by only 0.1 % (from 95.3 to 95.4%). In addition, the scaling exponent of wing length, although significant, was very low (-0.175). Therefore, adding wing length adds little to the predictive accuracy of F and equation 10 can be considered sufficient for proper prediction.

Body size scaling and thermal sensitivity of the inter-burst period (P_{int}) and burst period (P_b) are shown in Figs. 3.13 and 3.14. As expected, P_{int} and P_b were negatively correlated with temperature. The respective temperature estimates were -0.052 and -0.023 (CI_{95}: -0.055-0.049 and 0.54-0.64) corresponding to apparent Q_{10} of 0.30 and 0.59 (CI_{95}: 0.28-0.32 and 0.54-0.64). Contrasting with F, they were related to body size (M_{alive}, M_{dry} or WL). This is a somewhat unexpected outcome if we take into account that burst frequency did not depend on body size and that F = 1 / (P_{int}+P_b). However, (P_{int} + Pb) can remain independent of body size when the dependencies of both periods compensate one another. This seems to be the case since P_{int} slightly decreased whereas P_b slightly increased with increasing body size (Table 3.2 to 3.4). The respective estimates for M_{alive}, M_{dry} and WL were -0.194 (CI_{95}: -0.32 to -0.071), -0.157 (CI_{95}: -0.26 to -0.050) and -0.392 (CI_{95}: -0.74 to -0.045) for P_{int} and 0.428 (CI_{95}: 0.26 to 0.59), 0.368 (CI_{95}: 0.22 to 0.51) and 1.197 (CI_{95}: 0.74 to 1.65) for P_b.

P_{int} differed between age groups (Tables 3.2 to 3.4) whereas P_b did not (P = 0.87, 0.58 and 0.60, Tables 3.2 to 3.4). The inter-burst period P_{int} lasted for longer in 6-day-old mosquitoes. On the basis of multiple regression analyses, P_{int} and P_b can be predicted using the following equations (for 6-day-old female *An. gambiae* the value in brackets needs to be added to the intercept in equ. 12-14):

$$\log_{10} P_{int} = 2.266\ (+0.073) - 0.194 \times \log_{10} M_{alive} - 0.052 \times T \qquad (12)$$

$$\log_{10} P_{int} = 2.184\ (+0.075) - 0.157 \times \log_{10} M_{dry} - 0.052 \times T \qquad (13)$$

$$\log_{10} P_{int} = 2.419\ (+0.073) - 0.392 \times \log_{10} WL - 0.052 \times T \quad (14)$$

$$\log_{10} P_b = 1.243\ + 0.427 \times \log_{10} M_{alive} - 0.023 \times T \quad (15)$$

$$\log_{10} P_b = 1.430\ + 0.367\ \times \log_{10} M_{dry} - 0.023 \times T \quad (16)$$

$$\log_{10} P_b = 0.752\ + 1.197 \times \log_{10} WL - 0.023 \times T \quad (17)$$

The explanatory power of the equations to predict P_{int} with a r^2 of 87% is remarkable whereas P_b can be predicted with less accuracy on the basis of body size and temperature (r^2 = 47%).

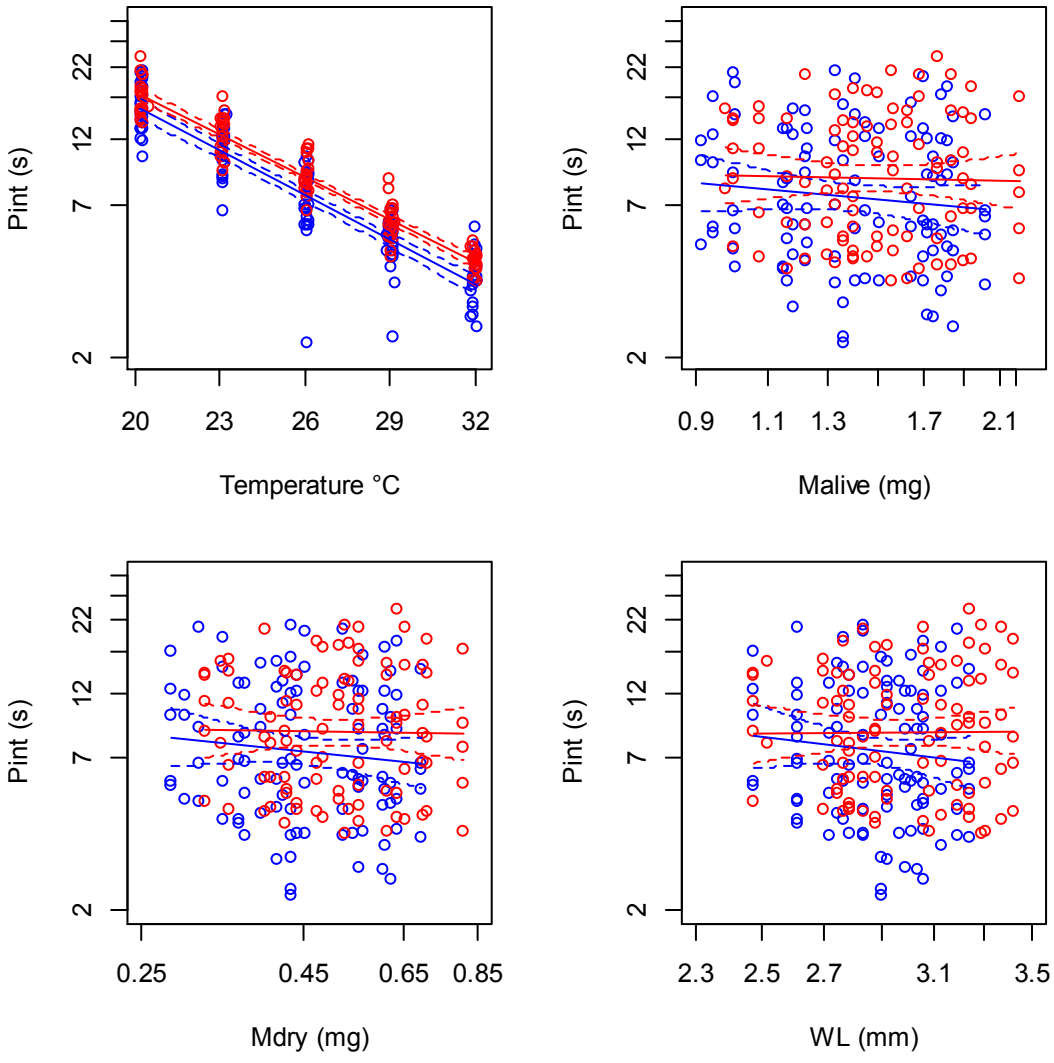


Figure 3.13 Relationships of inter-burst period (P_{int}) with temperature and size (M_{alive} or M_{dry} or WL) in female *An. gambiae*. Solid lines represent regressions for each age (blue: 3 days and red: 6 days) and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4.

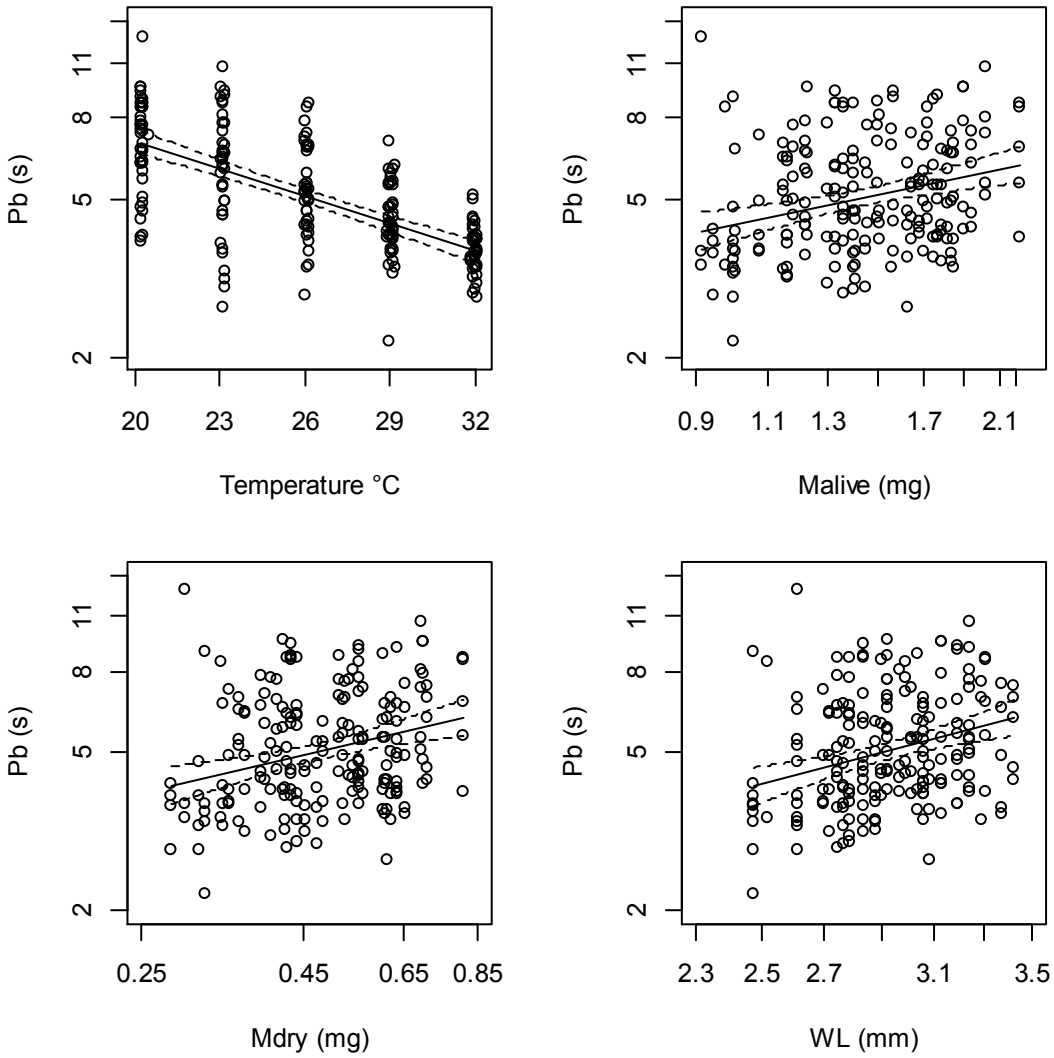


Figure 3.14 Relationships of burst period (P_b) with temperature and body size (M_{alive} or M_{dry} or WL) in female *An. gambiae* (pooled data for the two age groups). Solid lines represent regressions and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4.

3.3.5 Volume of individual expired CO_2 bursts

Knowing $s\dot{V}CO_2$, $i\dot{V}CO_2$ and F, burst volume (V_b) could be determined by dividing $b\dot{V}CO_2$, ($s\dot{V}CO_2$-$i\dot{V}CO_2$) by F. V_b varied in the nanoliter range (Table 3.1) and temperature and mosquito size were both found to significantly affect burst volume (Tables 3.2 to 3.4). Figure 3.15 also shows that the variation in burst volume is predominantly affected by body size of individual female *An. gambiae*. V_b decreases slightly with increasing temperature according to an apparent Q_{10} of 0.76 (95% CI: 0.68-0.82) and increases strongly with body size. Using

the estimates of multiple regression analyses, V_b can be predicted with reasonable accuracy (r^2 = 55 to 60%) using the following equations:

$$\log_{10} V_b = 0.678 + 1.315 \times \log_{10} M_{alive} - 0.012 \times T \qquad (18)$$

$$\log_{10} V_b = 1.257 + 1.150 \times \log_{10} M_{dry} - 0.012 \times T \qquad (19)$$

$$\log_{10} V_b = -0.754 + 3.504 \times \log_{10} WL - 0.012 \times T \qquad (20)$$

Both age groups expired single bursts of similar volume (P = 0.35, 0.06 or 0.09 depending on the body size parameter employed in the regression; Tables 3.2 to 3.4). Since it can be expected that V_b strongly depends on amplitude (A) and burst period (P_b), this outcome is consistent with the finding that A and P_b do not differ between age groups (see above). If both A and P_b can alone explain V_b, then V_b should be related to $A \times P_b$ (both of these variables already include body size and temperature variations (see equations 7 to 9 and 15 to 17). This assumption is close to reality considering that the linear regression between V_b and $A \times P_b$ results in a r^2 value of 84% with a slope of 0.63 (Fig. 3.16).

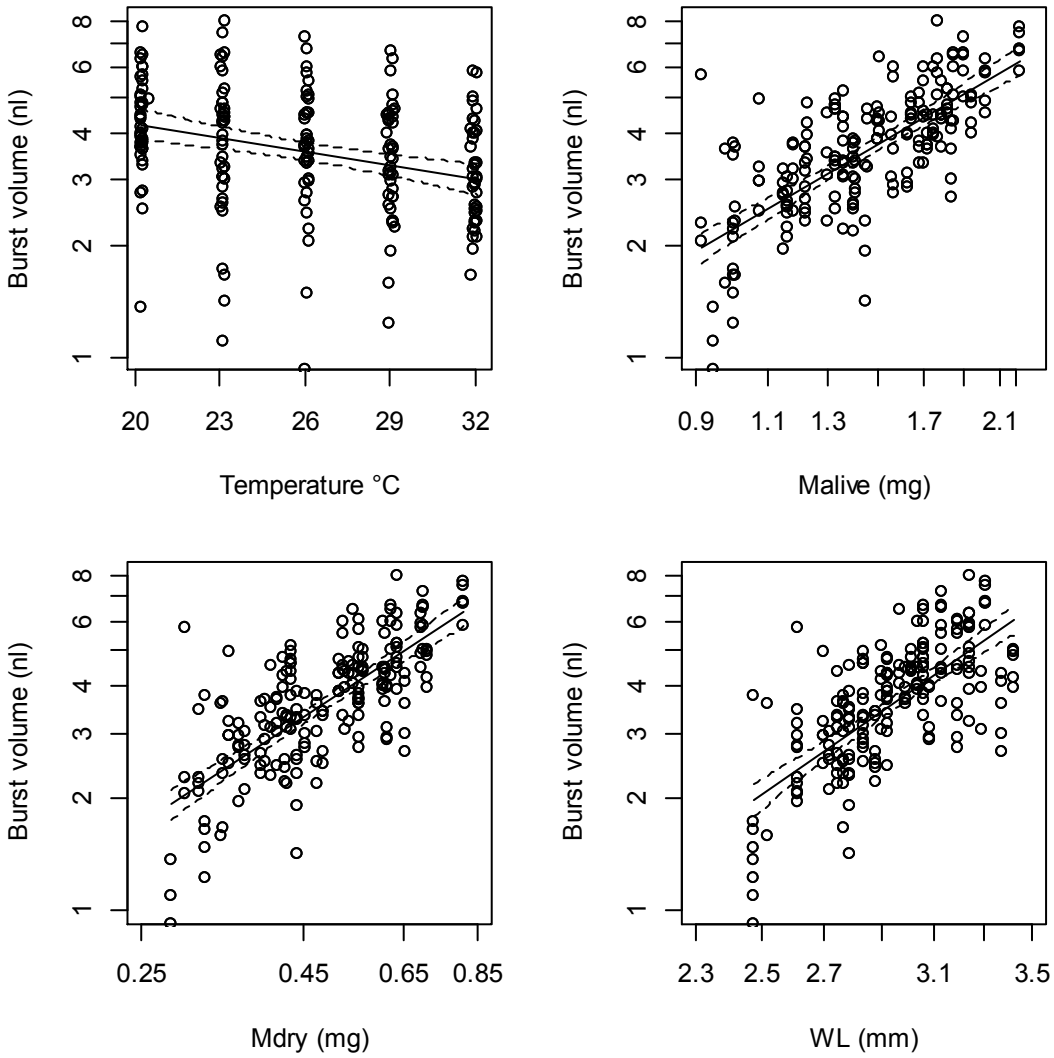


Figure 3.15 Relationships of burst volume (V_b) with temperature and size (M_{alive} or M_{dry} or WL) in female *An. gambiae* (pooled data for the two age groups). Solid lines represent regressions and dashed lines their respective CI_{95} limits. All axes are in logarithmic scale. Regression lines are drawn on the basis of the statistics in Tables 3.2 to 3.4.

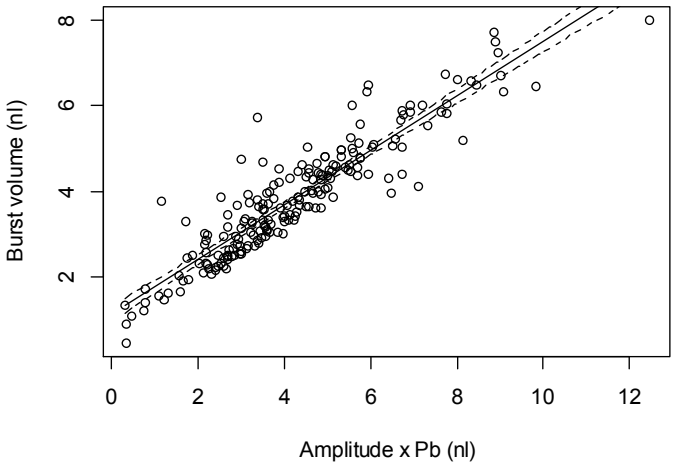


Figure 3.16 Relationship between burst volume (V_b) with amplitude (A) times burst period (P_b) in female *An. gambiae*. (pooled data for the two age groups). The solid line represents the regression $V_b = 1.16 + 0.63 \times (P_b \times A)$, r^2 = 84% and dashed lines the CI_{95} limits.

3.4 Discussion

3.4.1 Precision of the flow-through respirometry system

For smaller mosquitoes (weighting around 1-2 mg) such as *An. gambiae*, *An. arabiensis* (Gray and Bradley, 2005) and *Culex tarsalis* (Gray and Bradley, 2003) the GEP has been measured with FTR using 50-100 ml/min flow rates and have been described as cyclic. However, as nicely shown in Gray and Bradley (2006a), when the flow rate or the temporal resolution of the FTR system is insufficient the GEP may appear only cyclic when in fact it is both cyclic and discontinuous. In the latter study, the GEP of the winter mosquito *Culiseta inornata* (a larger mosquito species of mass ~ 7-8 mg) was investigated using various flow rates and at different temperatures. A higher FTR flow rate and small animal chamber serve to reduce the residence time of the airflow in the gas analyser and the mosquito chamber time constant permitting a higher temporal resolution and avoids the blurring effect of the inter-burst periods that approach the value of the baseline. In the FTR system used here the flow rate was 250ml/min, the animal chamber volume was 0.5 ml and the volume of the detection chamber of the LI-7000 was 11.86 ml. As described by Bartholomew et al. (1981), variation in the partial pressure of gases in the mosquito chamber can be described by the following equation:

$$(P_{CO2})_t = (P_{CO2})_0 \times e^{-F/V(t)}$$

where $(P_{CO2})_0$ and $(P_{CO2})_t$ represent, respectively, the instantaneous partial pressure at time 0 and partial pressure exiting the mosquito chamber at time t. F and V represent, respectively, the flow rate through and the volume of the mosquito chamber. A useful value of this equation is the time constant which is represented by V/F (in this case 0.5/250 = 0.002 min or 0.12 s). Assuming that gas mixing is instantaneous, the time required to wash out 99.3% of a single CO_2 burst in the chamber corresponds to –ln[(100-99.3)/100] or 5 times the time constant (5 × 0.12 = 0.6 s). Here the residence time in the detection chamber of the infrared gas analyser is much more relevant. The LI-7000 has a long narrow tube as a detection chamber with a volume of 11.86 ml. In this detection chamber the flow is laminar and the residence time is 2.8 s (=11.86/250) (for further explanations see Bartholomew et al., 1981 and Gray and Bradley, 2006a). In other words, the temporal resolution of the FTR system used here is in the range of 3 seconds. At the higher temperature of 32°C, very few measured periods (P_b or P_{int}) were below or around 3 seconds in duration, meaning that the flow rate of 250 ml/min was appropriate. A higher flow rate would alter the precision of CO_2 measurement by the gas analyser which was operating just above the limit when one considers that the lowest measured emission rates of CO_2 approached the baseline fluctuation range (the baseline had a mean of 0.005 µl hour^{-1}, CI_{95} of -0.38 to 0.39 µl hour^{-1}). It can therefore be concluded that the observed pattern is close to reality although the few extreme events (short P_b and P_{int} or low $i\dot{V}CO_2$) need to be interpreted with caution.

3.4.2 Gas exchange pattern of female *An. gambiae*

At rest, the GEP of female *An. gambiae* was characterized by well controlled (by the nervous system) and regular frequency of CO_2 bursts accompanied by clearly distinguishable inter-burst periods. The CO_2 burst event probably resulted from coordinated and synchronized spiracle opening. This regulation could be the by-product of thoracic compression or head movements, but this is rather unlikely considering that spiracle opening has been observed not to coincide with such movements in mosquitoes (Krafsur, 1971 and own observations). At lower temperatures, few traces showed $i\dot{V}CO_2$ levels almost undistinguishable from zero. Mosquitos in general but smaller ones in particular, have a rather high surface to volume ratio (long abdomen, legs, proboscis and wings). Therefore, it cannot be totally excluded that this low emission rate, even if close to zero, hides some CO_2 diffusion across the cuticle. That body size estimates for $i\dot{V}CO_2$ are slightly lower than those for $s\dot{V}CO_2$ is very supportive for the presence of diffusion across the cuticle. Nevertheless, it can be concluded that *An. gambiae* has the ability to respire both cyclically and discontinuously. Insects show a

diversity of GEPs ranging from continuous to barely cyclical, strongly cyclical and discontinuous gas-exchange cycles (Chown and Nicholson 2004). Strongly cyclical GEPs are not always easily distinguished from true DGC. DGC is distinguished from cyclical GEP by inter-burst periods composed of a C-phase where CO_2 expiration is negligible followed by an F-phase in which the spiracle flutters before it finally reopens (Lighton, 1996; Marais et al., 2005; Chown, 2011). If the GEP of active *An. gambiae* can be qualified as continuous (Fig. 2.3), it can be admitted that *An. gambiae* employs all 3 GEP types described so far in the literature. Among the 8 individuals that displayed DGC, both age groups as well as entire body size ranges (M_{alive} from 0.91 mg to 2.22 mg) were represented. However, these recordings were made at the lower experimental temperature (mostly at 20°C and eventually also at 23°C) and when the mosquito, compared to others, presented a particularly low $s\dot{V}CO_2$ (ranging from 0.64 to 1.18 µl $hour^{-1}$). It can be concluded that just as in bigger mosquito species such as *Culiseta inornata* as shown in Gray and Bradley (2006b), a smaller mosquito species like *An. gambiae* also displays this highly regulated form of breathing. Earlier findings have suggested that insects display a continuum of all 3 patterns transitioning from DGC that operates when metabolic demand is lowest (at rest and at lower temperatures) to cyclical as soon as temperature increases and to continuous as soon as the insect becomes active (Contreras and Bradley, 2009, 2010). This earlier suggestion regarding GEP seems also to apply to the main malaria vector *An. gambiae* and can probably be concluded as being common to all mosquitoes when we consider earlier work on *Aedes aegypti*, *An. gambiae*, *An. arabiensis*, *Culiseta inornata* and *Culex tarsalis* (Krafsur, 1971; Gray and Bradley, 2003, 2005, 2006a and b) and my own single recordings for *An. stephensi* and *An. atroparvus* (see Appendix 8.2.2).

3.4.3 Temperature sensitivity of the gas exchange pattern

$s\dot{V}CO_2$ increases with temperature with a Q_{10} of 2.13 (see Chapter 2, 2.3.5, equ. 1 to 3). $s\dot{V}CO_2$ can here be decomposed in an inter-burst level that increases faster with temperature (Q_{10}=2.47) and a burst volume (V_b) that decreases with temperature (Q_{10}=0.76). Meanwhile burst frequency (F) increases with a Q_{10} of 2.57 and consequently both inter-burst (P_{int}) and burst periods (P_b) decrease with temperature (Q_{10} = 0.30 and 0.59, respectively). Both F and $i\dot{V}CO_2$ increase faster than $s\dot{V}CO_2$ (equ. 10 and 4 to 6) but this effect is compensated by the fact that both P_{int} and V_b decreases with increasing temperature (equ. 12 to 14 and 18 to 20). It is important to note that burst volume can be estimated accurately on the basis of burst amplitude and period (A and P_b, see Fig. 3.16 for rationale) and that the reduction in burst

volume is merely due to a strong decrease in burst period (equ. 15 to 17) that overrules an increasing burst amplitude (equ. 7 to 9) with temperature.

Admitting that the inter-burst period is constituted of both C and F phases it is well possible that the C-phase fades out and that the F-phase becomes more and more important with increasing temperature. Unfortunately, the data did not allow proper distinction between the two phases but it is quite possible since $i\dot{V}CO_2$ had a higher apparent Q_{10} than $s\dot{V}CO_2$ (2.47>2.13). Moreover, Lighton et al. (1993) showed that in a desert ant the C-phase stays stable and accounts for only a small proportion of the inter-burst period (14%) whereas the F-phase becomes exponentially important with increasing temperature. For a C-phase or period of no gas exchange two conditions need to be met: the PO_2 needs to be above the threshold that triggers spiracle opening and endotracheal PCO_2 needs to be below the threshold that triggers spiracle opening (Chown, 2011). In *An. gambiae*, it is possible that these conditions are never met above a certain temperature or a certain metabolic demand and thus explain a fade out of the C-phase. Meanwhile if we consider the temporal resolution (in the range of 3 seconds, see 3.4.1) the FTR system used may have failed in detecting a hypothetical C-phase, in particular if it stays stable and accounts for a small portion of inter-burst period.

That burst volume decreases with increasing temperature has been observed in other insects such as ants, carabid beetles and in pupae of several lepidopterans (Buck and Keister, 1955; Schneiderman and Williams, 1955; Lighton, 1988b; Quinlan and Lighton, 1999; Vogt and Appel, 2000; Duncan and Dickman, 2001). With the present findings, *An. gambiae* can be added to this list. As proposed by Lighton (1988b), higher temperatures may lead to a decline of CO_2 solubility and pH in the haemolymph that reduces its buffering capacity leading to more frequent but smaller volume of CO_2 excretion events. It must, however, be noted that other insects succeed to keep burst volume constant whilst temperature increases (Lighton and Wehner, 1993; Davis et al., 1999; Chappell and Rogowitz, 2000; Shelton and Appel, 2001). These findings suggest that further mechanisms may regulate the burst volume when temperature increases but the one presented above seems most plausible for *An. gambiae*.

As stated in Chapter 2, ectotherm biodiversity is mostly concentrated in the tropics and the non-linear effect of temperature on MR exacerbates small changes in the higher temperature range (Dillon et al., 2010). For this reason, tropical mosquitoes seem to modulate their GEP by augmenting burst frequency much faster than mosquitoes in colder environments. In support of this hypothesis Fig. 3.17 shows an interspecific comparison of CO_2 burst frequency

as related to temperature in mosquitoes using data I collected during this PhD thesis research and data extracted from published sources.

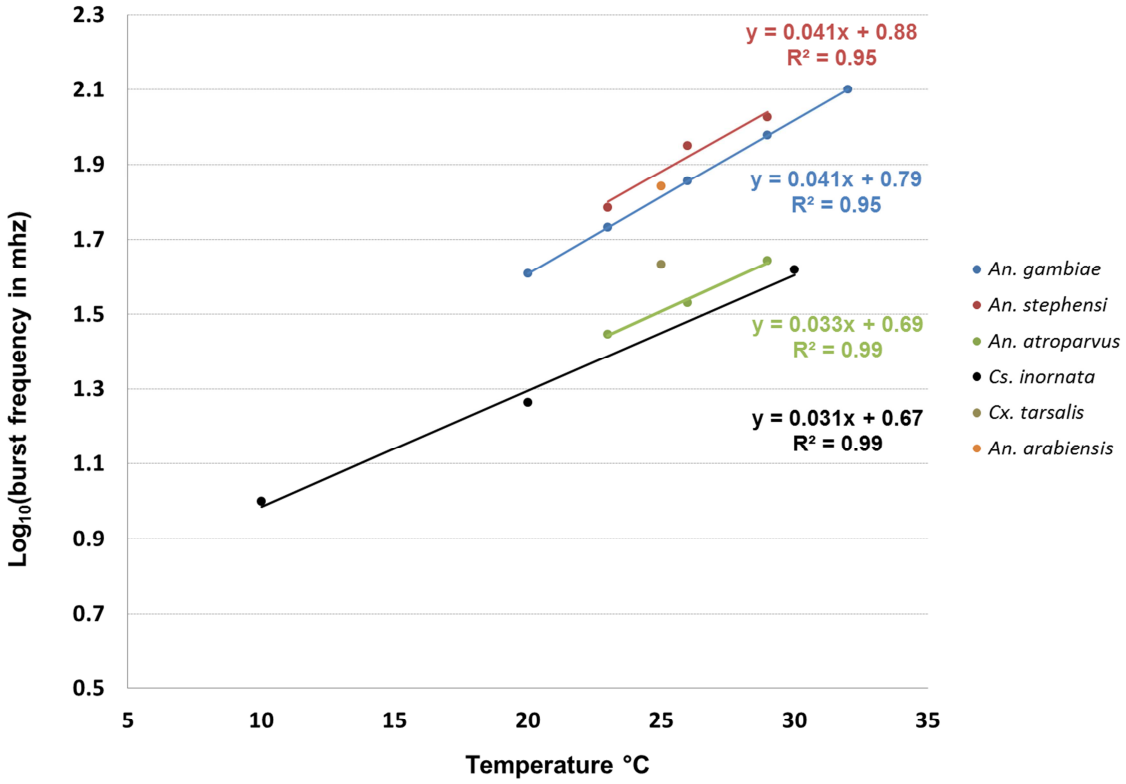


Figure 3.17 Interspecific comparison of thermal sensitivity of CO_2 burst frequency in 6 mosquito species. Tropical mosquitoes such as *An. gambiae*, *An. stephensi* and *An. arabiensis* increase burst frequency much faster (differing slopes corresponding to apparent Q_{10}= 2.57 to 2.04) than species more tolerant to a temperate climate such as *An. atroparvus* and *C. inornata.* Data source: *An. gambiae* (see equ. 10), *An. stephensi* and *An. atroparvus* (Appendix 8.2.2), *Culiseta inornata* data from Gray and Bradley (2006b), *An. arabiensis* data from Gray and Bradley (2005) and *Culex tarsalis* data from Gray and Bradley (2003). Note that Gray and Bradley (2005) saw no difference between *An. gambiae* and *An. arabiensis* in terms of CO_2 burst frequency.

3.4.4 Body-size scaling of gas exchange pattern parameters

In insects, the mass scaling of GEP parameters has been investigated only on a few occasions. Interspecific investigations in ants and beetles tend to show that both V_b and $s\dot{V}CO_2$ have similar mass scaling exponents resulting in independence of F to body mass (Lighton, 1991; Davis et al., 1999; Chappell and Rogowitz, 2000). Mass scaling of the other GEP parameters tend to vary between taxa and the intraspecific scaling exponent of GEP remains poorly investigated (Chown and Nicolson, 2004), in particular for smaller insect such as *An.*

gambiae. In some species, GEP parameters are not influenced by body mass (Bosch, M. et al., 2000). In some ants, both F and V_b increase with body size (Lighton and Berrigan, 1995). It must be remembered that for such a relation to be properly established a sufficient body/size variation range is a prerequisite (restricted body size variations make any statistical inference difficult). The present studied *An. gambiae* population covered a wide range of mosquito sizes (the biggest mosquito was more than 2 times greater than the smallest in terms of weight, see Table 2.1) and this size range seems to be sufficient to safely conclude that all GEP parameters are influenced by body mass with the exception of F. That burst frequency varies independently of body mass has also been shown for other taxa (see above). Clearly, over the observed range of temperatures and body sizes, temperature influenced $s\dot{V}CO_2$ most in *An. gambiae*. In most relations depicted in Tables 3.2 to 3.4, the absolute t-value for temperature estimates exceeds the absolute t-value for body-size estimates. An exception to this rule is burst volume (also $b\dot{V}CO_2$) and its amplitude. The variation observed for these two variables were most strongly correlated with body size. Both of these parameters had a mass exponent that clearly exceeded 1 (equ. 7 to 9 and 18 to 20). This means that both were relatively higher for bigger mosquitoes than for smaller ones. This could be readily explained by a relatively bigger tracheal volume in bigger individuals or a relatively larger tracheal network in bigger *An. gambiae*. Such a scaling effect has also been observed in bigger species of grasshopper that seem to use this strategy to withstand hypoxia in muscles (Whitman, 2008). It is quite impressive that such effects can also be observed by *An. gambiae*, a much smaller insect. Such adaptations are most likely to occur when insects possess particularly demanding organs or encounter demanding situations in terms of metabolism. In small mosquitoes such as *An. gambiae* this may be an advantage in many situations during adult life. For example, their low mass and long appendages render flight particularly demanding in terms of metabolism. It has recently been shown that mosquito fly unlike any other insect. Their wing stroke amplitude is much lower than in other insects (≈ 40°, 2 to 3 times lower than fruit flies, honey bees or hawkmoths) and they flap their wings, relatively to their size, with a rather high frequency around 800 Hz (Bomphrey et al., 2017). This particular way of flying has recently been proposed to be linked to their mating behaviour since males respond to the flight tone of flying female *An. gambiae* (Charlwood and Jones 1980). Here, the idea that flight is particularly demanding in terms of metabolism also finds supports in that wing length was associated with F although body mass was not (equ. 11). Another challenging situation in terms of metabolic demand encountered by female *An. gambiae,* is when it imbibes the blood meal at human body temperature. During a rather fresh night with a temperature of say 20°C,

within a short time, this small ectotherm encounters a thermal stress by ingesting human blood at a temperature of 37°C. By instantaneously filtering blood, it takes a large blood meal relative to its size and blood meal duration is extended (Briegel and Rezzonico, 1985; Briegel, 1990a, b; Horler and Briegel, 1995; Briegel, 2003; Fernandes and Briegel 2005). Added to this let us not forget that *An. gambiae* is both endophilic and endophagic. Both resting and blood feeding occurs under rather hypercapnic conditions.

3.4.5 Effect of age on the gas exchange pattern

Not much is known about the effect of aging on GEP parameters. Older *An. gambiae* had a lower $s\dot{V}CO_2$ than 3-day-old ones (Chapter 2). Modulation of this down regulation seems to be due to 1) a decrease in burst frequency (logically reflected in longer inter-burst periods) since burst volume and its constituting variables did not differ between the two age groups, and 2) to a substantial lowering down of $i\dot{V}CO_2$. This finding supports the fact that burst volume reflects the architecture of the tracheal network volume and there is no reason to think that it changes with age. Clearly older *An. gambiae* seem to exchanges gas less frequently (lower F) and more discontinuously (lower $i\dot{V}CO_2$) in a more controlled manner. It must, however, be said that a hypothetical and negligible CO_2 diffusion across the cuticle (see above) would also evolve with aging. Hardening and pigmentation may render the cuticle less permeable to CO_2. As suggested in Chapter 2, 3 day-old *An. gambiae* are probably not fully mature so it is possible that optimal GEP control by the nervous system is only reached after a defined age. The optimum age for host seeking is around six days (Jones and Gubbins, 1978). Full control of breathing seems to coincide with host seeking behaviour in *An. gambiae* which is not surprising if one considers the additional metabolic demand related to host seeking behaviour. Behaviours such has host sensing, sustained flight towards the host and warm blood uptake on the host are metabolically demanding and probably require optimal control of breathing.

3.4.6 Gas exchange pattern and water loss

To return to the 'hygric hypothesis' (see 2.4.4) and admitting that gas exchange is merely convective during a hypothetical F-phase (hidden in P_{int}) it should be expected that water loss merely occurs during the O-phase (P_b) and that such loss should be related to the CO_2 production rate during the O-phase ($=b\dot{V}CO_2$). However, multiple regression analyses taking into account the effect of age (6-day-old mosquitoes lost significantly more water than 3 day-old mosquitoes) showed no significant relation between these two variables (P=0.21). It is possible that mosquitoes that lost most water were more active and had a much higher MR

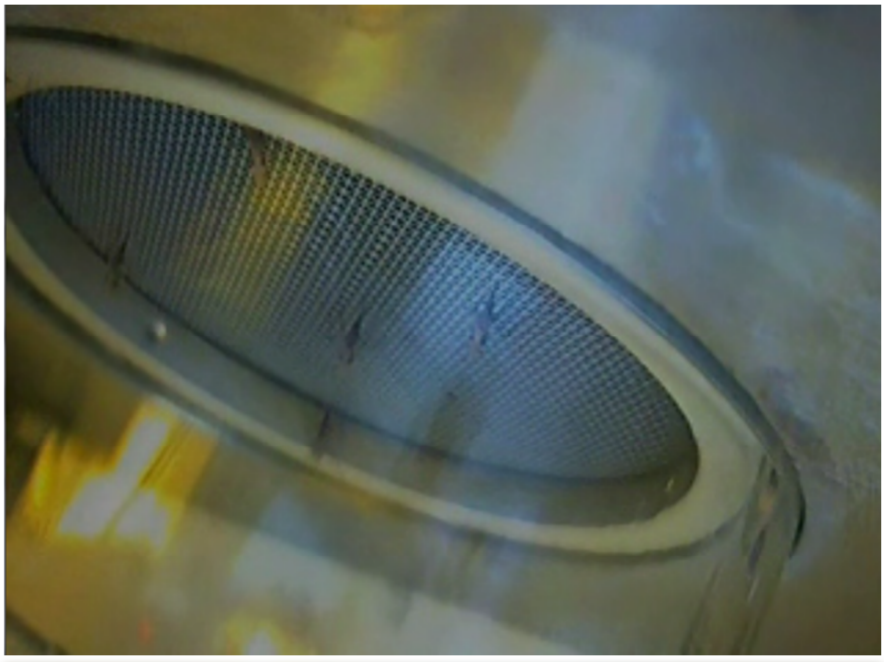
during respirometry. However, that should be translated into less periods at rest (valid record periods) for 6-day-old *An. gambiae*. However, no clear pattern of this kind can be extrapolated from Table 2.5. As already stated in Chapter 2, it really seems unlikely that mosquitoes have evolved discontinuous and cyclical gas exchange in order to prevent water loss. It is however possible that they reduce respiratory water loss in response to water stress, which would rather be a physiological response than an adaptive one.

3.4.7 On the origins of the gas exchange pattern employed by *An. gambiae*

According to the findings presented in this thesis and an earlier comparative study between *An. gambiae* and *An. arabiensis* (Gray and Bradley 2005), the 'hygric hypothesis' of Kestler (1984) does not really hold for *An. gambiae* or will at least remain very difficult to demonstrate. The possibility remains open for the other hypotheses (introduced in 3.1) although the 'strolling arthropod hypothesis' of Harrison et al. (2001) remains difficult to test unless some mosquitoes are more likely to be infected by any sort of abiotic or biotic agent and consequently display less discontinuous and cyclical gas exchange? As for the 'oxidative damage hypothesis' of Hetz and Bradley (2005), measuring the GEP of *An. gambiae* under hyperoxia would be necessary to test it. But let us return to the General Introduction of this thesis: the life cycle of adult mosquitoes and in particular that of *An. gambiae*. When *An. gambiae* emerges it is rather deplete of energy reserves, it uses multiple and concentrated blood meals to cope with this disadvantage, it is endophilic and endophagic and lives in the vicinity of human habitations due to its marked anthropophily (Chapter 1, see 1.4). During periods of activity, i.e. during blood or sugar foraging, it is likely that this mosquito exchanges gas continuously. The cyclical pattern is probably the rule at rest at higher temperatures and may even occur during rest for blood meal digestion or even during a slowly taken and undisturbed blood meal. A hypercapnic environment when resting indoors (endophily) might also be encountered and favour more cyclical and more discontinuous gas exchange (the 'chthonic hypothesis'; see Lighton and Berrigan, 1995). During aestivation, aged adults may also downregulate their MR via nervous input (Huestis et al., 2011; Huestis et al., 2012; Huestis and Lehmann, 2014) to minimize metabolic demand and help survive until the next blood meal (the 'neural hypothesis'; see Chown 2011, Matthews and White 2011). Basically, the panel of behaviours during adult life may dictate this small insect to use a GEP continuum (Marais and Chown, 2003; Contreras and Bradley, 2009 and 2010). Mechanistically, a true C-phase characterizing a genuine DGC may appear as soon as mosquito MR falls below a certain threshold (possibly around 1 µl CO_2/h), at lower

temperatures and during extended periods of rest or aestivation, independent of body size. In this sense the ‘neural hypothesis’ and ‘emergent property hypothesis’ (Chown and Holter, 2000; Förster and Hetz, 2010) is appealing since it, as nicely stated by Steven L. Chown (2011), ‘incorporates both a non-adaptive mechanistic component – interacting control systems – and an adaptive one – energy saving – so reuniting evolutionary and mechanistic explanations’. Although appealing, it cannot be excluded that other adaptive and mechanistic drivers act in concert with the latter. In any case, further research is needed on the role of the nervous system in the control of gas exchange (Matthews and White, 2010; Matthews, 2017).

4 Using equal CO_2 pulses in an olfactometer to uncover host choice odour cues



4.1 Introduction

It is well established that many Afro-tropical mosquito species orient to their hosts by using olfactory cues made up of complex mixtures of chemical compounds. This is particularly the case for night-active mosquitoes such as *An. gambiae* the main African malaria vector (Takken and Knols, 1999). Natural odour blends or synthetic compounds are often qualified as being 'attractive' or 'not attractive' after multiple behavioural tests. In the majority of the cases such conclusions are reached through experimental paradigms where the mosquitoes are confronted to conduits one with and one without the test odour. Mosquitoes ready to seek a host will naturally fly to the test odour source if it is attractive. For example, in a two arm olfactometer when mosquitoes are confronted to no odour versus a human hand most of them will fly into the olfactometer arm with the human hand, allowing the conclusion that a human hand is 'attractive' (Geier and Boeckh, 1999). However, the attractive nature of an odour blend is not necessarily the whole story. Simply because an odour is concluded to be unattractive does not exclude that it is perceived by the test animal, for example it may not be attractive in itself but still could be used by the mosquito as a signal to choose between two hosts. A good example of such a chemical cue for *An. gambiae* is lactic acid. This product is a poor attractant on its own. In contrast to as what is observed for *Aedes aegypti* the synergetic effect of lactic acid on carbon dioxide is not as strong for *An. gambiae* (Geier and Boeckh, 1999, Dekker et al. 2002). Humans carry higher lactic acid levels on their skin than non-human hosts (Smith et al., 1970), and although weak as an attractant itself, lactic acid was demonstrated to be an important odour-related host choice cue at both inter- and intra-specific levels for both *An. gambiae* and *Aedes aegypti* (Steib et al., 2001; Dekker et al., 2002). In a paper entitled 'Do choice tests really test choice?' Martel and Boivin (2011) addressed the importance of the experimental conditions needed to conclude that a stimulus is used by an animal to choose between two resources. Among others it was underlined that the level of perception of the two resource stimuli the test animal is confronted with should ideally be equal or at least maximized. If the animal only perceives one of the resources it will only exploit that one, in which case it may be premature to speak about a true choice. In the context of odour-mediated host seeking behaviour by *An. gambiae*, the resources could be two potent host (blood meals) between which *An. gambiae* may be faced to choose according to their odour signature. Carbon dioxide (CO_2) is probably the simplest infochemical that signals the presence of potential nearby hosts and it is known that mosquitoes respond to the intermittent nature of CO_2 stimulation (Gillies, 1980; Mboera and Takken, 1997). Bearing this

in mind, the aim here was to develop a new experimental paradigm by using an improved dual-choice olfactometer to test whether or not an odour is used by mosquitoes as a true choice cue whilst maximizing the level of perception of both resource cues (CO_2 and test odour). For this latter purpose equal pulses of CO_2 were applied in both arms of the olfactometer. The validity of the experimental paradigm is tested with lactic acid, a known chemostimulus eliciting host preference by *An. gambiae.*

4.2 Methods and Material

4.2.1 Mosquitoes

For details of the *An. gambiae* colony see under 2.2.1 in Chapter 2. Eggs were collected in an oviposition bowl filled with deionized water 2 days after blood meal ingestion. It took 2 days for larval eclosion. The larvae were reared in trays (25 x 32 x 6 cm) filled with 400 ml water. Each tray contained 250-300 larvae and were fed on Tetramin® fish food (Tetra, Blacksburg, VA, U.S.A.) according to the following feeding regime: day 1: 36mg, day 2-5: 72mg and day 6-7: 144mg. Approximately 800 pupae (sex ratio ~ 1:1) were collected and placed in Plexiglas® cages (35 x 35 x 55 cm) for adults to emerge. All pupae transformed to the imago stage by the following day (age = 0) allowing age determination for further behavioural tests in the olfactometer. For olfactometer tests we used non-blood-fed female *An. gambiae* aged 5-8 days during their last 6 h of scotophase, an age and a period during which their behaviour is most likely to be related to host seeking behaviour (Jones and Gubbins, 1978). Adults were provided with 10% glucose and water on cotton wicks *ad libitum*.

4.2.2 The olfactometer

The experimental setup consisted of an olfactometer made of Plexiglas® as described in Geier et al. (1999b). This type of olfactometer has already proven effective on many occasions to test the responses of mosquitoes to chemostimuli (Geier and Boeckh, 1999; Geier et al., 1999b; Bosch, O. J. et al., 2000; Steib et al., 2001; Dekker et al., 2002). In brief, this olfactometer consists of two stainless steel cylinders (11 cm diameter × 15 cm long) lined inside with a 1-mm thick Teflon liner (Angst and Pfister, Zürch, Switzerland) with a 7-cm downwind opening covered by fine polyethylene terephthalate netting (0.8 mm mesh, Sefar, Heiden, Switzerland) connected to the two upwind arms of the olfactometer (7 cm diameter × 17 cm long). These arms lead to an intermediate rectangular chamber (16 × 22 × 7 cm) from which a tube (7 cm diameter × 53 cm long) reaches the mosquito release cage at the downwind end (Fig. 4.1). The airflow source (20 ± 2 cm/s in both upwind arms and 40 ± 2

cm/s in the downwind tube) originated from the institute's pressurized air system. The air was purified through a charcoal filter, humidified (~80% RH) and warmed to ~24 °C, equal in both arms. The olfactometer was surrounded with black curtains to avoid visual cues. Experiments were conducted under 1 kHz low fluorescent light conditions (<1 lux).

4.2.3 Mosquito testing procedure

Through a brief expiration female *An. gambiae* were activated in the rearing cage and lured to the experimenter's hand by placing it over the mosquito netting of the cage entrance. From there 18-23 female *An. gambiae* were drawn up with a mouth aspirator and put in a release cage (7 cm diameter × 11 cm long; Fig. 4.1) that was then plugged onto the downwind tube of the olfactometer where the mosquitoes were left to acclimatize in the airstream for 20 minutes. Immediately after stimulus onset, the rotating door of the release cage was opened and the mosquitoes were exposed to test conditions for 1 minute. The rotating doors of both upwind arms and the release cage were then closed (Fig. 4.1) and the number of mosquitoes contained in each compartment counted. All the experimental manipulations were performed with clean tissue gloves in order to avoid any contamination of the setup.

4.2.4 Odour delivery

To deliver CO_2 in a pulsed manner a gas tank with 1% CO_2 (Carbagas, Switzerland) equipped with a manometer was connected to a solenoïd valve (model V301, Sirai, Italy). The valve permitted delivery of CO_2 intermittently with 2s open, 2s closed corresponding to 15 cycles/min, which is approximately the CO_2 release rhythm of a human at rest. The outlet of the valve was equipped with a gas diffuser and inserted into the air supply upwind the split into the two arms of the olfactometer. The pressure level on the manometer was adjusted on a regular basis to obtain regular fluctuations in the range of 50 ppm in each arm of the olfactometer (Fig. 4.1). In order to monitor and evaluate CO_2 pulses with accuracy, the olfactometer was additionally equipped with a gas analysis system (Fig. 4.2). This system consisted of a pump (Subsampler TR-SS3, Sable System, Las Vegas) connected to an multivalve switcher (RM8 Multiplexer, Sable System) controlled and monitored with a computer (Expedata software, Sable System). The sampled air (flow: 1.4L/min) passed through an infrared CO_2 gas analyser (LI-7000) and humidity analyser (RH 300). The multivalve switcher was programmed to consecutively sample air during 20 s in each of the 3 compartments of the olfactometer (in both upwind arms and in the downwind tube) in a loop-wise manner. A typical recording of CO_2 fluctuations measured in the 3 compartments during an experiment is depicted in Fig. 4.3. In each of the 3 compartments of interest CO_2 pulses

could be evaluated using Expedata software by computing the following parameters: mean period between two fluctuations (P, in seconds), mean amplitude of the CO_2 fluctuations (A, in Δppm), mean level of carbon dioxide (L, in ppm) and the background increase (BI, in Δppm) measured as the difference between the mean level of carbon dioxide (represented by the dashed lines in Fig. 4.3) and the mean level of carbon dioxide measured prior to the experimental trial (represented by the dashed-dotted lines in Fig. 4.3). In addition, to reflect the differences of the measured values between each arm the ratios between the measured amplitude, period and background increase in both arms were also computed (denoted as A ratio, P ratio and BI ratio). Lactic acid was applied in one of to two arms in a randomized manner by bubbling charcoal-filtered air (airflow S1 or S2, Fig. 3.1) through a 250 ml Erlenmeyer holding 10 ml of L-(+)-lactic acid solution (90 % aqueous solution; Fluka, Switzerland) at a flow rate of 40ml/min. The output of the Erlenmeyer roughly equals the highest rates of lactic acid released by a human hand (Smith et al., 1970; Geier et al., 1999a). Lactic acid was chosen as the test stimulus as it has been demonstrated to be a poor attractant on its own but to be used by two anthropophilic mosquitoes species (*Aedes aegypti* and *Anopheles gambiae*) as a host choice cue (Steib et al., 2001; Dekker et al., 2002).

4.2.5 Test series and their objectives

Three series of experimental trials were performed. In a first series no odour was applied in either arm of the olfactometer in order to test that no mosquitoes responded to the olfactometer airflow to which they were acclimatized for 20 min prior to the experimental trial (N=18 cages and 350=mosquitoes). In a second experimental series, mosquitoes were exposed to equal CO_2 pulses in the two arms of the olfactometer (Fig. 4.3) to test if this enhanced and sustained upwind flight and if mosquitoes were distributed equally in each arm of the olfactometer (N=81 cages and n=1631 mosquitoes). The third series of tests was performed by randomly applying lactic acid in one of the two arms in addition to CO_2 pulses (N=10 cages and n=204 mosquitoes). Since lactic acid has been demonstrated to be a host choice signal for *An. gambiae*, the outcome of this experiment should be similar to the previous ones except that the mosquitoes should not be equally distributed in both arms. The experiment applying equal pulses of CO_2 into each arm of the olfactometer was performed on a daily basis as a positive control. Typically, during an experimental day, the responsiveness of mosquitoes from two release cages was tested with the CO_2 pulses presented alone. Added to these two control tests, mosquitoes from 6-10 releases cage could be tested with the desired

test odour added to the airflow in one arm. In total, an experimental day permitted the assessment of the responsiveness of mosquitoes from 8-12 release cages.

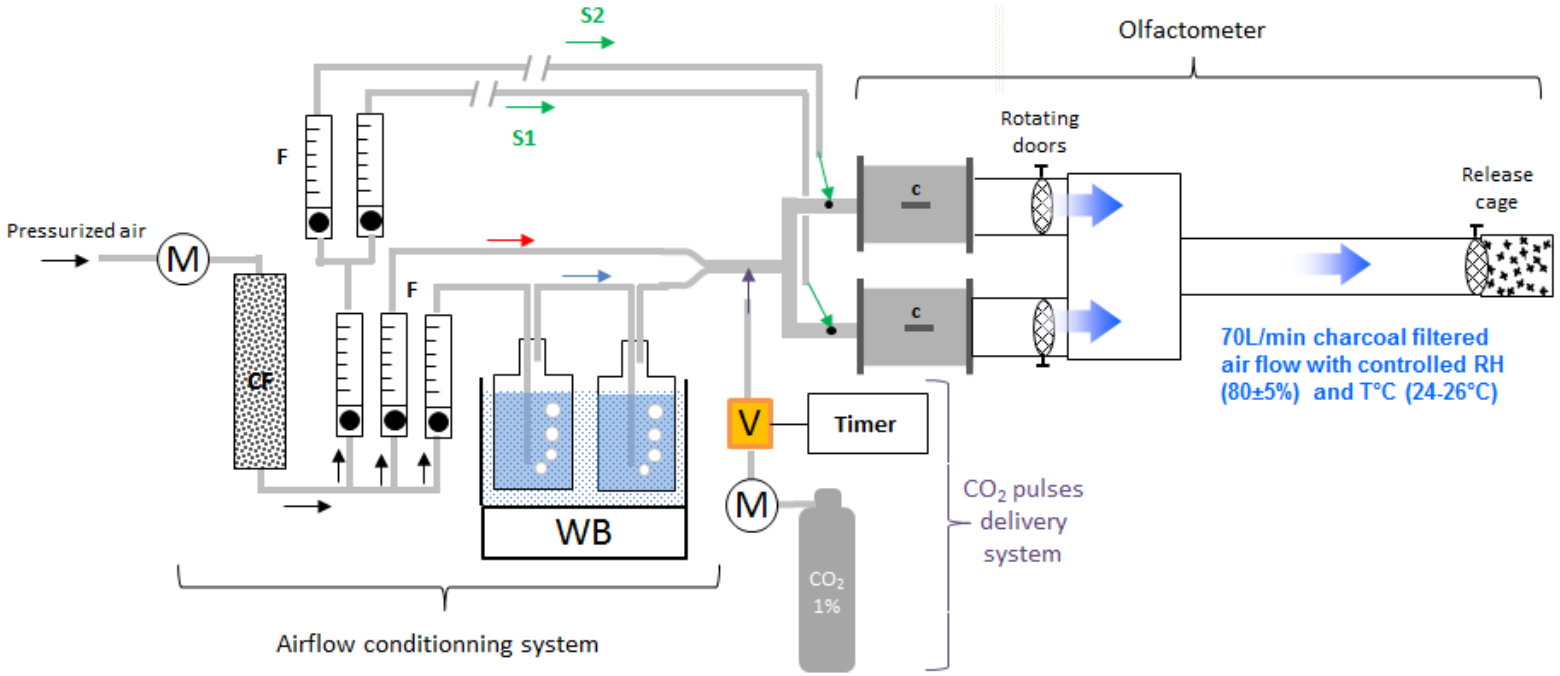


Figure 4.1 Scheme of the two-arm olfactometer. The airflow source originates from the institute's pressurized air system (**black arrows**) and is purified through a charcoal filter (**CF**). Pressures are controlled by manometers (**M**). The humidified and temperature-controlled airflow of the olfactometer was achieved by mixing a water vapour saturated airflow (**blue arrow**, ~ 15 L/min) and a dry airflow (**red arrow**, ~ 55 L/min). Each flow rate was controlled with a flowmeter (**F**). The saturated airflow passed through gas-wash bottles filled with deionized water and placed in a water bath (**WB**). The temperature of the airflow was controlled by adjusting the temperature of the water bath (**WB**). Two odour-delivery systems are available for experimentation: 1) by using charcoal filtered air that passes through gas-wash bottles containing the odorant substrate (**S1** and **S2**) or 2) by using the cleft in the stainless steel cylinders (c) to insert a sand-blasted glass slide impregnated with the desired chemostimulus. CO_2 delivery system: valve for CO_2 pulses (**V**), CO_2 delivered upwind of the split into two olfactometer arms (**purple arrow**).

The bigger number of mosquitoes from cages tested only with CO_2 pulses presented in the airstreams results from pooling all the data for these positive controls including those controls that accompanied other test odour series presented in following Chapter 5. This large pool of data permitted proper evaluation of how CO_2 pulse characteristics (A, P, BI, A ratio, P ratio and BI ratio), airflow condition (T°C and RH %) and mosquito age affected the behavioural response variable.

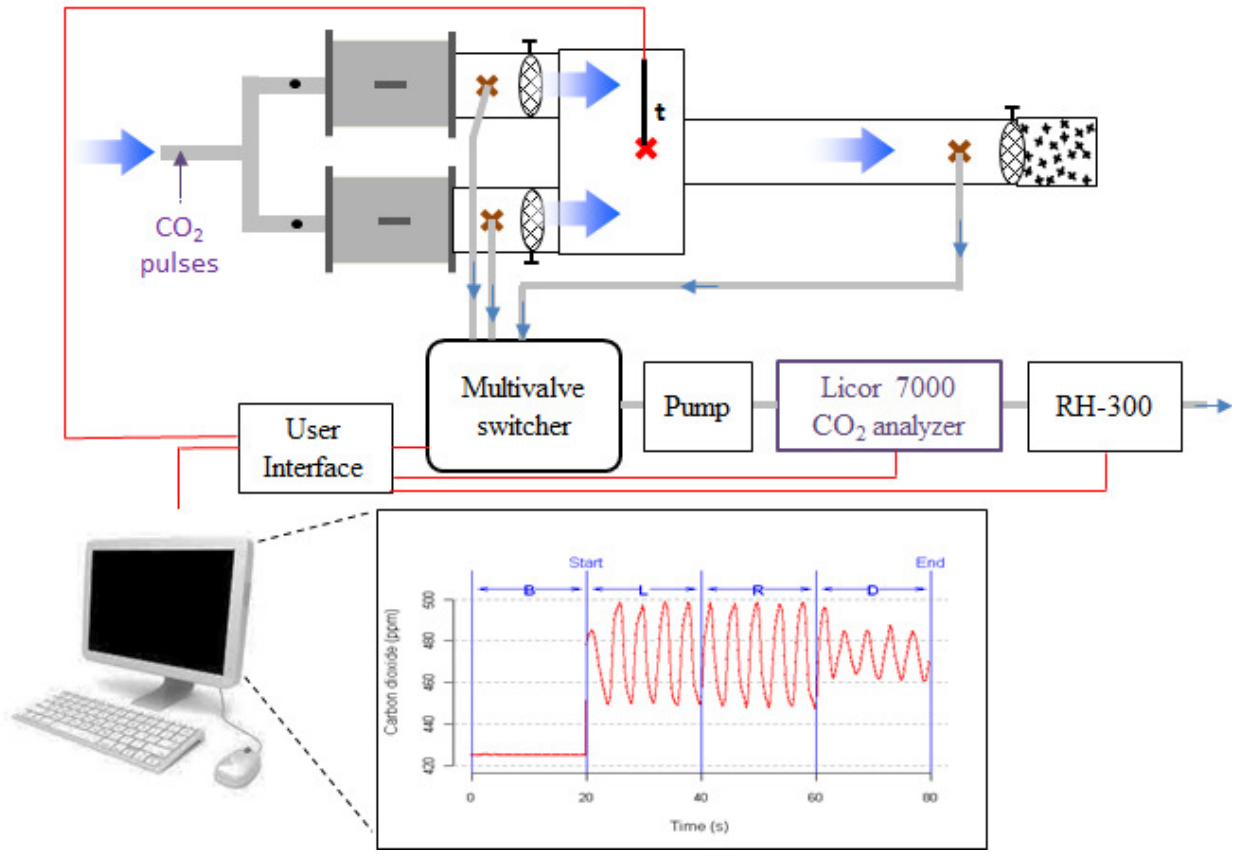


Figure 4.2 CO_2, humidity and temperature measurement system added to the olfactometer. Tubing is in grey and cabling in red between measuring devices and computer. Positions for air sampling to measure CO_2 and RH% (brown crosses) and position for T°C measurement (red cross); CO_2 measured with a LI-7000, humidity with an RH 300 and the temperature with a thermistor probe (t; see text). The multivalve switcher served to consecutively sample air during 20 s in each of the 3 compartments of the olfactometer (in both upwind arms and in the downwind tube) in a loop-wise manner.

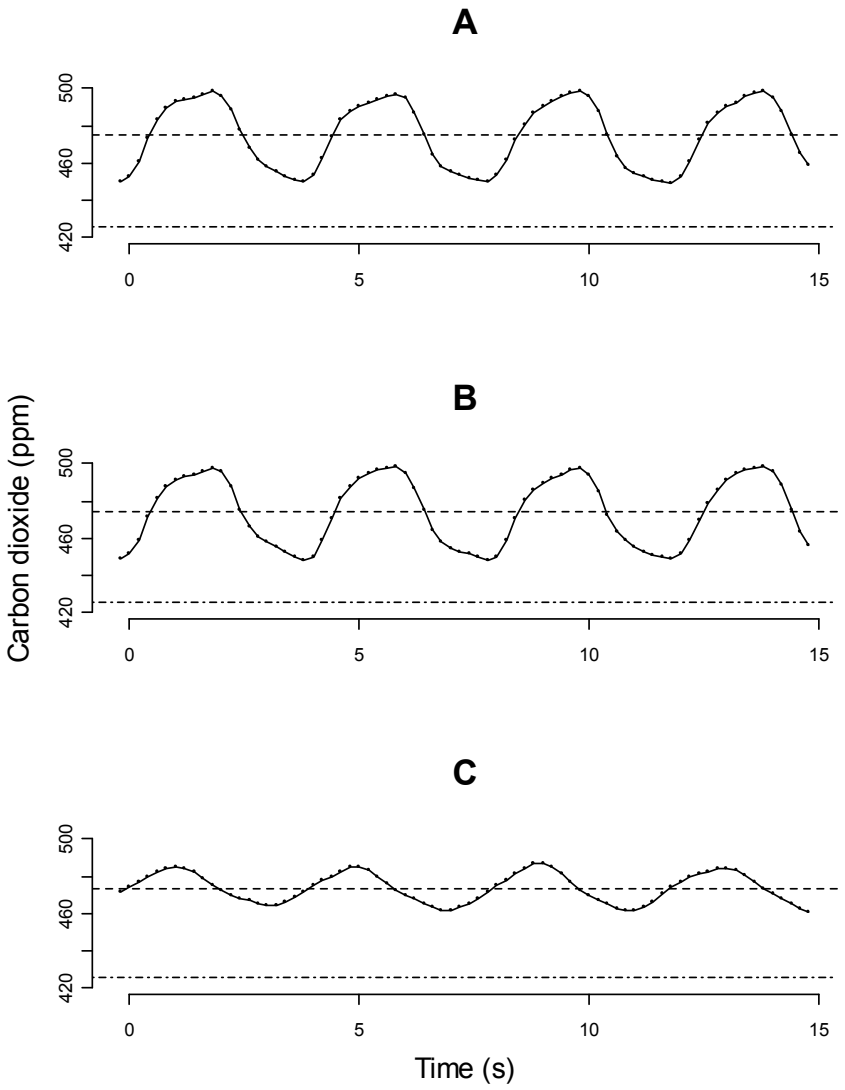


Figure 4.3 CO_2 pulses measured during an experiment in the dual choice olfactometer. Pulsing carbon dioxide into the olfactometer (upwind of the split into two arms) results in equal periodic fluctuations (~ 4s and ~ 50 ppm) in the two arms (**A** and **B**). At the downwind end, near the mosquito release cage, the mean CO_2 level and emission rate stays stable but the amplitude of fluctuations is roughly half of the 50 ppm in each arm (**C**). Dashed lines represent the mean level of CO_2 pulses and the dashed-dotted lines the mean level of CO_2 prior to the experiment (CO_2 level in the olfactometer airflow without CO_2 pulses added).

4.2.6 Data evaluation and statistics

The behavioural responses of mosquitoes were expressed in terms of two binomial response variables: **percentage of responding mosquitoes (R%)** – defined as the number of mosquitoes caught in both upwind arms divided by the number of mosquitoes that left the release cage, and **percentage of mosquitoes flying into each arm** – defined as the number of

mosquitoes flying into one or other arm of the olfactometer divided by the total number of mosquitoes (Fig. 4.4A). R% provides an evaluation of the number of mosquitoes that were activated and effectively reached both upwind arms (intensity of anemochemotactic upwind flight). The mean percentage of mosquitoes flying into each arm provides an evaluation of the preferred arm/test odour applied in an arm (degree of preference for one of the two arms or choice between the two arms). By using the number activated mosquitoes as denominator for computing R%, pertinent information is provided concerning the number of mosquitoes that left the release cage but did not reach the upwind arms of the olfactometer. Descriptive and inferential statistics were performed in R software (version 2.12.1, Copyright © 2010 The R foundation for Statistical Computing). To test whether or not the two binomial response variables differed from a 1:1 distribution for a given treatment proportion tests were made. To compare differences between treatments in each binomial response variable a generalized linear model (GLM) with a logit link function (binomial distribution) followed by a *post hoc* Tukey-Kramer analysis was used. With the large pool of experiments performed with CO_2 pulses applied alone in each arm of the olfactometer additional GLMs were applied to assess the effect of the airflow conditions, age and measurements characterizing CO_2 pulses on the two binomial response variables. For example, R% could be correlated with the measured temperature and humidity in the olfactometer, with the age of mosquitoes, or with A, P and BI measured in the downwind tube of the olfactometer. Furthermore proportions of mosquitoes flying into either arm of the olfactometer could be related to the computed A ratio, P ratio and BI ratio representing differences in CO_2 pulses between the two arms. A, P and BI measured in each arm were also compared by a t-test. In all statistical evaluations a significance threshold was set to $\alpha = 0.05$. Unless otherwise stated, means are always accompanied by ± standard errors.

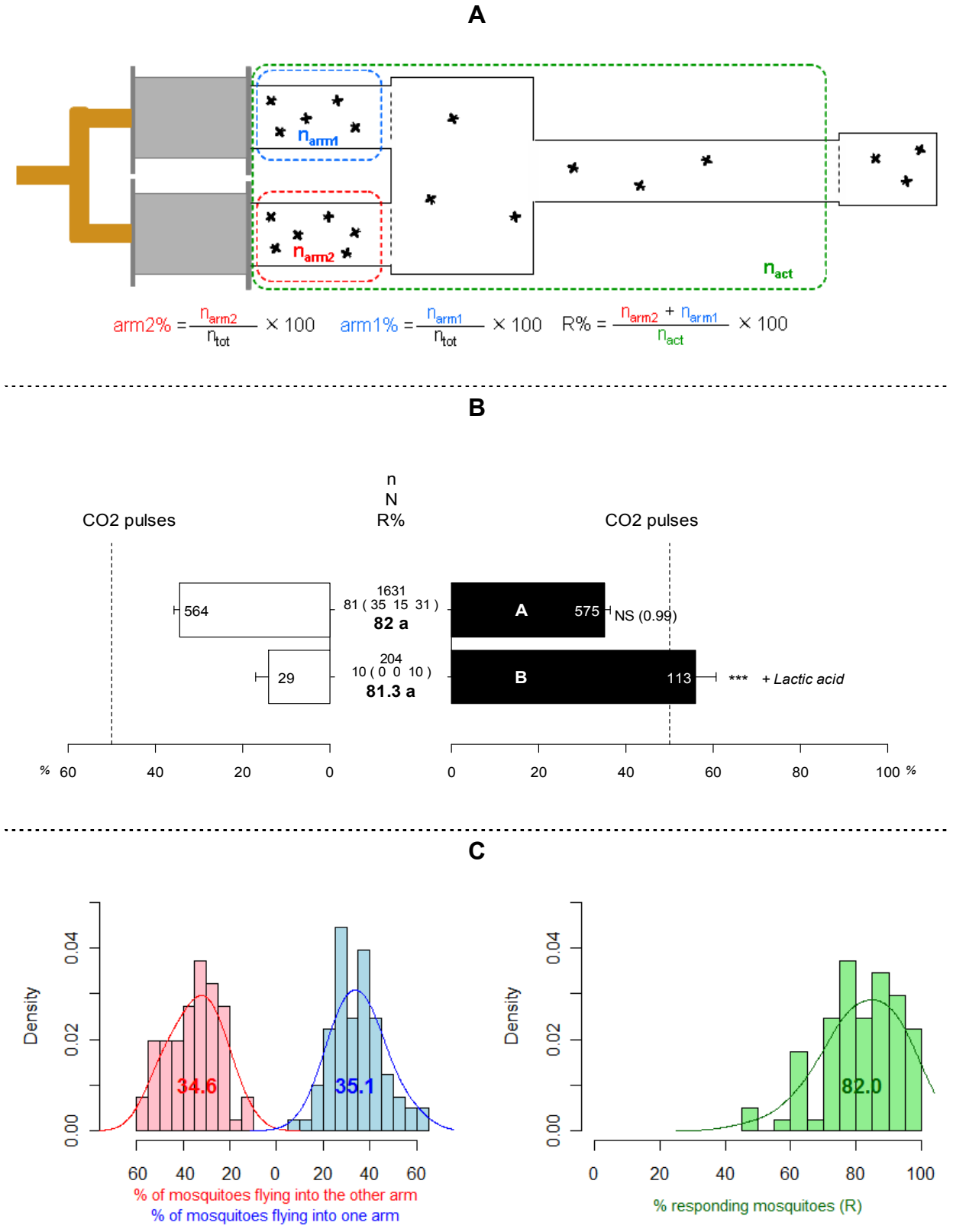


Figure 4.4 **A**: Data evaluation procedure: arm1% and arm2% are the percentages of mosquitoes flying into the corresponding upwind arms and R% the percentage of responding mosquitoes. **B**: Mosquito distribution in each arm (black and white bars) and response R% (bold value in the centre between each pair of bars) of *An. gambiae* after the one minute test period in the olfactometer to CO_2 pulses alone (upper pair of bars) and to CO_2 pulses

accompanied with lactic acid released into one of the two arms (lower pair of bars). For each treatment, n indicates the total number of tested mosquitoes and N the number of tested cages; presented in brackets are the number of cages where the majority of mosquitoes responded to one or the other arm (left and right digits) and the number of cages where an equal number of mosquitoes flew into each arm (central digit). The same lower case letter indicates no statistical significance in R% between treatments and different capital letters indicate a statistically significant difference in the distributions of mosquitoes between the two arms between treatments. **C**: Histograms with according density estimates for each response variable for the large dataset where only CO_2 pulses were added to the airstream in the olfactometer. Note the accuracy and regularity of the probability distributions.

4.3 Results

4.3.1 Mosquito responses in absence of any added chemical stimulus

Testing the response of mosquitoes to the airflow of the olfactometer without any added stimulus elicited very low upwind responses. Only 5.6% (±1.5) female *An. gambiae* flew into one arm and 4.9% (1.5) flew into the other arm (N=18 cages, n=350 mosquitoes). Of the mosquitoes that left the release cage only 20.4±4.9% reached one of the two upwind arms. The low percentage of mosquitoes reaching either arm of the olfactometer includes many tests where no mosquitoes (=zero events) were observed in one of the two arms after the one minute test period (61.1%) making it difficult for a statistical evaluation but, the data are self-explanatory. These results indicate that after an acclimation period of 20 minutes the response to the olfactometer airflow without any chemical stimulus added elicited very low or almost no response by *An. gambiae* females.

4.3.2 Mosquito responses to equal CO_2 pulses without and with lactic acid added

When confronted to equal CO_2 pulses in the two arms of the olfactometer, of the mosquitoes that left the release cage a combined 82.0% (±1.3) female *An. gambiae* flew upwind into the two arms of the olfactometer. The proportion of mosquitoes flying into each arm were equal and statistically undistinguishable: 34.6% (±1.2) flew into one arm and 35.1% (±1.2) into the other arm (proportion test: P=0.99; Fig. 4.4 B). When confronted with equal CO_2 pulses in both arms but with lactic acid added in one arm, the percentage responding *An. gambiae* was 81.3% (±3.3). This R% was statistically undistinguishable from the one obtained when only CO_2 pulses were added (p=0.73, Fig. 4.4). However, the proportion of mosquitoes flying into the arm with lactic acid was significantly higher than the proportion flying into the arm without lactic acid (53.6±4.7 versus 10.1±2.3 %, proportion test: P<0.001; Fig. 4.4 B).

Moreover, the binomial distribution between the two arms observed in the experiments with lactic acid was significantly different from the binomial distribution recorded where only CO_2 pulses were presented to the mosquitoes ($p<0.001$; Fig. 4.4B).

4.3.3 CO_2 pulses characteristics and their effect on the response of *An. gambiae*

The variables A, P and BI (Table 4.1) characterizing the CO_2 pulses in experiments without and with lactic acid added could be maintained stable and only varied within a very narrow range. In each treatment A, P and BI were equal in both upwind arms (Table 4.1, t-test: all $p>0.05$). Despite A, P and BI varying within a narrow range and statistically undistinguishable by t-tests it still is possible that these small variations may have affected the responses of mosquitoes. However, using the large dataset from the multiple experiments performed with only CO_2 pulses presented in each arm of the olfactometer no significant correlation was found between the percentage of responding mosquitoes R% and the CO_2 pulse parameters measured in the downwind tube (Fig. 4.5 A-C, all $p>0.05$). Similarly, the percentage of mosquitoes flying in either arm was not correlated with the ratios in A, P and BI reflecting the differences between the CO_2 pulses measured in each arm (Fig. 4.5 D-F, all $p>0.05$). This is confirmed in the probability distribution of both response variables (% mosquitoes in each arm and R %) as most of the data are located within a narrow range depicting regular distributions (Fig. 4.4 C).

Table 4.1 The measured variables A, P and BI characterizing the CO_2 pulses in each upwind olfactometer arm and near the release cage (downwind) for each treatment.

		CO_2 pulses versus CO_2 pulses			*CO_2 pulses versus CO_2 pulses + lactic acid*		
		each upwind arm		downwind	each upwind arm		downwind
A (Δppm)		48.10±0.36	47.97±0.42	22.54±0.22	*50.03±1.67*	49.53±1.25	22.85±0.36
P (s)		4.01±0.01	4.01±0.01	3.99±0.01	*4.01±0.02*	4.04±0.02	4.01±0.02
BI (Δppm)		45.78±0.39	45.82±0.37	45.42±0.39	*44.92±1.66*	45.20±1.51	44.91±1.56
t-test	A	0.81			0.81		
	P	0.76			0.31		
	BI	0.95			0.90		

The CO_2 pulse characteristics measured in the arm where lactic acid was added are in italic.

4.3.4 The effect of temperature and humidity on the response of *An. gambiae*

Although the temperature and the humidity of the airflow of the olfactometer also varied only within a narrow range (80±5 RH% and 24±2 T°C), to be able to compare different treatments further analysis was performed to ensure that these two parameters had no effect on the

responses of mosquitoes. Using the large dataset from multiple experiments where mosquitoes were only stimulated with CO_2 pulses, testing for a relation between the measured temperature (including T°C measured in the olfactometer, in the laboratory and the difference between the two) and the percentage of responding mosquitoes indicate nothing significant (Fig. 4.6 A-C, all $p>0.05$). Similarly, the effect of the humidity variations in the olfactometer airflow between the cages tested had no effect on the response of mosquitoes (Fig. 4.6 D, $p> 0.05$).

4.3.5 The effect of age on the response of *An. gambiae*

In the large dataset from multiple experiments where mosquitoes were only exposed to CO_2 pulses in the olfactometer mosquitoes had an age ranging from 5 to 8 days. Since mosquitoes were only tested during the scotophase this resulted in 3 age classes or categories. After testing for a relation between age and responsiveness of mosquitoes R% no significant relation was found (Fig. 4.7, $p>0.05$).

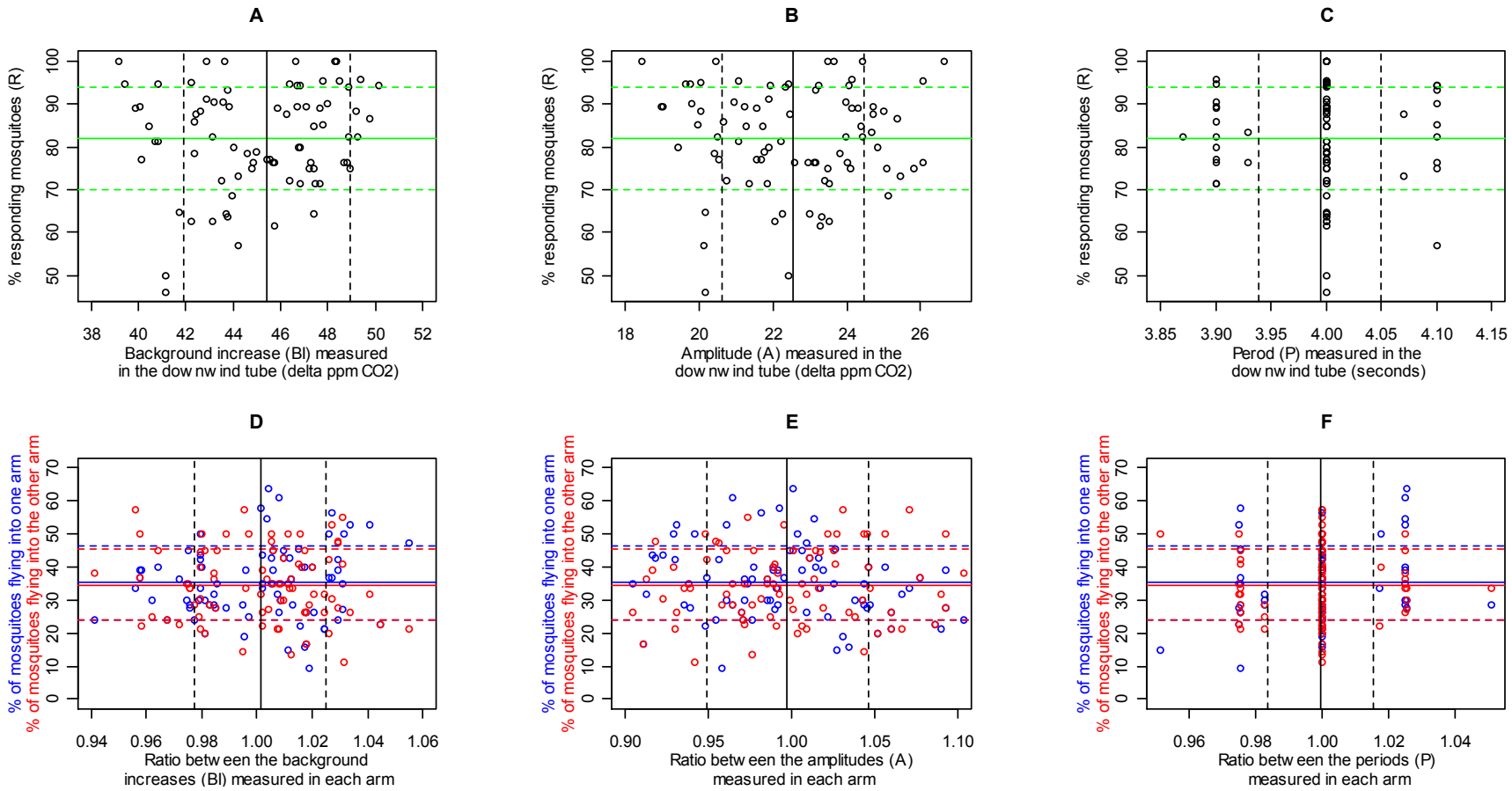


Figure 4.5 Relation between behavioural response variables and CO_2 pulse features measured in the olfactometer. Percentage of responding female *An. gambiae* R% to equal CO_2 pulses (in green) in relation with BI (**A**, p=0.18), A (**B**, p=0.79) and P (**C**, p=0.88) measured in the downwind tube. Percentages of mosquitoes flying into one (red) and the other arm (blue) of the olfactometer in relation with the BI ratio (**D**, p=0.23), the A ratio (**E**, p=0.09) and the P ratio (**F**, p=0.17) for these CO_2 pulse parameter between arms. Solid lines represent the means and the dashed lines ± one standard deviation around the mean.

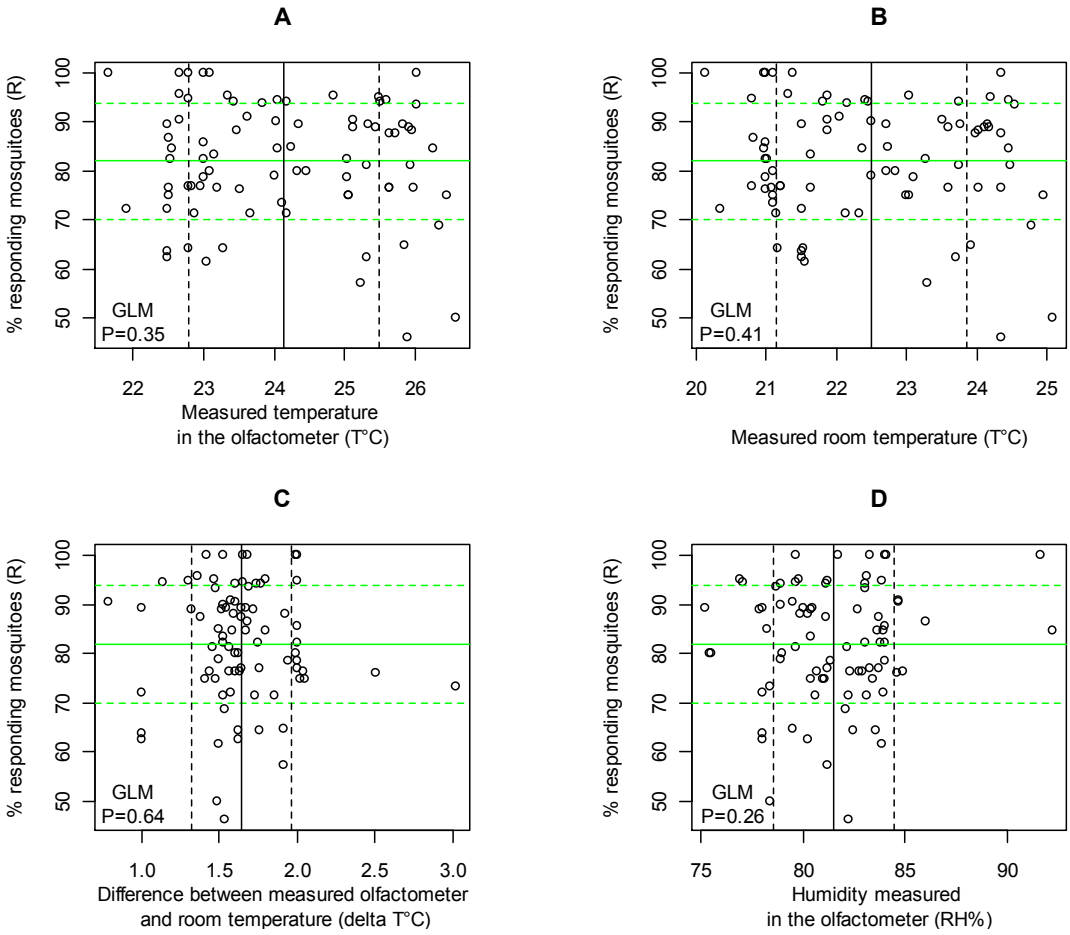


Figure 4.6 Relation between the percentage of responding female *An. gambiae* R% to equal CO_2 pulses in relation with olfactometer airflow temperature (**A**, p=0.35), laboratory temperature (**B**, p=0.41), and the difference between the two (**C**, p=0.64) and olfactometer airflow humidity (**D**, p=0.26). Solid lines represent the means and the dashed lines ± one standard deviation around the mean.

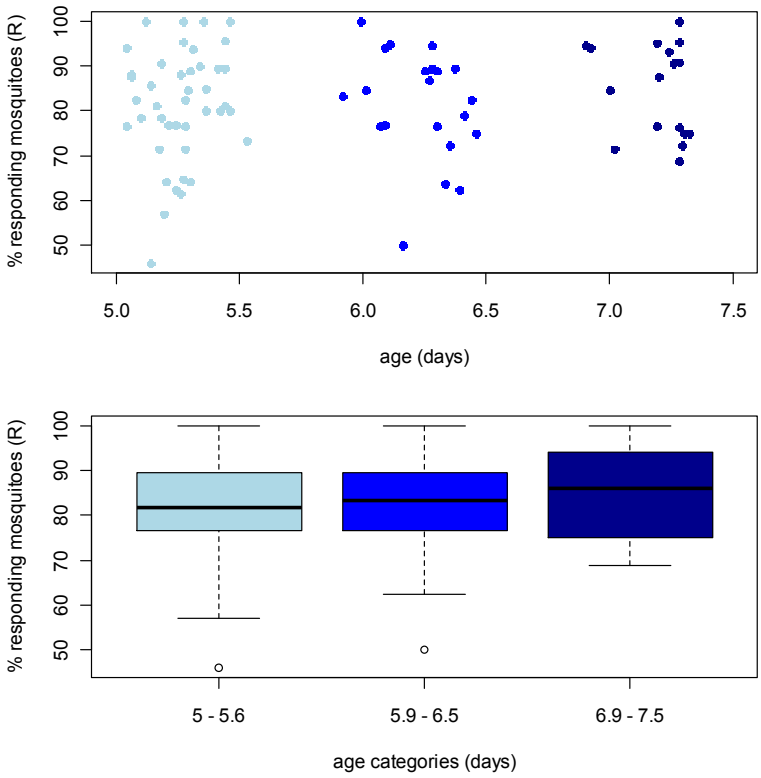


Figure 4.7 Relation between age and the percentage responding female *An. gambiae* R% to equal CO_2 pulses (p=0.52). Lower figure: age clusters represented by boxplots.

4.4 Discussion

The olfactory-driven behavioural sequence leading *An. gambiae* to human hosts starts with activation followed by sustained upwind flight (anemochemotaxis). In the dual-choice olfactometer experiments presented here this behavioural step was evaluated with R%. During sustained upwind flight additional infochemicals input may redirect the flight of *An. gambiae* to its preferred host. Here the preference for an arm emitting test odour was used as a proxy to evaluate this behavioural step.

4.4.1 Testing the olfactometer experimental paradigm with lactic acid

The importance of lactic acid as a host related infochemical for mosquitoes is not new. For the first time, almost half a century ago, lactic acid was shown to affect the behavioural response of the yellow fever mosquito *Aedes aegypti*, another anthropophilic species (Acree et al., 1968; Geier et al., 1996; Geier and Boeckh, 1999). However, evidence for the importance of lactic acid in odour-mediated host seeking behaviour by *An. gambiae* was highlighted more

recently. In Braks et al. (2001) after testing a wide dose range of lactic acid emitted by traps, only one dose caught significantly more *An. gambiae* than the control trap. Moreover and interestingly, a sweat extract also caught significantly more mosquitoes. After removal of lactic acid from the same extract it remained attractive indicating that lactic is sensed but not a prerequisite for *An. gambiae* to find its host. Subsequently, and contrasting with the above findings of Braks, in the same type of olfactometer as used here, Dekker et al. (2002) found that lactic was not attractive on its own and that its attraction was only slightly increased by the addition of CO_2 compared to findings with *Aedes aegypti* where lactic acid and CO_2 were shown to be strong synergists. Due to its particularly high concentration in human sweat compared to other non-vertebrate hosts, lactic acid was proposed and shown to be an important contributor to the marked preference for humans by *An. gambiae* (Dekker et al., 2002). A cow rubbing extract could be rendered as attractive as a human skin rubbing extract by completing its lack in lactic acid content, and differential attractiveness elicited by exposing *An. gambiae* to human fingers of different persons could be compensated by adding lactic acid indicating that this chemical plays an important role in both the inter- and intraspecific host selection (Dekker et al., 2002). The findings presented in this thesis confirm those of Dekker et al. (2002) between the two treatments (1. CO_2 pulses versus CO_2 pulses and 2. CO_2 pulses versus CO_2 pulses + lactic acid) in that the responsiveness R% or the intensity of sustained upwind flight of mosquitoes remained the same between treatments but the preference shifted towards the arm where lactic acid was added indicating that this infochemical is rather signal for choice as opposed to an attractive one.

4.4.2 Using CO_2 as sensitizer in the olfactometer

Use of CO_2 pulses as a background stimulus in the olfactometer offers advantages in many respects. First of all, in the vicinity of a breathing host such as a human atmospheric CO_2 fluctuations occur continuously and the experimental paradigm used here, even if quite artificial, is closer to reality since continuous and rhythmic changes in CO_2 stimulation indicates the presence of a nearby host. It is well known that mosquitoes respond to the intermittent nature of CO_2 stimulation (Gillies, 1980; Mboera and Takken, 1997) and the ability of numerous mosquito species to sense small CO_2 increments has also been demonstrated by electrophysiological recordings on the CO_2 receptor neurons located in sensilla on the maxillary palps (Grant et al., 1995 and my own electrophysiological measurements; see appendix 9.3.1). Using the same type of olfactometer as the one used here it has been shown that mosquitoes are more responsive to filamentous and turbulent CO_2

plumes compared to homogenous CO_2 plumes. Homogenous plumes only elicit initial activation followed by a quick adaptation to the newly prevailing CO2 level and are as such far less effective than filamentous or turbulent plumes in maintaining sustained upwind flight by both anthropophilic mosquito species *Aedes aegypti* and *An. gambiae* (Geier et al., 1999a; Dekker et al., 2002). However, in these studies, the intermittent nature of the CO_2 stimulation was not controlled as accurately as presented in this thesis. In our assay the intermittency of the CO_2 pulses was held within a range below 100 ppm and characterized by regular periodicity and increases over the background level. In Dekker et al. (2005) CO_2 has been described as a 'releaser' or 'sensitizer' for skin odour. *Aedes aegypti* was responsive to a skin odour plume generated with a human arm by marked upwind flight but when the same odour was diluted 100 fold the mosquitoes were no longer responsive. However female *Aedes aegypti* were able to orient upwind towards the diluted odour after a brief exposure to a CO_2 pulse indicating that CO_2 acts as a sensorial releaser for other host-related chemicals. In this thesis, by using CO_2 pulses, rhythmic and permanent sensitization is maintained throughout an experiment and so maximizes the detection resolution of any test odour to which mosquitoes may be responsive.

4.4.3 Testing a true choice by mosquitoes in the olfactometer

Another advantage offered by the experimental paradigm used here resides in the fact that it is a more appropriate approach when the experimenter wants to reveal the role of an infochemical as a host choice odour cue. The panel of infochemicals released by a host is complex, consisting of several hundred compounds. Meanwhile, host seeking behaviour is the result of complex sequence of behaviours which are not always dissociable. Each infochemical released by the host plays a different role in the complex sequence of behaviours leading to successful host location by the mosquito. It is therefore important to keep in mind that any experimental setup or paradigm only measures a small portion of this complex sequence. The experimental paradigm presented in this thesis is not an exception to this rule but has the advantage that it allows the experimenter to assess the involvement of an infochemical in host selection. The majority of studies with two-arm olfactometers aiming to identify infochemicals used by the insect vector to find its host are based on an approach where mosquitoes are confronted to no odour in one arm versus the test odour in the other. In the case of lactic acid or any hypothetical infochemical tested as a choice cue, such an approach would fail in highlighting the responsiveness of the mosquitoes to the test product since lactic acid has no behavioural effect on its own. Accordingly, when an experimenter

wants to reveal or express a choice component in an experimental paradigm the level of perception of the two offered stimuli should be maximized. As mentioned in the Introduction to this chapter, Martel and Boivin (2011) proposed to distinguish between 'apparent' from 'true' choice tests by underlining that for a true choice to happen both resources, here two odorous stimuli indicating a potential host for a blood-meal, need to be perceived by the tested animal. In this perspective presenting CO_2 pulses as a background stimulation offers a valid approach for discovery. A true choice can only occur if the insect needs both stimuli because the effective neuronal mechanism underlying a choice process can only be activated by presence of two sensory inputs. In a typical experimental situation as presented here mosquitoes are first confronted to CO_2 pulses at the downwind end of the olfactometer which elicits sustained upwind flight of a high percentage of the tested mosquitoes as it indicates the presence of a potential host upwind. On reaching the intermediate compartment of the olfactometer mosquitoes are faced with a choice between two potential stimulus sources, one with an extra stimulus added. The equal CO_2 pulses coming from each arm render mosquitoes aware of two potent stimulus sources. This contrasts to an experimental paradigm consisting of testing no odour stimulus in one arm versus a test odour in the second arm where the insect ends up only sensing one possibility and will only exploit that one. This is never the situation of a 'true' choice. In the context of true choice testing, the response variable R% is very informative for in the case of lactic acid the level of R% did not change compared to the where only CO_2 pulses were tested in the two arms. This indicates that lactic acid is rather an infochemicals used by *An. gambiae* to differentiate between two potential hosts as opposed to an infochemicals which acts as an attractant. If lactic acid had served as an attractive infochemicals enhancing anemotaxic oriented flight behaviour R% would have increased.

4.4.4 Control of CO_2 pulses

In order to be able to use CO_2 pulses as background to maximize the level of perception of an odour stimulus it is also important to ensure that the nature of the pulses defined by their amplitude, periodicity and increase over the background level of $C0_2$ be reproducible within a narrow range. The CO_2 pulse characteristics stayed stable throughout the experiments accounted for in this thesis and their values were statistically undistinguishable between the two arms of the olfactometer. Moreover, within the slight variations recorded between sets of experiments no significant relation with R% should be observed since the observed effect of any added stimulus could then be ascribed to those slight differences between olfactometer arms and not to the added effect of test stimulus in itself. The data presented here clearly

eliminated this as a possibility. Analysis of the large dataset from multiple experiments performed only with CO_2 pulses no relation was found between the CO_2 pulse variations and the responses of mosquitoes. This outcome confirms the experimental paradigm as a valid method by its repeatability.

4.4.5 Control of temperature and humidity in the olfactometer airflow

Temperature and humidity could be maintained within a reasonable range (24±2 T°C and 80±5 RH %) within the airflow of the olfactometer. It has been shown that mosquitoes possess and use highly fine-tuned thermohygroreceptors in many ecological circumstances. Temperature and humidity have been demonstrated to be potent physical host cues for mosquitoes (Kellogg, 1970; Kröber et al., 2010) and *An. gambiae* shows microclimate preferences to optimize its survival strategy (Kessler and Guerin, 2008). Moreover, in Chapters 2 and 3 it was shown that temperature and humidity to some extent affect the GEP and cost of living (reflected by the mean CO_2 production rate) of *An. gambiae*. Over the range of variation of the humidity and temperature measured across experiments the thermohygro-sensitivity of mosquitoes justified the necessity to verify if variations in temperature and humidity affected the behavioural response of *An. gambiae* in the olfactometer. However, the response of *An. gambiae* did not seem to be affected by these airstream parameters. These findings are important in the context of testing additional test odours and to perform appropriate statistical comparison between treatments. Although mosquitoes are known to be highly sensitive to temperature and humidity cues the outcome of the experiments described in this thesis is not surprising because the tested mosquitoes always underwent an acclimation period of 20 minutes to the olfactometer airstream prior to an experiment. In other words, the tested mosquitoes had enough time to adapt to the new ambient conditions encountered in the olfactometer.

4.4.6 The effect of age of mosquitoes on their responses in the olfactometer

The biggest dataset resulting from multiple experiments with only CO_2 pulses presented also allowed gauging the effect of the age of mosquitoes on olfactometer responses. It could be expected that younger mosquitoes may not be mature enough or physiologically disposed to seek a host resulting in differences in the responses of mosquitoes between treatments that could be ascribed to differences in age. However age did not influence the responsiveness to equal CO_2 pulses of mosquitoes. This outcome was expected since the age classes for olfactometer tests were chosen on the basis of prior studies indicating that from day four most adult *An. gambiae* are responsive to host cues (Jones and Gubbins, 1978; Arsic, 2008 and

personal observations). Mosquito were reared in a standardized manner with optimal water depth and optimal feeding regimes according to (Briegel, 2003) resulting in adult emergence within a defined period, allowing accurate age determination (± 6 h). The rearing methodology should not be neglected and it surely contributed to the homogeneity of the resulting behavioural responses by *An. gambiae* in the olfactometer.

5 Response of *An. gambiae* to notable specificities of human axillary odour

5.1 Introduction

An. gambiae is the main malaria vector in sub-Saharan Africa (WHO 2008). There is evidence that its high vectorial capacity, among other important physiological, behavioural and ecological factors, is due to its marked preference for human hosts (Garrett-Jones, 1964). Added to this anthropophily, the vector is also characterized by its preference for certain human types. In mosquitoes the complex sequence of behaviours leading to host location is strongly olfactory driven (Takken and Knols, 1999). Behavioural and electrophysiological investigations have revealed several kairomones found in host effluvia that alter the human host seeking behaviour of *An. gambiae*. Carbon dioxide (CO_2) a major component of expired human breath is well known for its role as an activator and sensitizer for anemochemotactic upwind flight behaviour, particularly when presented intermittently (Gillies, 1980; Mboera and Takken, 1997; Dekker et al., 2005; McMeniman et al., 2014). Ammonia and volatile organic compounds such as aliphatic carboxylic acids, lactic acid, 1-octen-3-ol, and 4-methylphenol identified in human sweat emanations have also been shown to be important human host cues for *An. gambiae* (Cork and Park, 1996; Knols et al., 1997; Braks et al., 2001; Dekker et al., 2002; Smallegange et al., 2005; Smallegange et al., 2009; Okumu et al., 2010). There is evidence that selective human host-seeking behaviour of *An. gambiae* relies in the difference of odour emitted by humans compared to other vertebrate hosts (Costantini et al., 1993; Mboera et al., 1997; Costantini et al., 1998; Dekker and Takken, 1998; Dekker et al., 2001 and 2002) and certain human types compared to others (Knols et al., 1995; Dekker et al., 2002; Mukabana et al., 2002). Although it is established that *An. gambiae* is influenced in its choice between humans over other vertebrate hosts by infochemicals, the identity of the products and their point of action in the complex behavioural sequence leading the vector to its final preferred host remains poorly investigated.

Fresh and incubated human sweat has already been tested on several occasions to show that incubated sweat is significantly more attractive than fresh sweat (Braks and Takken, 1999; Meijerink et al., 2000; Braks et al., 2001). These studies have underlined the importance of skin bacteria in the odour-mediated host seeking behaviour of *An. gambiae.* The involvement of human skin bacteria in the chemical ecology of mosquitoes has also been review more recently in Verhulst et al. (2010a) and Smallegange et al. (2011). Humans present high variation in their skin microbiotic profile. Earlier studies indicate that variation in the microbial community composition is more stable over time on single individuals than

between individuals (Gao et al., 2007; Fierer et al., 2008; Grice et al., 2008; Costello et al., 2009). Past research suggests that there is a correlation between microbial composition (in terms of diversity, quantity and density) and individual body odour profile (Xu et al., 2007) with a clear correlation between underarm sweat odour intensity and bacterial density (Leyden et al., 1981; Labows, 1982; Austin and Ellis, 2003; Taylor et al., 2003; James et al., 2004; Rennie et al., 2007). Single individuals can even be differentiated by the odour profile emitted from their hands (Curran et al., 2007). Skin bacteria play a key role in metabolizing skin secretions into volatile compounds constituting odorous effluvia emitted by humans, and there is evidence that individual humans bear they own microfloral community and hence chemical signature.

With this in mind, recent work in mosquito chemical ecology has brought attention to the role of human skin bacteria in the host-vector interaction between humans and *An. gambiae*. In a wind tunnel, traps baited with blood agar plates incubated with skin bacteria from human feet or a reference strain of *Staphylococcus epidermidis* caught significantly more *An. gambiae* than a control (Verhulst et al., 2009). Headspace analysis of air of blood agar plates colonized by skin bacteria collected on human feet revealed 14 volatile compounds of which five were also found when the same media was inoculated with *Staphylococcus epidermidis* alone (Verhulst et al., 2009). Later on, the same research group investigated the role of four additional skin bacteria spp. (*Bacillus subtilis*, *Brevibacterium epidermidis*, *Corynebacterium minutissimum* and *Pseudomonas aeruginosa*) (Verhulst et al., 2010b). With the exception of *Pseudomonas aeruginosa* the four other bacteria spp. were shown to produce semiochemicals that affect the behavioural response of *An. gambiae*. Further experiments revealed that *Corynebacterium minutissimum* is capable to metabolize particularly attractive VOCs for *An. gambiae*. Headspace analysis combined with behavioural assays also allowed the identification of five attractive and one repellent volatile compound for *An. gambiae* (Verhulst et al., 2010b).

The axillary underarm region is endowed with apocrine and eccrine glands. Apocrine glands are mainly found in the axillary region (Wilson, 2009). They become active only at puberty and their activity slows down with aging. They have also been hypothesized to play a role in human pheromone production (Stoddart, 1990; Van Toller and Dodd, 1993). Earlier studies revealed compounds of steroidal origin (5α-androst-16-en-3-one, 5α-androst-16-en-3α-ol and 4,16-androstadien-3-one) and volatile carboxylic acids (3-methyl-2-hexenoic acid and 7-octenoic acid) as key constituents of human-characteristic axillary odour (Brooksbank et al.,

1974; Zeng et al., 1991; Zeng et al., 1992; Van Toller and Dodd, 1993; Zeng et al., 1996). Recent analytical work has led to the identification of further human specific odorous compounds. The proteinaceous liquid excreted by apocrine glands contains, among other molecules, water soluble compounds which are unique to humans. These compounds are transformed by the axillary microflora into (R)/(S)-3-methyl-3-sulfanylhexan-1-ol (MSH) (Hasegawa et al., 2004; Natsch et al., 2004; Troccaz et al., 2004) and (R)/(S)-3-hydroxy-3-methylhexanoic acid (HMHA, the hydrated analogue of 3-methyl-2-hexenoic acid) (Natsch et al., 2006). HMHA is released from a glutamine conjugate by the action of a zinc-dependent aminoacylase from Corynebacteria (Natsch et al., 2003), whereas MSH is derived from a cysteinyl-glycine-S-conjugate by the action of Staphylococci (Starkenmann et al., 2005). Both compounds and their precursors are suspected to be unique to human odour physiology (Natsch et al., 2004; Natsch et al., 2006) and as such, are potential candidates to explain the highly selective and anthropophilic odour-mediated host seeking behaviour of *An. gambiae*. However, to our knowledge the behavioural sensitivity of *An. gambiae* to MSH and HMHA has never been investigated. The aim here was to examine the response of *An. gambiae* to these two compounds in the dual choice olfactometer. Additionally, the response of *An. gambiae* were tested to odorous samples resulting from the inoculation of sweat from the human axillary region with the three bacteria spp. *Staphylococcus epidermidis*, *Corynebacterium jeikeium* and *Staphylococcus haemolyticus*. The two latter mentioned species play a major role in the constitution of human axillary odour and both have the ability to produce HMHA and MSH (Troccaz et al., 2004; Troccaz et al., 2009). To investigate the responsiveness of *An. gambiae*, the developed olfactometer experimental paradigm presented in Chapter 4 was used.

5.2 Methods and Materials

5.2.1 Mosquitoes, the olfactometer and testing procedure

For behavioural experiments non-blood fed female *An. gambiae* aged 5-8 days were tested during their last 6 h of scotophase. They were reared and maintained in the same manner as described in section 4.2.1 of Chapter 4. The olfactometer is described in section 4.2.2 of Chapter 4. The mosquito testing procedure is the same as described in section 4.2.3 of Chapter 4.

5.2.2 Collection of axillary sweat and odour regeneration with bacteria

Axillary sweat samples used to generate bacterial/specific odour were kindly provided by Firmenich SA, Geneva (Dr. Myriam Troccaz and Dr. Christian Starkenmann). The same sweat samples were used as in Troccaz et al. 2009. The sterile sweat samples originated from a collection campaign on 49 humans volunteers. Human apocrine and eccrine secretions were collected from the axillae of 24 men and 25 women, all Caucasians, who used a sauna over three winter seasons from November 2004 to April 2007. The average sweat yield was 5 times greater for males at 11.8 ± 1.2 ml (mean ± standard error of the mean) than for females at 2.4 ± 0.5 ml, whereas the average protein content was similar (0.22 ± 0.06 vs. 0.16 ± 0.03 g/l for males and females, respectively). The glucose content was 2–5 times higher for males than for females (3.6 ± 0.9 g/l and less than 1.0 ± 0.1 mg/l in male and female apocrine secretions, respectively). Glucose is an important source of carbon for Gram-positive bacterial growth. The average pH was lower in females at 7.5 ± 0.3 versus 8.0 ± 0.3 in males (results taken from Troccaz et al. 2009).

To regenerate bacteria-specific axillary odour, a 400 µl sterile sweat sample (either male or female) was inoculated with a 50 µl bacterial solution for 30 min. at 37°C prior to a test in the olfactometer (Table 5.1). This procedure gave the sample its typical odour depending on the bacterium species and gender origin of the sterile sweat. It also made it easier to ensure availability whenever an adult *An. gambiae* population was ready for a series of tests in the olfactometer. Further details on the volunteer pool and the procedure to obtain sterile sweat samples, as well as the preparation of bacterial solution of each species are provided in Troccaz et al. 2004 and 2009. Table 5.1 provides an overview of the material used to generate bacterial and gender-specific axillary odour.

Table 5.1 Sweat sample composition used to produce sweat odorous after incubation

ID	Bacteria	Bacterial solution (µl)	$NaPO_4$ buffer, 0.1 M, pH 6 (µl)	Sterile male sweat (µl)	Sterile female sweat (µl)
1	*C. jeikeium*	50	0	0	400
2	*St. haemolyticus*	50	0	0	400
3	*St. epidermidis*	50	0	0	400
4	None [a]	0	50	0	400
5	*C. jeikeium*	50	0	400	0
6	*St. haemolyticus*	50	0	400	0
7	*St. epidermidis*	50	0	400	0
8	None [b]	0	50	400	0

[a] and [b] : female and male 'odourless controls' respectively.

5.2.3 Odour delivery

The CO_2 pulse stimulation used as a background sensitizer was delivered and monitored in the same manner as described in Chapter 4. To deliver the odour generated by the sweat samples (Table 5.1) into each arm of the olfactometer, charcoal-filtered compressed air (at 150ml/min) passed through two 500ml glass gas-wash bottles each containing the desired sweat sample. The bottles were placed at position S1 and S2 as indicated on Fig. 4.1 (Chapter 4). Both stimulation bottles were placed in the centre of a heating plate (30 ±1°C) in order to maintain bacterial activity. The sweat sample vial was placed in the centre of the base of the bottle. The inlet tube of the gas-wash bottle reached the top of the sweat sample in the vial to ensure odour flush out. To economize the odour produced by the sweat samples, the air flow was turned off between tests. The chemical compounds (R)/(S)-3-hydroxy-3-methylhexanoic acid (HMHA) and (R)/(S)-3-methyl-3-sulfanylhexan-1-ol (MSH) were kindly provided by Firmenich SA, Geneva, Switzerland (Dr. Christian Starkenmann). Dilutions ranging from ~10^{-5} to 10^{2} ng/µl of both chemicals were made using tertyl-butyl methylether (TBME, 99%, Merck) as solvent. For olfactometer tests, 10 µl of a given dilution of either HMHA or MSH was applied on a sand-blasted glass slide and, after allowing the solvent to evaporate, the slide was inserted randomly into one of the upwind clefts of the olfactometer (for cleft positions, see Fig. 4.1, Chapter 4). Another sand-blasted glass slide on which 10 µl pure TBME was applied served as a control and was inserted into the other arm.

5.2.4 Test series and objectives

Four different sets of olfactometer experiments were performed.

1. An. gambiae *responses to female (or male) sweat incubated without any bacteria against female (or male) sweat incubated with one of three bacteria species*

In order to evaluate if one of the three bacterium species metabolized odourless sweat to provide an odour to which *An. gambiae* was responsive, sterile sweat was tested against sweat incubated with a given bacterium species. One subset of olfactometer trials was performed using female (Fig. 5.1; sweat samples 1-4, Table 5.1) and the other using male (Fig. 5.2, sweat samples 5-8, Table 5.1) sweat as the substrate. Within each of these experimental subsets, a separate series of tests was performed for each bacterium species.

2. An. gambiae *responses to female against male sweat both incubated with the same bacterium species*

Since bacterial transformation of sterile female and male sweat could result in the generation of two different odour profiles it might also be expected that *An. gambiae* responds differentially when confronted with these two treatments simultaneously. Consequently, another set of olfactometer trials was performed where mosquitoes were presented with odour generated by bacterial transformation of female sweat against male sweat incubated with the same bacterium (3 intermediate pairs of bars, Fig. 5.3; sweat samples 1-3 and 5-7, Table 5.1). As a control, sterile female sweat was tested against sterile male sweat (bottom pair of bars, Fig. 5.3; sweat samples 4 and 8, Table 5.1). In addition, the additive effect of the odour generated by each bacterium species was tested (upper pair of bars, Fig. 5.3) where the odour generated by all the three bacteria species incubated independently in female sweat (sweat samples 1, 2 and 3 placed in the same gas-wash bottle) was tested against the odour resulting from the same procedure but using male sweat as a substrate (sweat samples 5, 6 and 7 placed in the same gas-wash bottle).

3. An. gambiae *responses to HMHA and MSH*

Since both *C. jeikeium* and *St. haemolyticus* have the ability to metabolize sweat to produce the unique human volatile compounds HMHA and MSH, concentration series of both HMHA and MSH were tested using TBME as a solvent to ascertain if these compounds elicit any behavioural response from *An. gambiae*. Pure solvent was used as a control.

For test series with sweat samples, 9-10 cages were tested within one experimental day. As indicated above, the gas-wash bottles containing the sweat samples were only flushed during the experimental trial in order to economize the odour resulting from the incubation. This permitted to use a single sweat sample for the 9-10 cages tested. A minimum of 6 cages with 18-23 mosquitoes per cage were tested to assess the response of mosquitoes to HMHA and MSH. As demonstrated in Chapter 4, CO_2 pulses as a background in the olfactometer was demonstrated as being effective to render mosquitoes more sensitive to odour and was used in the testing of the sweat samples and pure compounds with a view to present the mosquitoes with a true choice. As such, all the described test series were performed using CO_2 pulses as described in Chapter 4 as a background sensitizer.

5.2.5 Quantification of behaviours and statistical analysis of data

Evaluation and statistics were performed as described in Chapter 4. In brief, the mean response of mosquitoes (R%) and the percentage of mosquitoes flying into each arm of the olfactometer were calculated. Within treatments, proportion tests were applied to gauge if the percentage of mosquitoes flying into each arm differed significantly. Between treatments differences in R% and distribution between the two arms (degree of preference) were assessed using a GLM followed by a *post hoc* analysis (for more details see Chapter 4). A large control dataset (N=81 cages, n=1631 mosquitoes) where the experiments were performed by applying only CO_2 pulses was evaluated in Chapter 4. This large dataset also includes the controls for experiments described here. Using this large dataset it was demonstrated that the CO_2 pulse structure was equal in each arm of the olfactometer and this structure could be maintained at very stable levels across experimental trials. This stability was also reflected in the behavioural response of *An. gambiae*. Over the range of variation (Table 4.1, Chapter 4) in the CO_2 pulse structure measured in the olfactometer no correlation was found with the behavioural responses of *An. gambiae* (Fig. 4.5, Chapter 4). In the experiments described in this chapter, the CO_2 pulses accompanying the treatments were produced in the same manner as described in Chapter 4. It can therefore be assumed that slight variations in the CO_2 pulses accompanying treatments had no influence on the responses of mosquitoes to test stimuli.

5.3 Results

5.3.1 The effect of each bacterium species on the response of *An. gambiae*

When sterile female sweat was tested against female sweat incubated with one of the three bacteria species more mosquitoes flew into the arm bearing the odour resulting from the bacterial inoculated substrate. This was highly significant for all three bacteria species and resulted in the following mean percentages of mosquitoes flying into each arm: 61.6±4.9% versus 16.0±2.7% (prop. test: $P<0.001$) with *St. epidermidis*, 63.4±4.8% versus 27.4±4.6% (prop. test $P<0.001$) with *C. jeikeium,* and 52.0±3.7% versus 17.3±3.4% (prop. test $P<0.001$) with *St. haemolyticus* (Fig. 5.1, pair of bars). These preferences, represented by the distribution of mosquitoes in the two arms of the olfactometer, did not differ significantly between treatments (Fig. 5.1, same capital letters). For the responsiveness of female *An. gambiae* R%, 83.8±4.8% left the release cage in experiments with *St. epidermidis*, 99.4±0.6% with *C. jeikeium* and 85.1±3.6% with *St. haemolyticus*. The response elicited by *C. jeikeium* was significantly higher than by either *Staphylococcus* species (Fig. 5.1, difference in lower case letters).

The outcome of a similar experiment but using male instead of female sweat as a substrate was very similar but differed in that the preference by *An. gambiae* for the arm with odour resulting inoculation of sweat with bacteria was less marked (lower differences in percentages between arms of the olfactometer, Fig. 5.2). The mean percentage of mosquitoes flying into each arm was: 51.7±2.9% versus 21.9±2.1% with *St. epidermidis* (prop. test: $P<0.01$), 56.8±2.9% versus 30.3±3.4% with *C. jeikeium* (prop. test $P<0.01$), and 55.3±3.6% versus 36.8±4.1% with *St. haemolyticus* (prop. test $P<0.05$). As with female sweat, these preferences did not differ significantly between treatments (Fig. 5.2, same capital letters). The responsiveness (R%) differed in that both *C. jeikeium* and *St. haemolyticus* elicited a higher response with mean R% values of 95.6±1.9 and 98.0±4.1%, respectively, significantly higher than the 84.5±2.6% elicited by *St. epidermidis* (Fig. 5.2, different lower case letters).

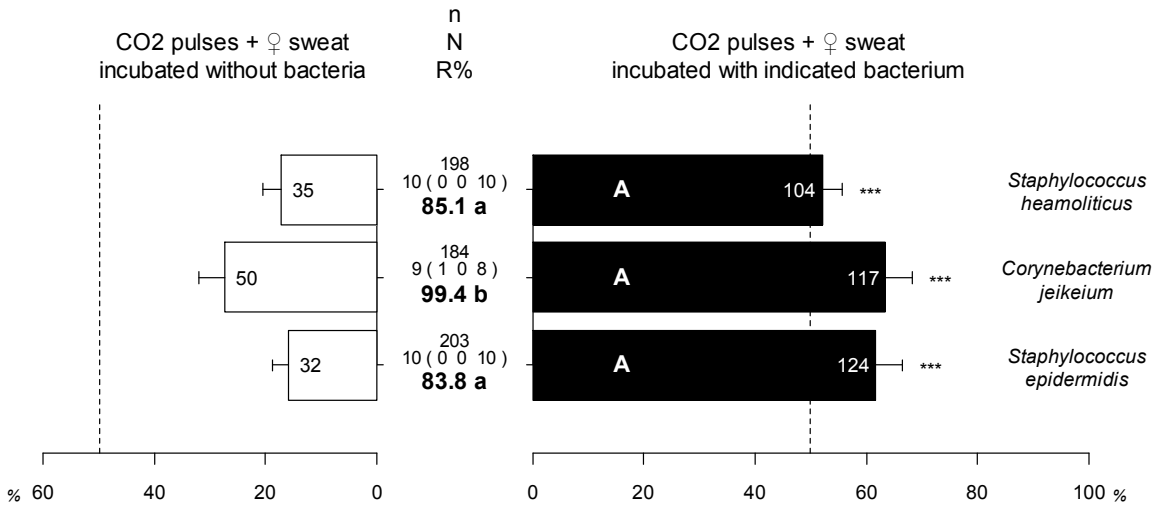


Figure 5.1 Distribution of female *An. gambiae* in the two arms of the olfactometer and response (R%) of the mosquitoes after the one minute test period in the olfactometer to the effluvium from sterile female sweat (odourless control) in one arm and the odour generated from female sweat incubated with one of three bacteria species in the other arm, in the presence of CO_2 pulses as background sensitizer. Different lower case letters indicate significance in R% between treatments and different capital letters indicate when the distributions of mosquitoes between the two arms differ between treatments. For more details of the description of the values depicted in this and the following figures see Fig. 4.4B of Chapter 4.

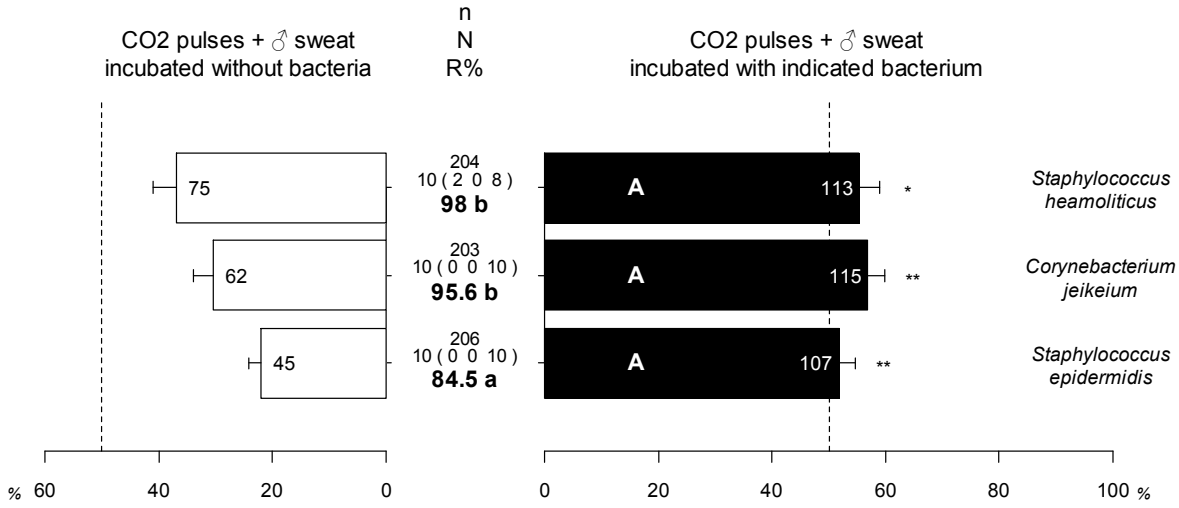


Figure 5.2 Response of female *An. gambiae* to the effluvium from sterile male sweat in one arm (odourless control) and to that of male sweat incubated with one of three bacteria species in the other arm, in the presence of CO_2 pulses as a background sensitizer. For further explanation see legends to Figures 4.1 and 4.4B of Chapter 4 and legend to Figure 5.1.

5.3.2 Response to female sweat tested against male sweat both incubated with the same bacterium species

Presenting mosquitoes in the olfactometer with female sweat ('female odourless control') tested against sterile male sweat (male odourless control) induced no preference: 33.6±4.0% of the mosquitoes chose the arm with female sweat and 35.3±3.5% the arm with male sweat (P=0.77, Fig. 5.3). When both the female and male sweat where incubated with the same bacterium species mosquitoes always preferred the arm with male sweat (Fig. 5.3). Using *St. epidermidis* to produce sweat odorous resulted in 49.51±5.9% of mosquitoes flying into the arm with male sweat odour and only 28.1±5.5% into the arm conveying odour from female sweat (prop. test P<0.001). The same trend was observed for the two other bacterial species. For *C. jeikeium* the distribution of mosquitoes in the two arms was 64.4±2.7% and 25.9±3.3%, and with *St. haemolyticus* 52.3±3.0% and 27.6±2.5% (prop. test P<0.001 and P<0.05, respectively). When the odour produced by each bacterium was combined, male sweat was also preferred over female sweat (49.1±3.6% versus 20.0±3.0%, prop. test P<0.001). Comparisons between treatments for the distribution of mosquitoes between the two arms revealed significant differences. The preference for the arm where odour resulted from the bacterial transformation of male sweat proved to be particularly marked when

performed with *C. jeikeium* as with the combination of the three bacteria spp. with male sweat (Fig. 5.3, differences in capital letters). In terms of the total response of mosquitoes (R%), only the experiment performed with *C. jeikeium* showed a significantly higher response as compared to the experiment where only the sterile sweat samples were compared to each other (Fig. 5.3, differences in lower case letters).

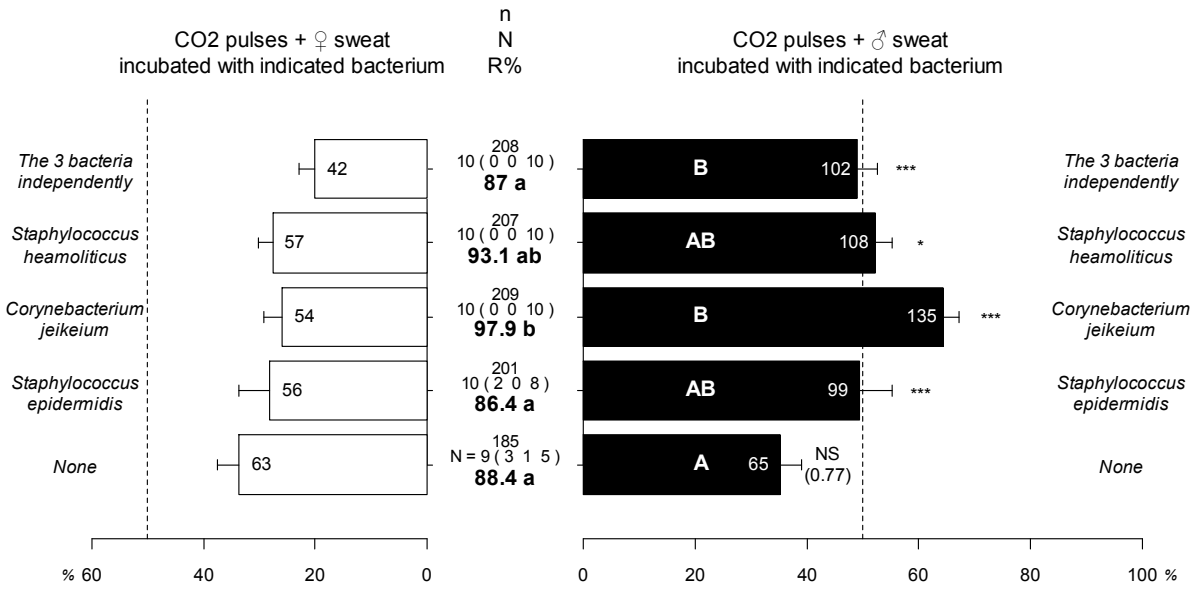


Figure 5.3 Response of female *An. gambiae* to odour generated with female sweat in one arm and with male sweat in the other arm of the olfactometer in the presence of CO_2 pulses as a background sensitizer. Lower pair of bars, sweat samples incubated without bacteria (odourless control); three next higher pairs of bars, female and male sweat samples both incubated with the same bacterium sp.; and the top pair of bars, odour from female and male sweat samples incubated with all three bacteria species. For further explanation see legends to figures 4.1 and 4.4B of Chapter 4 and legend to Figure 5.1.

5.3.3 Response to 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol

Tests with HMHA revealed dose-dependent effects on *An. gambiae* in the olfactometer over the range of tested doses (Fig. 5.4). The dose at which the highest preference for the arm with HMHA was measured was 1 ng/µl TBME. At this dose, 45.8±3.4% of the mosquitoes flew into the arm with HMHA whereas 34.1±4.0% flew in the control arm but this difference was not significant (prop. test P=0.13). At the highest dose tested (100 ng/µl), HMHA was clearly repellent (19.7±2.1% versus 54.1±5.1%, prop. test P<0.001, Fig. 5.4) and the distribution of

mosquitoes between the two arms was also significantly different form the distribution measured at the other tested doses of HMHA (Fig. 5.4, difference in capital letters). Although HMHA did not elicit a clear preference at 1 ng/µl, the total response (R%) was highest at this dose and could clearly be distinguished from the experiment where mosquitoes were exposed to CO_2 pulses alone (92.9±2.2% versus 82.0 ±1.3%, different lower case letters, Fig. 5.4). The R% measured at the other tested doses was also higher but could not be statistically distinguished from the control (CO_2 pulses alone).

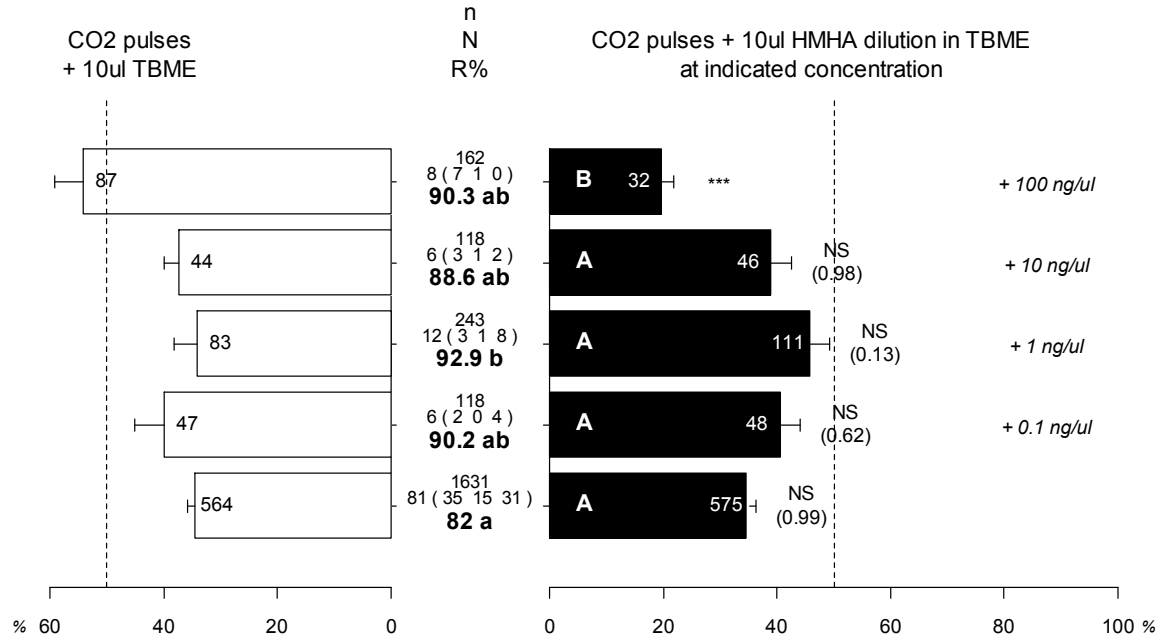


Figure 5.4 Response of female *An. gambiae* to 3-hydroxy-3-methylhexanoic acid (HMHA) applied onto sand-blasted glass slides at the indicated concentrations. The bottom pair of bars shows the results of experiment with CO_2 pulses alone in each arm of the olfactometer (control). For further explanation see legends to Figures 4.1 and 4.4B of Chapter 4 and legend to Figure 5.1.

When the same experiment was performed with MSH, the highest dose (10 ng) elicited a slight preference which was only marginally significant (53.4±5.2% versus 31.7±3.6%, prop. test $P<0.05$, Fig. 5.5). In terms of preference, no differences were found between the different doses tested (Fig. 5.5, same capital letters). As noted for HMHA but more marked with MSH, the total response of mosquitoes (R %) was higher than in the control (CO_2 pulses alone) and those differences are significant at all doses tested with the exception of the 0.1 ng/µl dose (Fig. 5.5, difference in lower case letters).

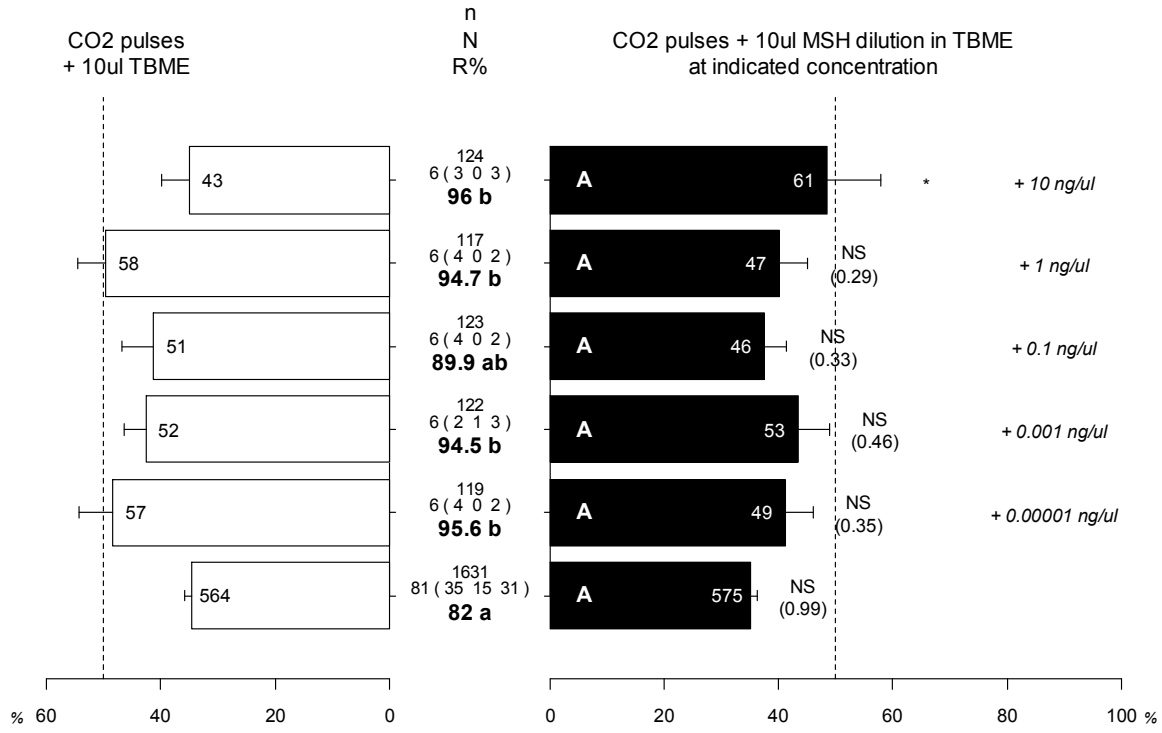


Figure 5.5 Response of female *An. gambiae* to 3-methyl-3-sulfanylhexan-1-ol (MSH) applied on sand-blasted glass slides at the indicated concentrations. The bottom pair of bars shows the results of experiment with CO_2 pulses alone in each arm of the olfactometer (control). For further explanation, see legends to figures 4.1 and 4.4B of Chapter 4 and legend to Figure 5.1.

5.4 Discussion

In brief, the findings of this study show that axillary sweat originating from two representative human pools is rendered more attractive by the action of each bacterium species that inhabits the armpits of humans. *An. gambiae* showed no preference for sterile male or female sweat. Irrespective of the bacteria species used as inoculum, sweat originating from the male pool was more attractive than sweat from the female pool. Compared to the two other species, *C. jeikeium* converted sweat into volatile compounds that rendered mosquitoes particularly responsive. This species also elicited the strongest degree of preference towards male sweat over female sweat. The two metabolites (MSH and HMHA) that are suspected to be unique to humans did not elicit a preference by *An. gambiae* in the olfactometer. However, MSH (at almost all doses tested) and HMHA (at one tested dose) elicited a higher overall response (intensity of anemochemotactic upwind flight) from the mosquitoes.

5.4.1 The action of bacteria on freshly collected axillary sweat and its effect on *An. gambiae*

The results of this study demonstrate that human axilla bacteria play an important role in odour-mediated host-seeking behaviour by *An. gambiae*. Sweat incubated with one of the three bacteria species was always more attractive than sweat of the same gender incubated without any bacterium (Fig. 4.1 and 4.2). These results are in line with the pioneer work of Shelley et al. (1953) who showed that freshly collected sweat is odourless. They also confirm findings from earlier behavioural and electrophysiological investigations where it was shown that fresh sweat is not or only slightly attractive to *An. gambiae* (Braks and Takken, 1999; Meijerink et al., 2000; Braks et al., 2001). The present study led to the identification of three bacteria species (*St. epidermidis, C. jeikeium* and *St. haemolyticus*) that inhabit the axillary region of humans and metabolize sweat to produce odorous compounds to which *An. gambiae* responds. Axillary sweat is populated by a diverse community of bacterial species and it could be expected that this diversity is required to maximize the attraction of *An. gambiae*. However, here it is shown that incubating axillary sweat inoculated with a single bacterium species is sufficient to elicit a strong behavioural response by *An. gambiae*. This observation underlines the importance of the three tested species in odour-mediated host-vector interactions of *An. gambiae*.

5.4.2 Bacterial and sweat specific effect on the response of *An. gambiae*

In the experiment where mosquitoes were offered to choose between sweat of the same gender but where one sample was incubated with one of the three bacterium species, no bacterium-specific effect was observed it terms of degree of preference. However, the overall responsiveness of the mosquitoes (R %) was particularly high in tests performed with *C. jeikeium* as inoculum and this was the case when either male and female sweat was used as substrate. The same effect was observed with *St. haemolyticus* but only when male sweat was used as substrate. *Staphylococcus* and *Corynebacterium* genera represent a large proportion of the microbial community that inhabit the human axillary region with one of these two genera being dominating in most individuals (Shehadeh and Kligman, 1963; Leyden et al., 1981; Troccaz et al. 2015). Remarkably, this outcome is in line with human sensory analysis performed on the same sweat incubations by Troccaz et al. (2004; 2009), where *St. epidermidis* was evaluated as the least sweat-like odour producer compared to *C. jeikeium* and *St. haemolyticus*. The high responsiveness of *An. gambiae* to *C. jeikeium* inoculated substrates is also of particular interest since earlier investigations have correlated human axillary odour intensity with higher *Corynebacterium* population density (Leyden et al., 1981; Jackman and Noble, 1983). Moreover, in Verhulst et al. (2010b) differential attractiveness of *An. gambiae* to different bacteria species grown *in vitro* revealed a *Corynebacterium* species (*C. minutissimum*) that produces particularly attractive VOCs for this insect vector of disease (Verhulst et al. 2010b). It seems that *Corynebacterium* spp. play a role of particular importance in the odour-mediated and anthropophilic host-seeking behaviour of *An. gambiae*.

The sterile sweat samples originated from collections on 49 humans volunteers (24 men and 25 women). This representative male and female sweat pool allowed conducting experiments where mosquitoes were offered to choose between male and female sweat both incubated with the same bacterium species. Surprisingly the mosquitoes always chose male inoculated sweat, independent of the bacterium species used. This result was confirmed when the additive effect of odour resulting from incubation of sweat samples with each bacterium spp. was presented simultaneously. That this result originated from an intrinsic attractive nature of male sweat can be excluded because when offered to choose between male and female sweat incubated without any bacterial inoculum no preference by the mosquitoes was observed. The action of bacteria was required to render male sweat more attractive. This outcome can result from two effects or a combination of both. Firstly, sweat originating from the male pool, in terms of its quantitative composition, provides a better substrate for bacteria resulting in male sweat

producing odours for *An. gambiae* in higher amounts than inoculated female sweat. The sweat samples showed notable differences in pH and glucose content which can be an important source of carbon for Gram-positive bacteria (Troccaz et al., 2009). Since male sweat contained more glucose than female sweat it cannot be excluded that bacterial growth was higher in which case the observed differential response would have a quantitative origin. Secondly, the presence of a male specific product(s) could be responsible for the more attractive nature of the incubated male sweat. Since they are the result of gene expression, proteins are likely to be the substrate metabolized by bacteria into such hypothetical sex specific products. Since the protein level of both male and female sweat samples were similar (Troccaz et al., 2009) this remains plausible. To reach a conclusion as to the origin of the higher response of *An. gambiae* to male sweat, a detailed chemical analysis needs to be made in order to establish whether the differential response of the mosquito originates from sweat gender specific product(s) or a difference in the amounts of odour products. On this matter, it is also important to note that researches working on axillary sweat odour collected from nearly 200 adults could identify a gender-specific odour signature (Penn et al., 2007, Zeng et al. 1996). On the other hand, studies have suggested that individual differences in underarm odour are mainly quantitative (Bernier et al., 2002; Curran et al., 2005).

In terms of bacterial specific effect, the results obtained in the experiments where male sweat was tested against female sweat are in accordance with those where the same sweat was used in each tube of the olfactometer. In olfactometer tests where male and female sweat samples were both inoculated with *C. jeikeium* elicited the strongest response (R% approached 100%) at the downwind end of the olfactometer. Moreover, the difference in odour resulting from male and female sweat incubation with this bacterium species elicited the highest degree of preference for male sweat. Male sweat inoculated with *C. jeikeium* elicited the most distinct response from *An. gambiae*. In Troccaz et al. (2009) human sensory analysis showed that when incubated with *C. jeikeium* male sweat had a particularly intense sweat-like odour. This result underlines that both the microfloral composition and sweat constituents contribute to the selective odour-mediated host seeking behaviour of *An. gambiae*. The odour specificity rendering a person more susceptible to biting by *An. gambiae* is probably induced by the combination of an adequate sweat composition and accompanying bacterial profile.

5.4.3 Response of *An. gambiae* to 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol

Adult humans, via their apocrine glands, excrete water soluble compounds which are unique to humans. These compounds are transformed by the axillary microflora (Corynebacteria and Staphylococci) into MSH and HMHA. In Chapter 4 it was shown that the experimental set-up used here provides an adequate approach to test infochemicals such as lactic acid that may be involved the both *An. gambiae*. The response of *An. gambiae* to different doses of these two compounds was therefore tested. At lower doses *An. gambiae* showed no preferential behaviour for the arm of the olfactometer bearing either of these test compounds. At higher doses that are questionable in terms of physiological relevancy HMHA was clearly repellent and MSH slightly attractive. From these findings it cannot be concluded that either of these two compounds (as shown for lactic acid in Chapter 4) as used by *An. gambiae* to differentiate between two odour sources. However, it must be noted that the overall responsiveness of mosquitoes (R%) was significantly higher than in control experiments at almost all the tested doses, including lower ones of MSH, and for one dose of HMHA. Those results suggest that these compounds may play a role in activating mosquitoes at a distance and recruited them preferentially. Although *An. gambiae* is known to be anthropophilic, behavioural studies investigating responses of this mosquito sp. to human-specific odour cues remain scarce. Costantini et al. (2001) described the behavioural and electroantennogram responses of *An. gambiae* to 3-methyl-2-hexenoic acid and 7-octenoic acid, two other compounds that are specific to humans and important constituents of axillary odour. Mixed together, these two compounds were found to inhibit the response to known long range attractants such as CO_2 and 'whole human odour' indicating that such compounds interrupt chemoanemotactic upwind flight to signal to the vector that it is close to the host. This inhibitory effect on the behaviour of *An. gambiae* observed for 3-methyl-2-hexenoic acid and 7-octenoic acid has also been observed at the electrophysiological level in recordings from single antennal and palpal olfactory receptors cells (Qiu et al., 2006; Lu et al., 2007; Carey et al., 2010). In the proximity of the host, the mosquito is confronted with higher levels of odour cues and in this context the clearly repellent effect of HMHA observed here at the higher dose of 100ng/µl may be relevant. As *An. gambiae* approaches its host at the level of the upper part of the body it is possible that HMHA elicits a host-specific descending response leading it to its preferred biting sites, namely the feet and ankles (Dejong and Knols, 1995). To confirm this hypothesis, recordings of the entire sequence of host approach behaviours in 3D would be appropriate.

5.4.4 The role of human axillary odour in the host seeking behaviour of *An. gambiae*

Due to its selective habit to feed on the feet and ankles (Dejong and Knols, 1995; Dekker et al., 1998) it might be tempting to conclude that *An. gambiae* uses foot odour to orient to its host. In the situation of standing humans the vector has often been reported to approach its host in the upper part of the body followed by a descending movement to the feet (Lewis et al. 1969, Dark et al. 1975, and personal observations). Our results show that *An. gambiae* strongly responds to axillary odour and in particular to the incubation of sweat with *C. jeikeium*, an important contributor to the generation of axillary odour. Human axillary odour is thus an important cue for the vector to locate a human host.

Underarm odour harbours many host specific constituents and so presents properties that could explain the selective host-seeking behaviour of *An. gambiae*. For example, apocrine glands only become active at puberty, indicating that human odour profile changes with age (Stoddart 1990). This is of special interest since *An. gambiae* has on several occasions been documented to prefer adults over children (Muirhead-Thomson, 1951; Boreham et al., 1978; Bryan and Smalley, 1978; Carnevale et al., 1978; Port et al., 1980). When it comes to preference by the mosquito for a particular sex, the question remains controversial. In this study, male sweat was preferred over female sweat incubated with the same bacterium. However, even though both sweat pools may be representative, only three bacterium species were tested and it remains to be determined whether the difference in attractiveness had a quantitative or a qualitative origin. Using the same sweat samples, Troccaz et al. (2009) showed that through the action of axillary microflora female sweat seems to have the potential to liberate significantly more MSH relative to HMHA. Our olfactometer experiments with these two compounds did not provide data that could be correlated with this. In other words, *An. gambiae* was neither repelled by MSH nor strongly attracted to HMHA. Moreover, field studies where human gender preferences by *An. gambiae* and *Aedes aegypti*, another anthropophilic mosquito species, were investigated remain controversial (Rahm, 1956; Gilbert et al., 1966; Carnevale et al., 1978).

Past research suggests that the malaria parasite may both enhance the vectors blood feeding behaviour and the attractiveness of human hosts (Koella et al., 1998; Lacroix et al., 2005). Enhanced attractiveness of malaria infected humans even seems to be odour mediated (De Moraes et al., 2014; Berna et al., 2015; Kelly et al., 2015). More recently a key apicomplexan metabolite intervening in this odour mediated mechanism has been identified (Emami et al., 2017). Baring this in mind and on the basis of the findings of this thesis, it would definitely be

interesting to further investigate how malaria infection alters the composition of the microbial community involved in the production of axillary odour.

6 Conclusions

6.1 On respiratory physiology in *An. gambiae*

Female *An. gambiae* starts its adult life with few teneral reserves (Briegel, 1990b), it is rapidly able to blood feed after emergence (Briegel and Hörler, 1993; Hörler and Briegel, 1995). Blood ingestion is accompanied by prediuretic excretion resulting in increased blood meal volume and duration allowing more protein uptake (Briegel and Rezzonico, 1985). This mosquito also engages in multiple feeding at short intervals (Briegel and Hörler, 1993; Hörler and Briegel, 1995). The first blood meals may be used to compensate for low teneral reserves whereas nutrients from subsequent blood meals are eventually allocated to oogenesis (Briegel, 2003; Fernandes and Briegel, 2005). Sugar feeding can also contribute to metabolic demand of female *An. gambiae* in a domestic environment (Foster 1995; Gary and Foster 2004). However it has been shown that *An. gambiae* can replace sugar with increased blood feeding without suppressing reproductive fitness even if life span is reduced (Briegel and Hörler 1993; Gary and Foster 2001; Straif and Beier 1996). Strong flight performance can be maintained with blood feeding alone by *An. gambiae* (Kaufmann and Briegel 2004). Sugar feeding, seems to not occur during but between gonotrophic cycle if blood donor are not available and under environmental constraints (Gary and Foster 2006). In an endophilic and endophagic context metabolic demand seems to be particularly associated with blood feeding behaviour by female *An. gambiae* (Beier 1996). Baring this in mind the relationship between body size and temperature with resting metabolic rate established here is of particular relevance as it can be used as a proxy for biting frequency and eventually to model malaria transmission rate. Knowing that most of biodiversity on earth is located in the tropics and that the exponential relation between temperature and metabolic rate is particularly pronounced at higher temperature, the global metabolic impact resulting from climate change can be expected to have important consequences on malaria transmission rates. It must, however, be taken into account that a higher resting metabolic rate besides augmenting biting frequency may eventually lead to an increased mortality rate. There is nothing new in the fact that metabolic rate is strongly influenced by body size and temperature, something that is particularly true in small ectotherms like *An. gambiae* (Brown et al. 2004; Chown and Nicolson 2004).

In this thesis more than this simple relation is documented. For the first time the gas exchange pattern and its underlying components is described for a small mosquito. This is not only

relevant for *Anopheles* species, that include the main malaria vectors, but also more generally in terms of insect respiratory physiology as not much is known about the gas exchange pattern of smaller insects. A few investigations on mosquitos respiratory physiology using modern methods exist but none of them served to resolve the gas exchange pattern of a small mosquito in detail (Gray and Bradley 2003, 2005, 2006a and b; Huestis and Lehmann 2014; Huestis et al. 2011 and 2012). As pointed out by Gray and Bradley (2006a) when using the technique of flow-through respirometry, the investigator can manipulate 3 parameters in order to augment the temporal resolution of the measured gas exchange rate. The first one is the time constant of the test chamber which is negligible in the present case considering its volume and the flow rate at which the CO_2 production rate at rest was measured. The second is the volume of the detection chamber of the gas analyser that is much more limiting in the present study since it takes close to 3 seconds for a single CO_2 burst to navigate across the gas detection chamber. Finally, and not least important, temperature is also a critical parameter that can influence the temporal resolution of the system. In a situation of an apparent cyclical pattern, CO_2 burst frequency can be decreased by lowering temperature, resulting in prolonged inter-burst periods which will augment the chance of observing the lowest rate of gas exchange. I conducted my respirometry experiments with a small test chamber (0.5ml), a high flow rate (250ml/min) and also at lower temperatures (20°C) which allowed recording of inter-burst CO_2 production rates that were barely distinguishable from zero, suggesting the use of discontinuous gas cycles by *An. gambiae*. According to the definition provided in the literature (Chown 2011) it can even be admitted that *An. gambiae* is among the smallest insect using this highly regulated form of breathing observed to date (see Marais et al. 2005 for an overview for insects employing discontinuous gas exchange cycle studied to date). According to the present findings it must, however, be said that at rest and under median temperature levels encountered in its habitat, the rule is probably cyclical gas exchange in *An. gambiae*.

According to the gas exchange pattern observed by *An. gambiae* ranging from both discontinuous and cyclic to only cyclic to continuous when activity increases, it is concluded that this small mosquito employs all the three patterns described so far in the literature (Chown 2011). It can even be concluded that the observed gas exchange pattern was dependent on the level of the resting metabolic rate then cyclic and discontinuous gas exchange occurred when resting metabolic rate was lowest. Interestingly, cyclic and discontinuous gas exchange pattern was observed in mosquitoes of different sizes and ages. That the intraspecific diversity of gas exchange patterns employed should be dependent on the level of the metabolic rate has already been suggested (Contreras and Bradley 2009 and

2010). Considering its small size and the range of behaviours undertaken during adult life, from longer periods at rest during aestivation (very low MR) to flying to seek an appropriate host and eventually ingest warm blood (high MR), I propose that the ability to employ various GEP and modulating their components might be an advantage to adapt the respiratory gas exchange pattern used by the mosquito to metabolic demand depending on the situation encountered during adult life. This would suggest that the discontinuous and cyclic gas exchange patterns cannot be considered as a distinct and are used by more insects than previously thought (Marais et al 2005). Those insect species that have been reported to employ only cyclic and/or continuous gas exchange (Marais et al. 2005) may be worth retesting by applying FTR at higher flow rates and conducting respirometry at lower temperatures. It would also be interesting to perform a meta-analysis of those species that have been reported to use discontinuous and cyclical gas exchange patterns over a wide MR range (at intervals from the lowest to the highest MR at rest) compared to their size range and encountered thermal amplitudes in the field.

This thesis also presents a first quantification of the underlying components characterizing the gas exchange pattern of *An. gambiae*. By using an alternative evaluation method (Kernel Density Estimate), the body size scaling and temperature dependency of inter-burst CO_2 production rate and duration, CO_2 burst frequency, duration, amplitude and volume revealed notable and distinct gas exchange traits in female *An. gambiae*. With increasing temperature, it is shown that CO_2 burst frequency strongly modulates the gas exchange rate by increasing faster than resting metabolic rate. This discrepancy is almost compensated by smaller CO_2 burst volumes at higher temperatures suggesting a decrease in the haemolymph buffering capacity for CO_2 with increasing temperature. CO_2 burst frequency is independent of body mass whereas burst volume scales disproportionally with body size, suggesting a relatively larger tracheal volume in bigger mosquitoes. It is suggested that this adaptation may be used to withstand situations where metabolic demand is particularly high such as when flying or during blood ingestion. More generally, optimal control of metabolic rate seems to be acquired after a certain age. Downregulation is mainly achieved by downregulating CO_2 burst frequency with a prolonged inter-burst duration at lower gas exchange rates. Aging does not affect CO_2 burst volume and its underlying components (amplitude and duration). Respiratory water loss probably occurs but most probably accounts for only a small portion to overall water loss. In this study the mosquitoes that lost the highest amount of water had a rather water-conserving gas exchange pattern (6-day-old mosquitoes lost more water and had lower inter-burst CO_2 production rate and duration). In this sense the present findings are rather

supportive of those of Gray and Bradley (2005) who rejected the 'hygric hypothesis' in *Anopheles* and attribute desiccation resistance of *An. arabiensis* to higher water content at emergence compared to *An. gambiae*. Other adaptive explanations for the occurrence of discontinuous gas exchange cannot be excluded (reviewed and discussed in Chown et al. 2006; Chown 2011; Lighton, 1996 and 2007; Matthews 2017). It would ultimately be interesting to investigate further whether or not *An. gambiae* displays discontinuous gas exchange cycles under hypercapnia reflecting the environment surrounding human beings due to the endophilic and endophagic habits of *An. gambiae*. Such an experiment could verify the 'chthonic hypothesis' suggesting that discontinuous and cyclic gas exchange has evolved in insects that are confronted with hypercapnic environment (Lighton and Berrigan 1995). It would also be interesting to investigate the gas exchange pattern of *An. gambiae* under anaesthesia in order to understand the role of the nervous system in regulating gas exchange or under prolonged dormancy reflecting aestivation. Such experiments could lead to conclusions regarding the validity on the 'neural hypothesis' suggesting that this gas exchange pattern is a nonadaptive consequence of an adaptive downregulation of brain activity encountered during eventual longer periods of rest such as when aestivating (Matthews and Withe 2010; Chown 2011).

6.2 On the quest for identifying human specific odour cues used by *An. gambiae*

Compared to other mosquitoes, *An. gambiae* is particularly dependent on blood feeding to sustain its metabolism. Next to this internal driving force, another external one reinforces its vectorial capacity by increasing its interaction with humans: *An. gambiae* seems to preferentially blood feed on humans and even seems to prefer certain human types over others. There is evidence that the selective human host-seeking behaviour of *An. gambiae* relies on the difference of odour emitted by humans compared to other vertebrate hosts (Costantini et al., 1993; Mboera et al., 1997; Costantini et al., 1998; Dekker and Takken, 1998; Dekker et al., 2001 and 2002) and certain human types compared to others (Knols et al., 1995; Dekker et al., 2002; Mukabana et al., 2002). The idendity of the infochemicals behind this selective host seeking behaviour and their point of action in the complex behavioural sequence leading the vector to its final preferred host remains poorly investigated. In this regard this thesis contributes in filling this gap of knowledge in two ways. First, by developing an experimental paradigm that was able to gauge the intensity of

anemochemotactic upwind flight towards a test odour by simultaneously testing if the same odour might be used by the mosquito to choose between two hosts. Second, by identifying important biological agents that intervene in both the odour-mediated interspecific and intraspecific host selection behaviour of *An. gambiae*.

The olfactory-driven behavioural sequence leading *An. gambiae* to human hosts starts with activation followed by sustained upwind flight (anemochemotaxis). In the dual-choice olfactometer experiments presented here, this important behavioural step was evaluated with R%. During sustained upwind flight additional infochemical input may redirect the flight of *An. gambiae* to its preferred host. To evaluate this second behavioural step the preference for an arm emitting the test odour was used as a proxy. The dual choice olfactometer developed permitted to test the host seeking behaviour of *An. gambiae* in the presence of continuous, well-controlled intermittent carbon dioxide stimulation in both arm. This background stimulation simulated artificially the presence of two potential hosts whilst acting as a sensitizer. When presented intermittently, CO_2 is well-known for its effect as an activator for mosquitoes and synergist when combined with other odours (Gillies 1980; Mboera et al. 1997; Geier et al. 1999a; Dekker et al. 2002, 2005). In olfactory-driven host-seeking behaviour of mosquitoes, rapid CO_2 fluctuations have also been shown to function as a "releaser" of higher sensitivity or responsiveness of mosquitoes to the presence of a potential host (Dekker et al. 2005). In this manner, the number of responsive mosquitoes tested could be maximized. Certainly, working with appropriate airflow conditions including humidity, temperature and speed, using a test population of mosquitoes that was ascertained to be in a state to seek a host also contributed to the strength of the experimental paradigm. It was important to verify that this background stimulation was repeatable and generated a uniform (equal mosquito distribution in both arms) and repeatable response to ensure that any differential response resulted solely from the effect of the test odour. In a similar olfactometer to the one used here, Dekker et al. (2002) showed that lactic acid, a human eccrine signature, does not augment the responsiveness of *An. gambiae* to fluctuating CO_2 levels but is probably used by this vector of disease to discriminate humans from other vertebrate hosts or even between different humans. The experimental paradigm presented here consequently offers a valid approach to test human-related olfactory cues that, on the one hand, potentially influence the responsiveness of the mosquito to a suitable host as simulated with CO_2 pulses and/or, on the other, are used by the mosquito to direct its flight to its favoured host odour

source. These two aspects of the mosquito's behavioural responses are of course not mutually exclusive.

After confirming the validity of the experimental paradigm describe above, I used it to investigate the response of *An. gambiae* to human specific odour coming from the human axilla. Human axillary sweat originates from sebaceous, eccrine, and apocrine glands. The composition of this sweat is also unique among living organisms. The water-soluble forms of HMHA, a N-glutamyl derivative, and the soluble form of MSH, a cysteinyl-glycine-S-conjugate, have not been found to date in any other species or primate (Natsch et al. 2006). These precursors are excreted in various ratios and concentrations by humans. The microflora of individuals varies considerably (Troccaz et al. 2015) with the consequence that an individual who is a high producer of precursors can release more HMHA and MSH depending on the type of microflora present. Incubation of sterile sweat with isolated bacteria colonies was preferred in this study for the selective odour release profiles obtained: *S. epidermidis* for its low odour-producing pattern, *C. jeikeium* for its strong Nα-acylglutamine aminoacylase activity that liberates many carboxylic acids in addition to HMHA, and *S. haemolyticus* for it capacity to liberate MSH from sweat along with other sulfur-containing compounds (Natsch et al. 2006). Sweat incubated with one of the three bacteria species was always preferred over sterile sweat of the same gender although the degree of preference by *An. gambiae* for sweat inoculated with either of the bacteria species did not differ significantly. Nevertheless, the percentage of responding mosquitoes (R%) was consistently higher in experiments performed with *C. jeikeium* as inoculum, irrespective of whether male or female sweat was used as substrate. The R% was also higher with *S. haemolyticus* as inoculum but only when male sweat was used as a substrate. When *An. gambiae* was offered a choice between male and female sweat samples both inoculated with the same bacterium species mosquitoes systematically showed a preference for male sweat. In these experiments, the arm of the olfactometer conveying odour from male sweat incubated with *C. jeikeium* induced the strongest preference and the highest percentage of R%. This suggests that *C. jeikeium* with its strong aminoacylase activity that liberates carboxylic acids including HMHA from human sweat is of particular importance in the sensory ecology of *An. gambiae*. On the other hand, *S. epidermidis* with its low odour-producing capacity in sweat systematically induced the lowest percentage of responding mosquitoes. Sweat samples incubated with bacteria induced different R% values but always permitted discrimination between the olfactometer arms.

Overall, our findings demonstrate that axillary odour metabolized by the 3-tested bacteria species is sensed by *An. gambiae* and plays an important role in the odour-mediated behaviours of *An. gambiae* toward human hosts. It also shows that both the bacterium species, that is the agents of transformation, and sweat constituents, that is the substrate, can contribute to different behavioural criteria in terms of percentage responding mosquitoes R% and the preference by *An. gambiae* for one or other arm of the olfactometer. A diverse community of bacterial species dominated by Staphylococci and Corynebacteria populates axillary sweat (Shehadeh and Kligman 1963; Leyden et al. 1981; Troccaz et al. 2015) and this diversity may be required to maximize the behavioural response of *An. gambiae*. Nevertheless, here I show that incubating axillary sweat with a single bacterium species is sufficient to elicit a strong behavioural response in *An. gambiae*. The high responsiveness of *An. gambiae* to *C. jeikeium* sweat incubations is of particular interest since earlier investigators have correlated human axillary odour intensity with higher Corynebacterium density (Leyden et al. 1981; Jackman and Noble 1983). Moreover, in Verhulst et al. (2010a), the behavioural response of *An. gambiae* to different bacteria species grown *in vitro* revealed a Corynebacterium (C. minutissimum) that produces volatile organic compounds that are pertinent to the host odour-mediated behaviours of this mosquito. It seems that Corynebacteria play a particularly important role in odour-mediated and anthropophilic host-seeking behaviours of *An. gambiae*. *An. gambiae* chooses odours emanating from incubation of male sweat inoculated with different bacteria species over female sweat incubated with same bacteria. On the basis of this result it might be tempting to conclude that human males are more attractive. Such a conclusion would be premature as the generated odour profile came only form 3 bacteria species and cannot be considered as representative of the entire population that inhabits the underarm region of humans. What is important to bear in mind is that the sweat composition also plays an important role. The specificity of the preferred human host is probably the result of a combination of both a sweat composition signature and personalised bacterial population. To reach a conclusion as to the origin of the stronger response of *An. gambiae* to male sweat, a detailed chemical analysis would need to be made, thus providing an opportunity for further research on the topic.

Among other carboxylic acid and sulfur-containing compounds, *C. jeikeium* and *S. haemolyticus* produce two human-associated compounds, HMHA and MSH (Troccaz et al. 2009; Natsch et al. 2006), which have not previously been tested on *An. gambiae*. Accordingly, the responses of *An. gambiae* to different doses of these two compounds were tested as part of this thesis research. The proportion test indicated an effect for both MSH and

HMHA at the highest doses tested, namely a preference for the olfactometer arm bearing MSH and an avoidance response in the test with HMHA. However, the doses tested at 100 ng for MSH and 1 µg for HMHA are arguably too high in terms of physiological relevance. Nevertheless, and in contrast to lactic acid, it must be noted that the percentage of responding mosquitoes (R%) was higher than in control experiments (CO_2 pulses alone) at almost all doses of MSH tested, including the lower ones and for one dose of HMHA. In view of this, one might cautiously suggest that MSH may signal the presence of human hosts at a distance or play a role in interspecific host selection and might be less relevant in intraspecific host selection when *An. gambiae* approaches a human host. HMHA was clearly repellent at a higher dose and may indicate to *An. gambiae* that it is time to descend to its preferred biting site (feet and anckles; see Dejong and Knols, 1995) but this is still an open question.

7 References

7.1 Citations

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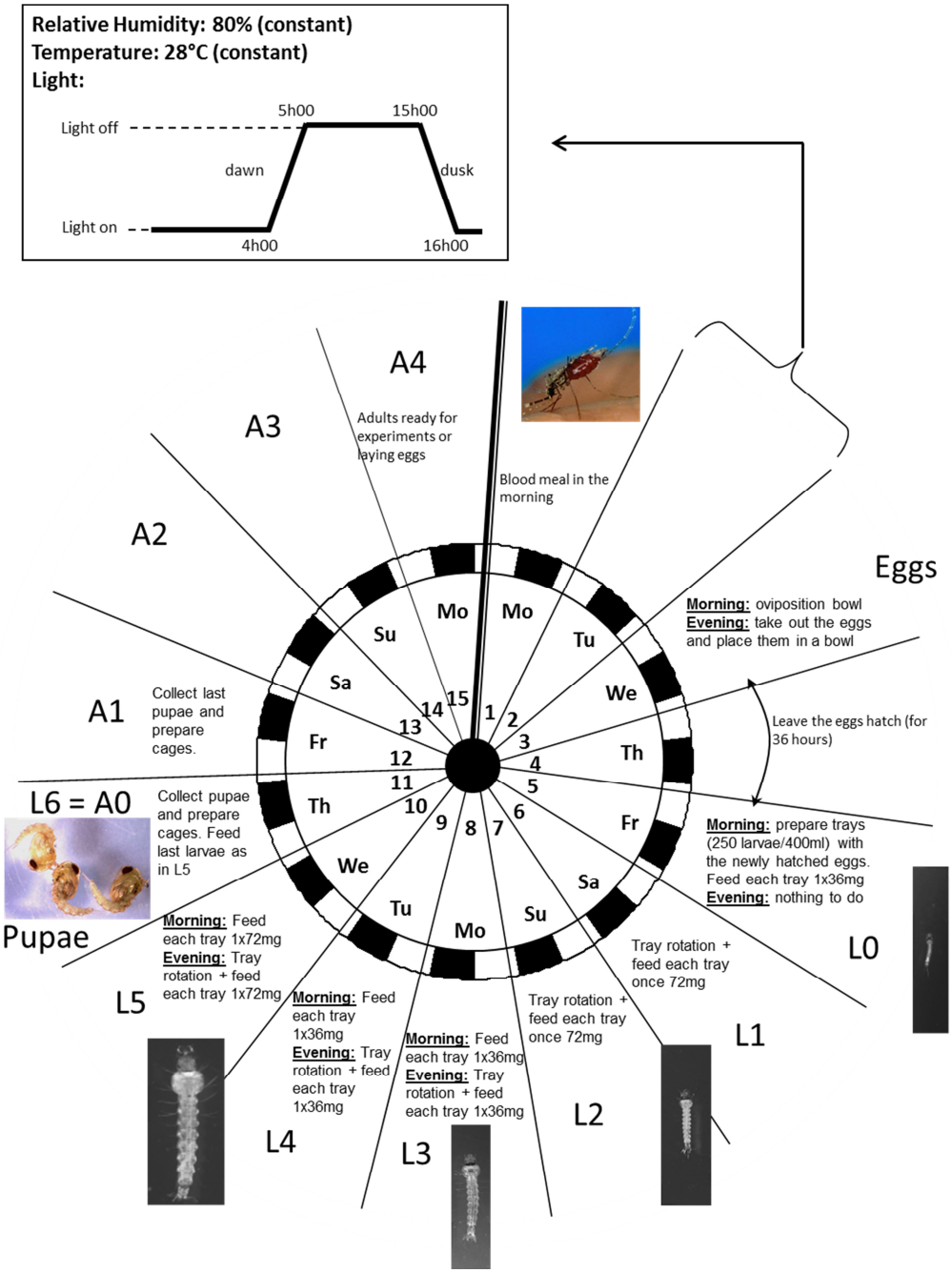
7.2 Publication resulting from the thesis

Frei, J., Kröber, T., Troccaz, M., Starkenmann, C. and Guerin, P. M. (2017). Behavioral response of the malaria mosquito, Anopheles gambiae, to human sweat inoculated with axilla bacteria and to volatiles composing human axillary odour. Chemical Senses **42**, 121-131.

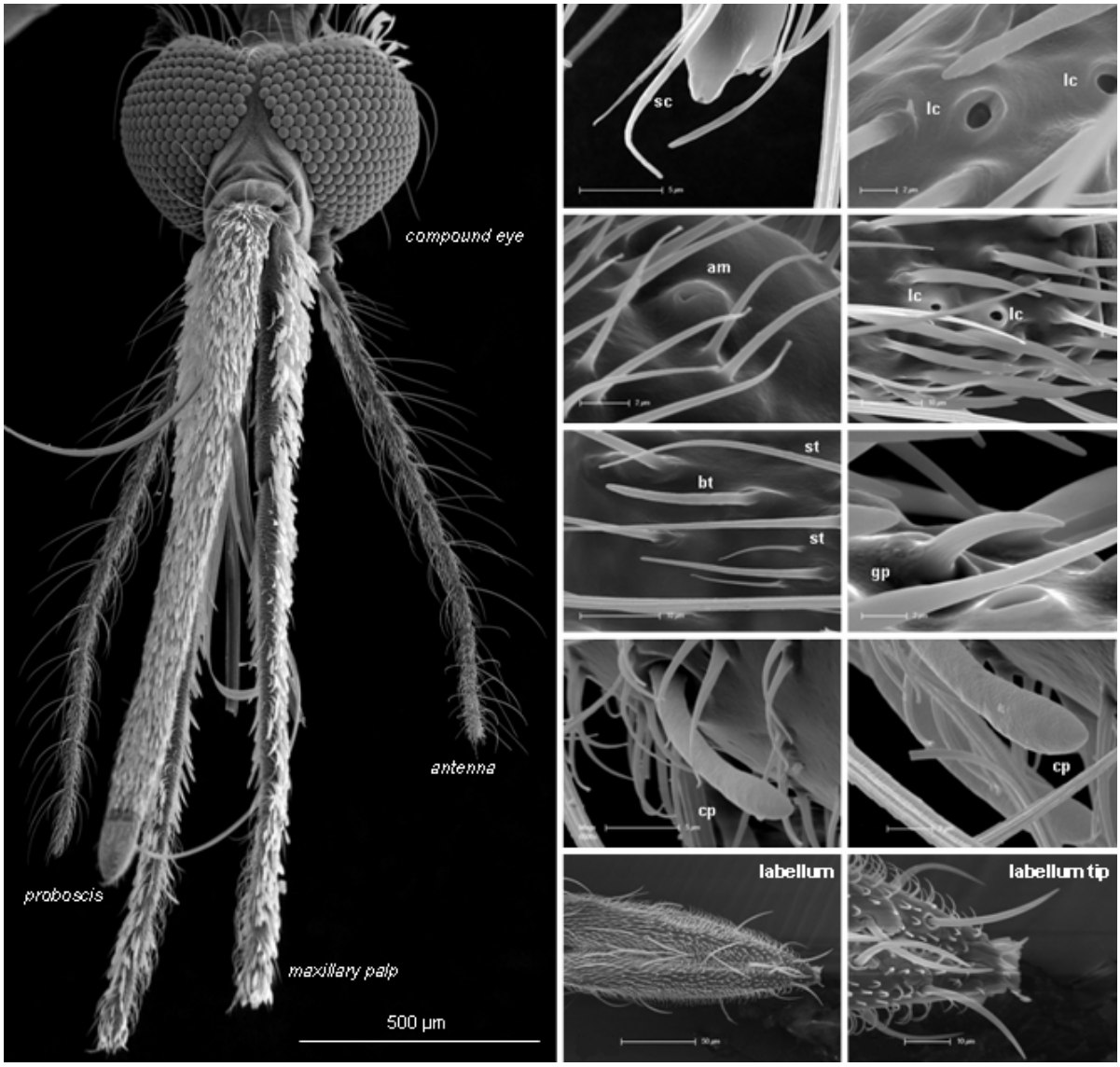
8 Appendices

8.1 Chapter 1

8.1.1 Protocol to maintain an *An. gambiae* colony in the laboratory



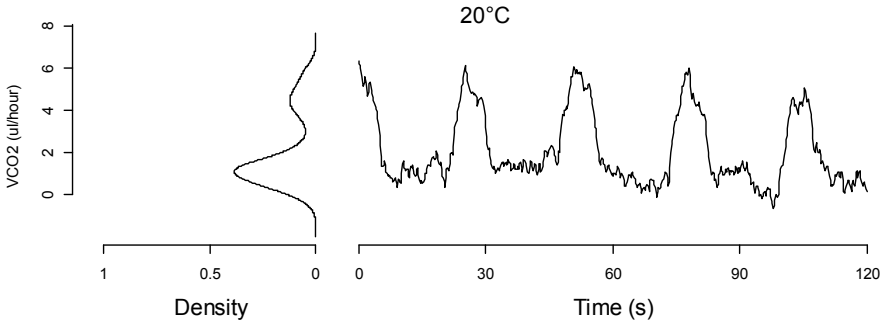
8.1.2 Scanning electron micrographs of the main sensory organs of *An. gambiae*

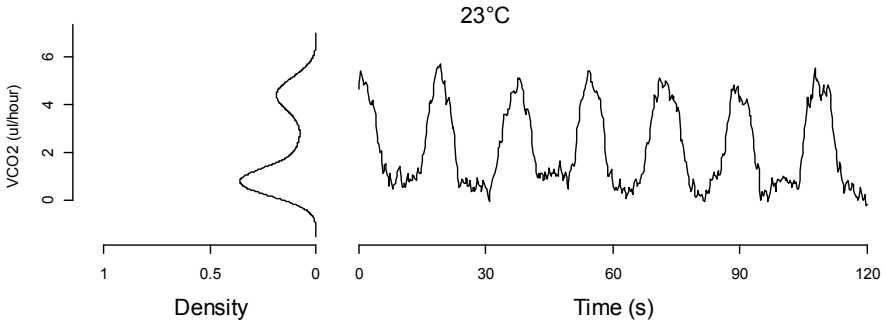


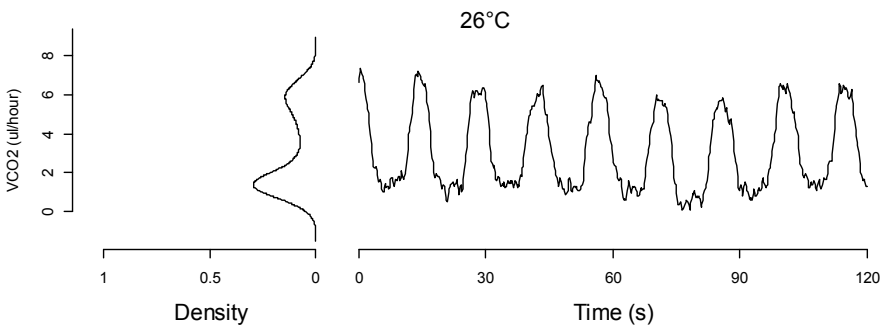
Main sensory organs of a female *An. gambiae*. To sense its host *An. gambiae* uses 3 main olfactory organs: the antennae, maxillary palps and the proboscis. The antennae carry numerous types of olfactory sensilla (upper six right-hand panels, scale bars: 5, 2, 2, 10, 10 and 2 µm) such as sensilla trichodea (st: sharp and bt: blunt), sensilla basiconica or grooved peg sensilla (gp), sensilla coeloconica (sc: small and lc: large) and sensilla ampullacea (am). The maxillary palps carry only one type of olfactory sensillum (fourth from top right-hand panels, scale bars: 5 and 2 µm): the multiporous capitated peg (cp). Although it was long thought that the proboscis was a gustatory organ only recent studies revealed that sensillae located at the end on the labellum (bottom right-hand pair of panels, scale bars: 50 and 10 µm) also possess olfactory receptor cells. These scanning electron microscopy (SEM) images were taken with a Philips XL20 (CSEM, Neuchâtel).

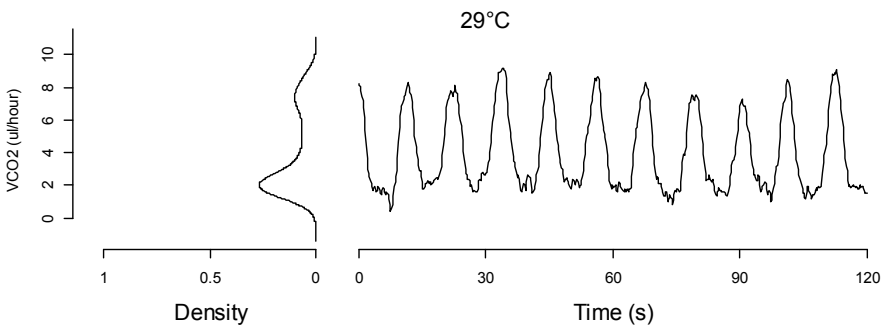
8.2 Chapters 2 and 3

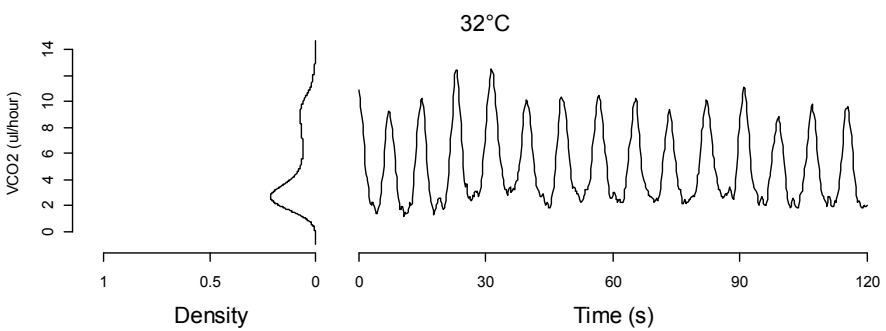
8.2.1 Extracts of FTR measurements from 43 *An. gambiae* with the corresponding KDEs.



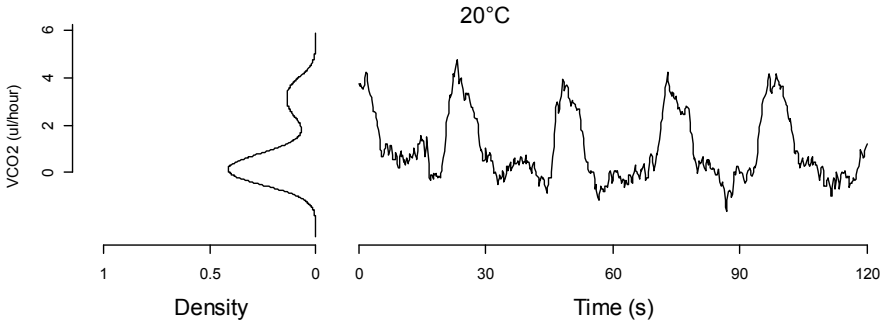


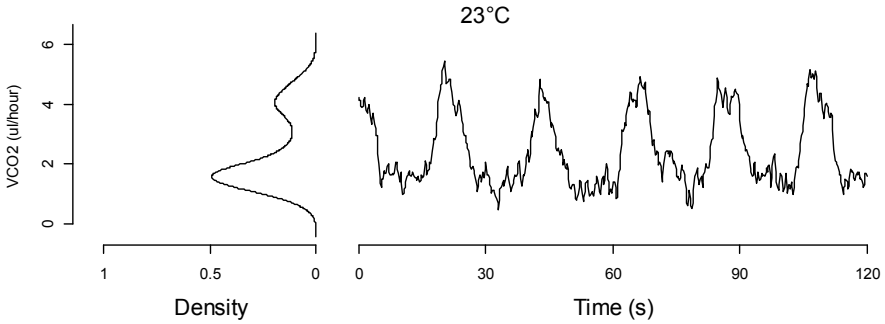


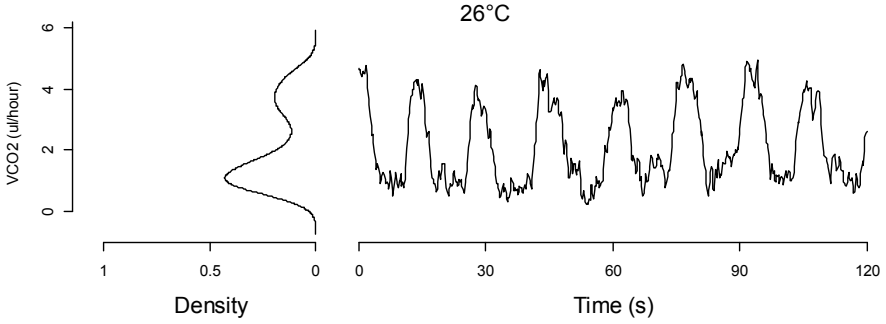


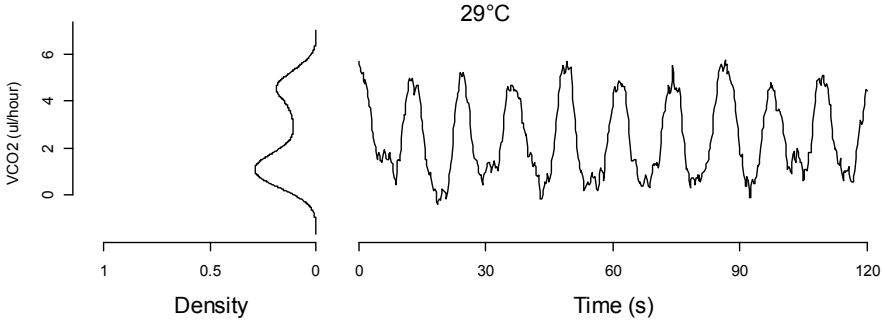


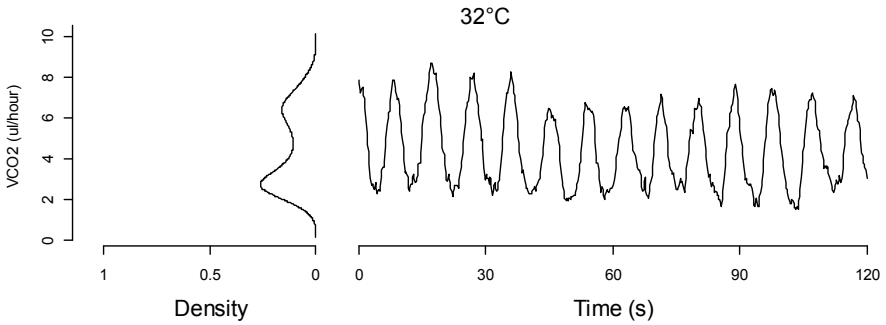
ID = u4
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.811
Living body mass (mg) = 2.2215
Wing length (mm) = 3.3075



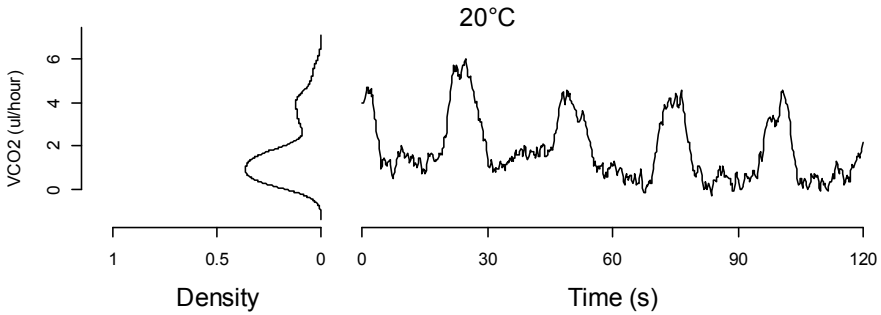


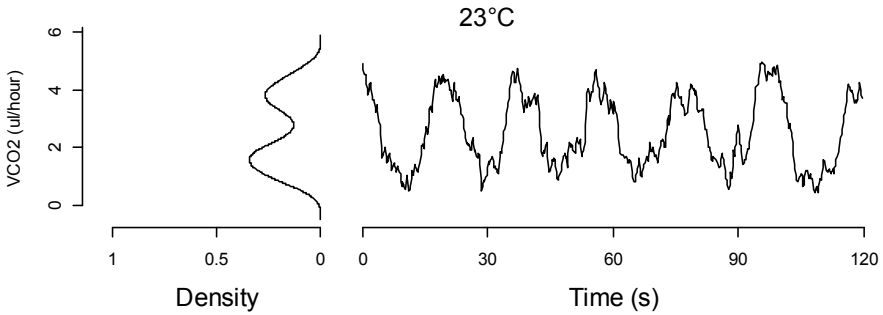


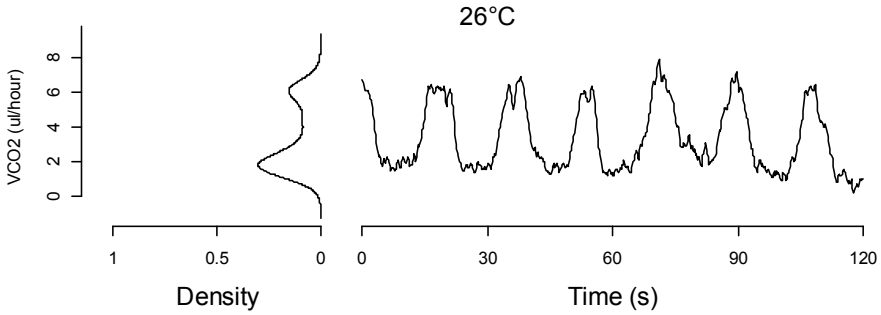


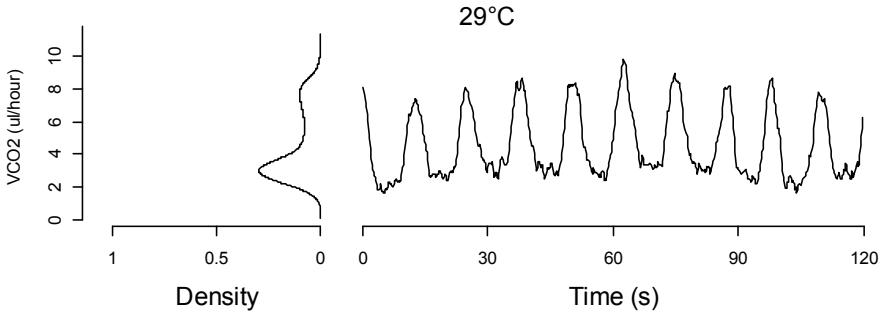


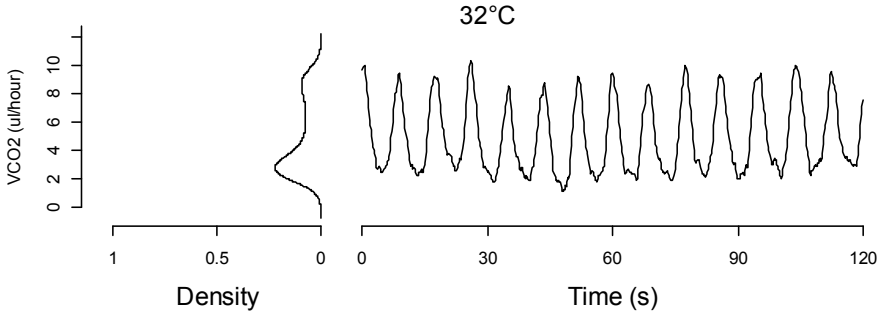
ID = u45
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.71
Living body mass (mg) = 1.949
Wing length (mm) = 3.42



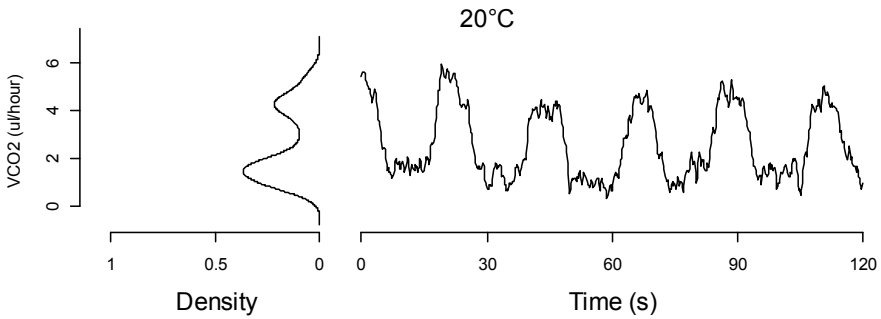


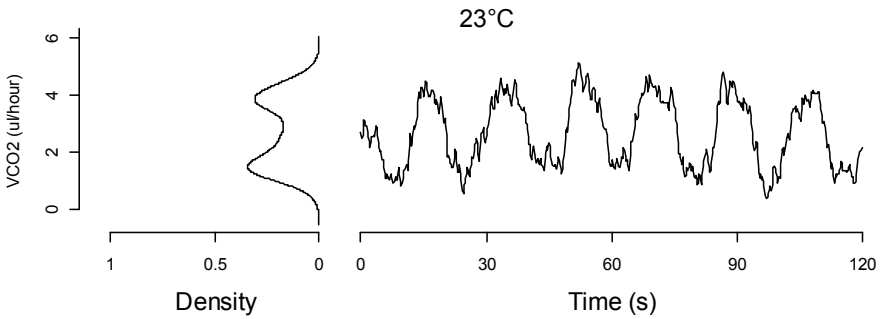


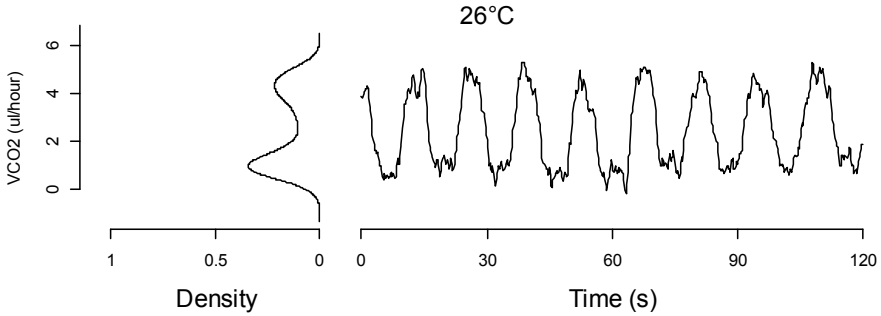


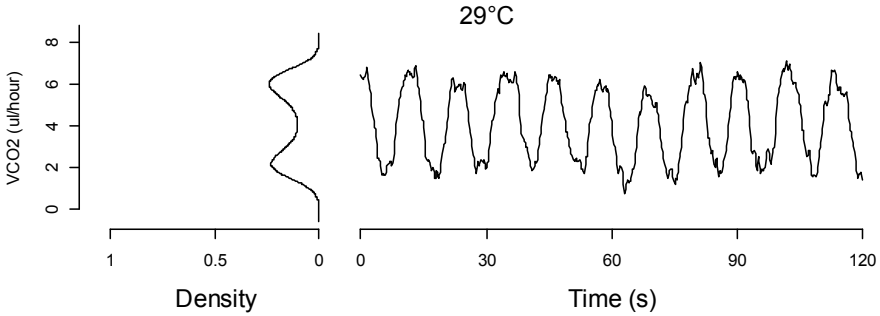


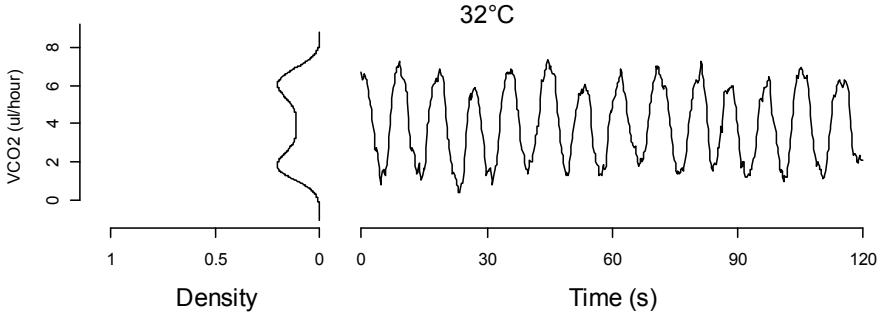
ID = u10
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.701
Living body mass (mg) = 1.9
Wing length (mm) = 3.1275



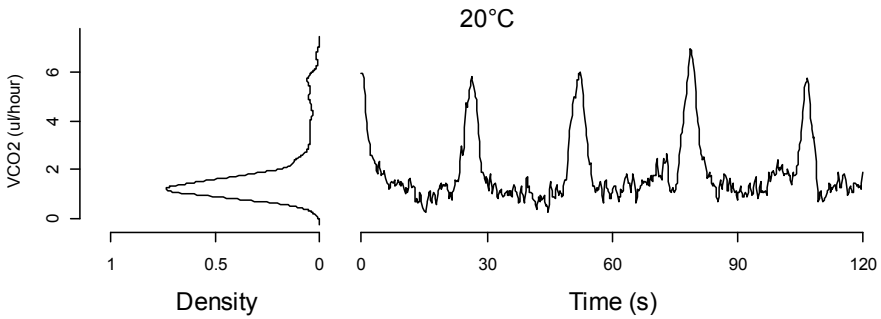


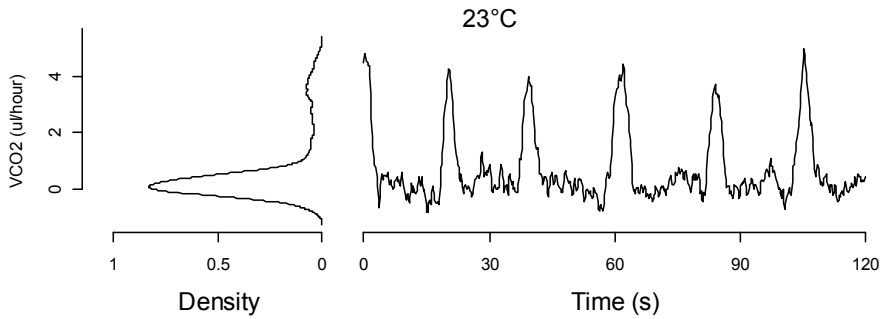


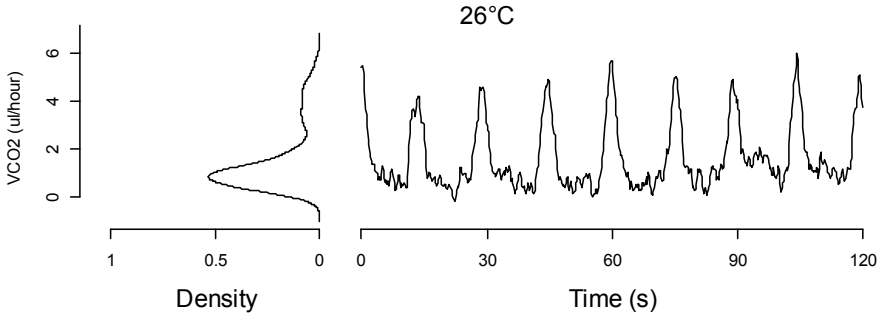


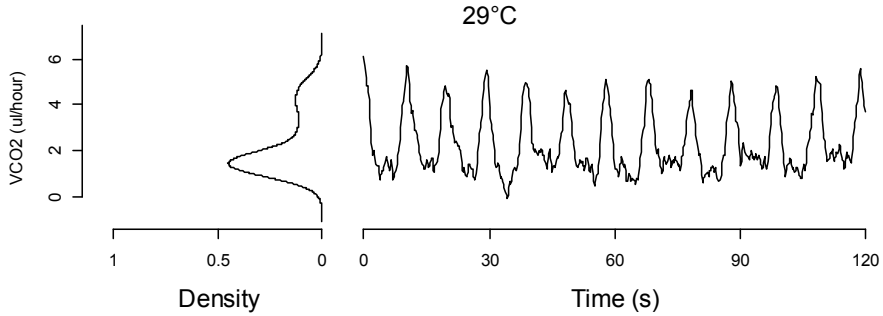


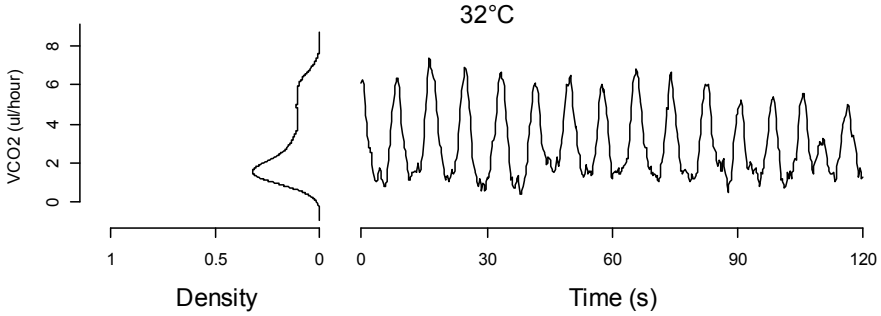
ID = u16
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.695
Living body mass (mg) = 2.0235
Wing length (mm) = 3.24



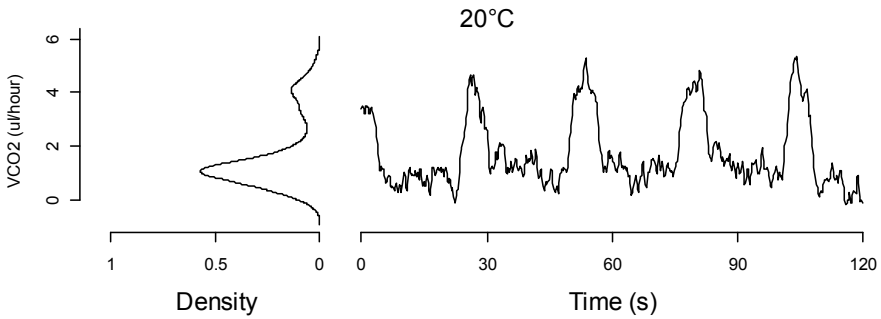


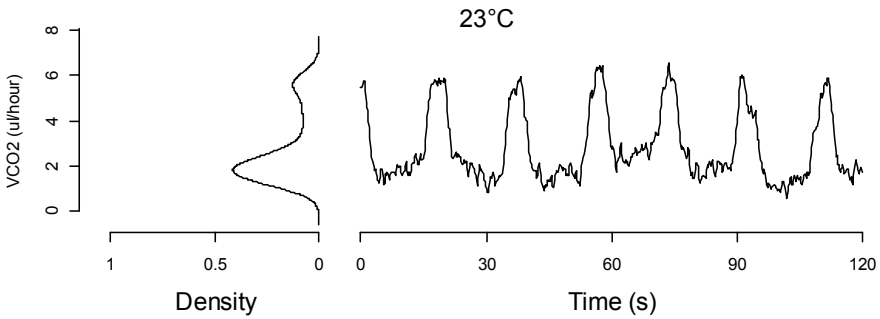


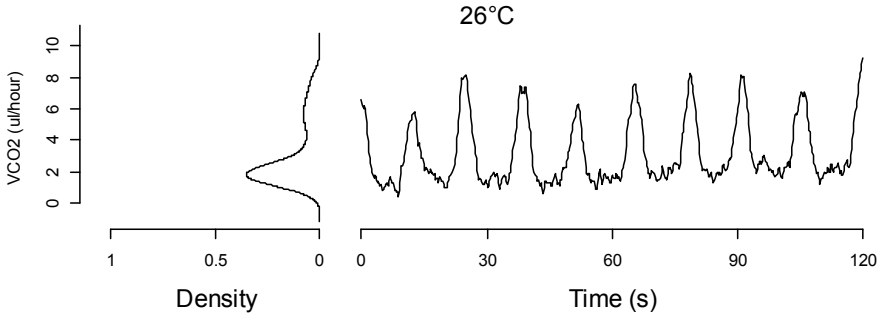


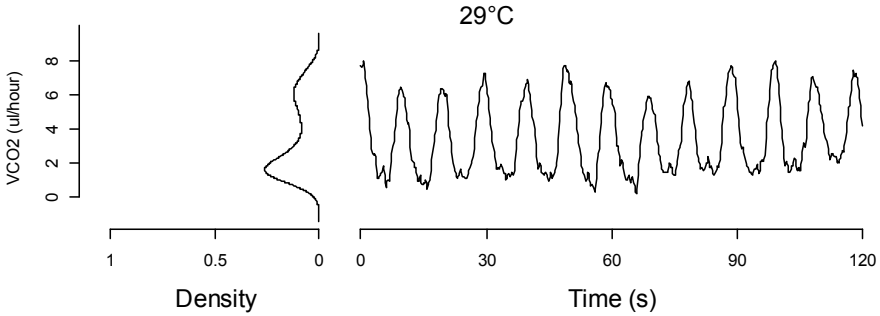


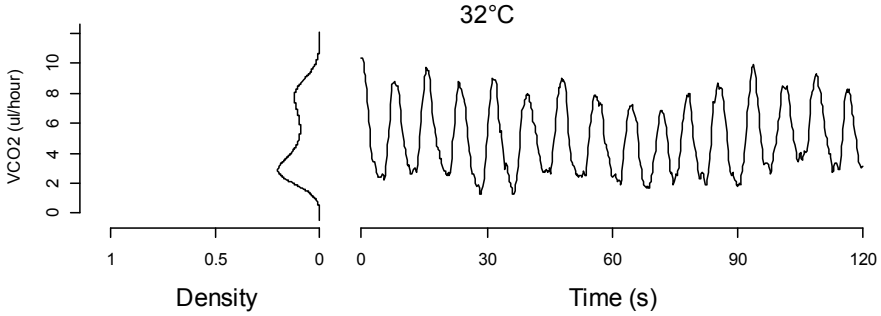
ID = d46
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.654
Living body mass (mg) = 1.837
Wing length (mm) = 3.375



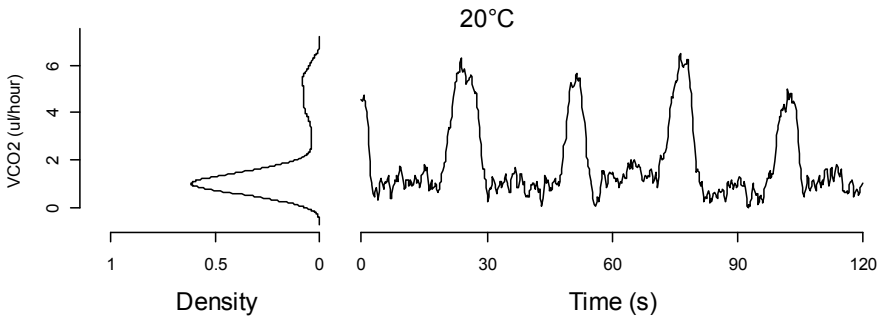


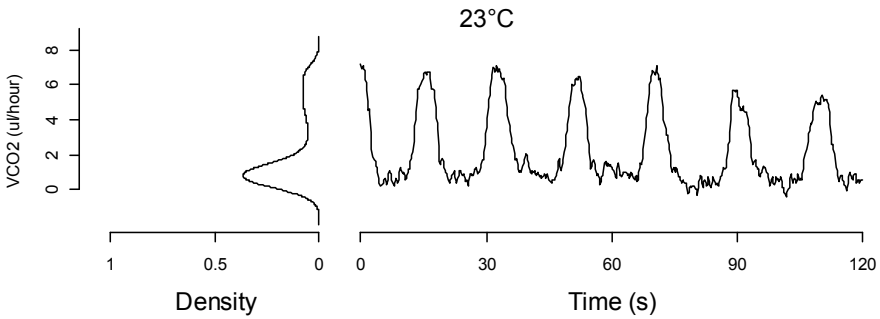


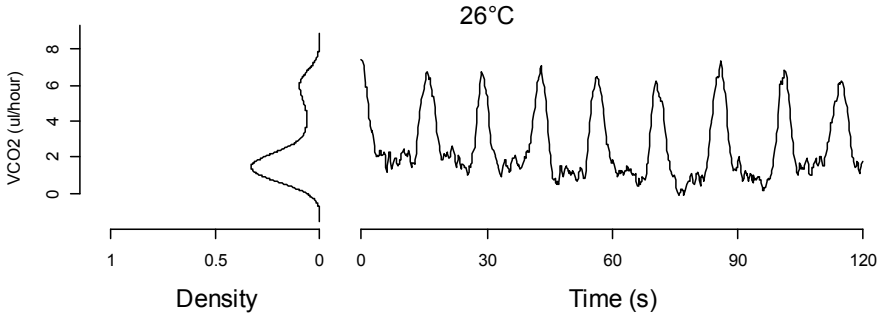


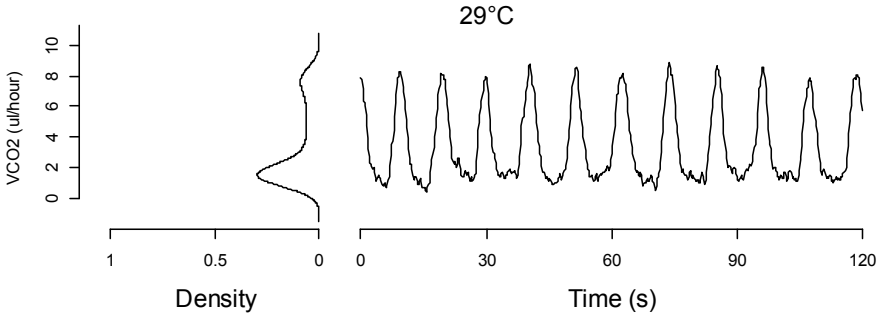


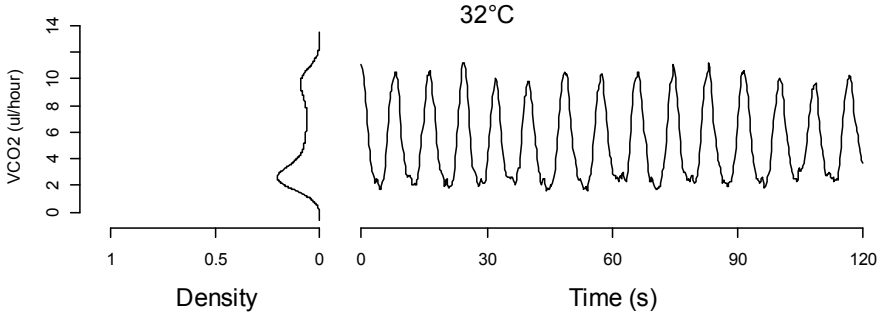
ID = d30
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.636
Living body mass (mg) = 1.816
Wing length (mm) = 3.06



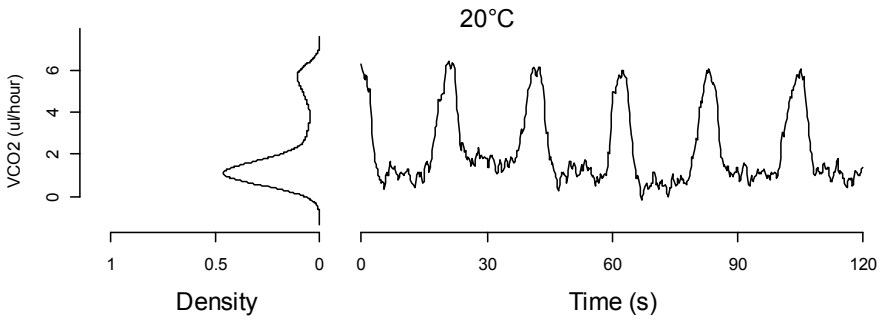


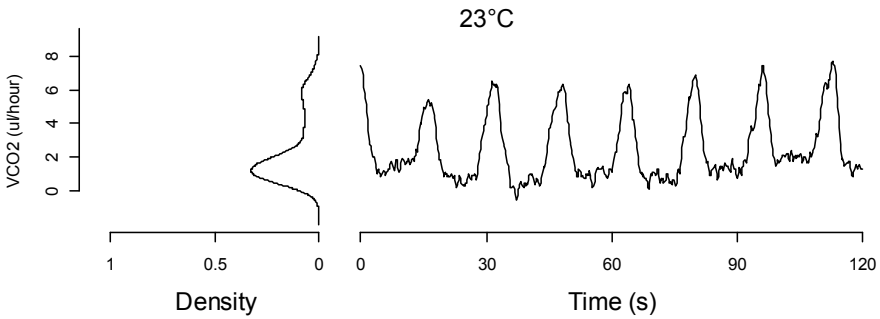


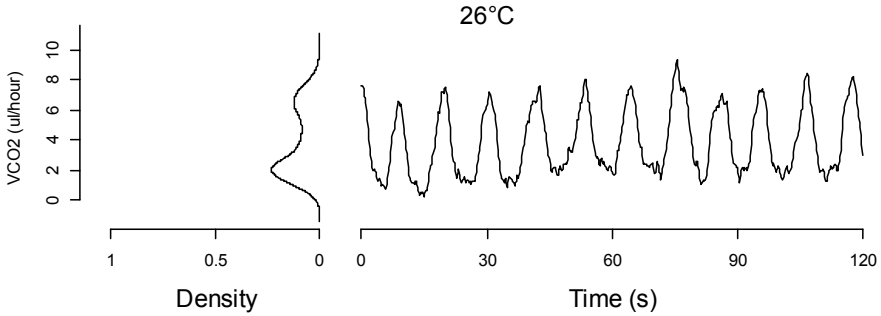


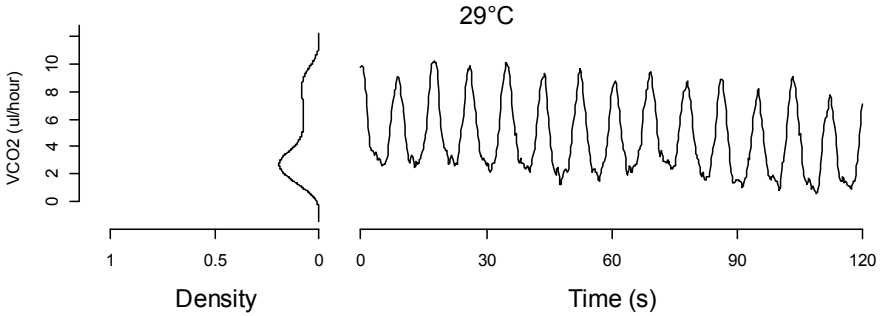


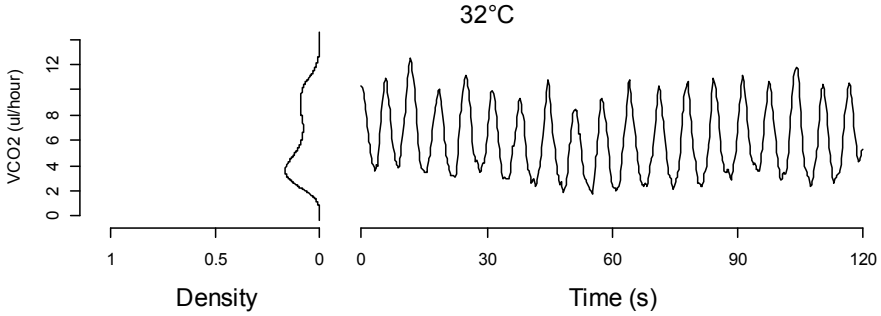
ID = d39
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.634
Living body mass (mg) = 1.7745
Wing length (mm) = 3.24



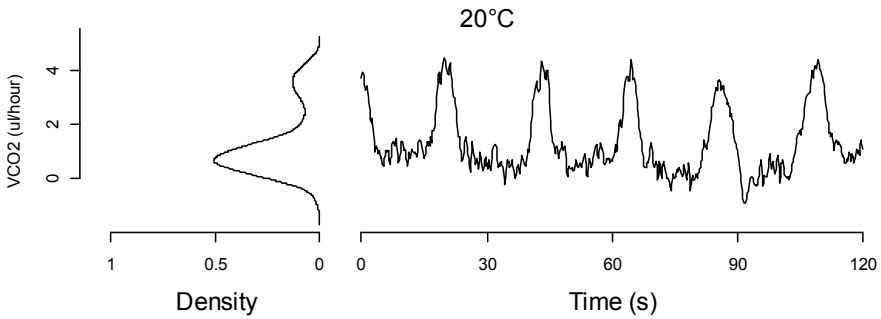


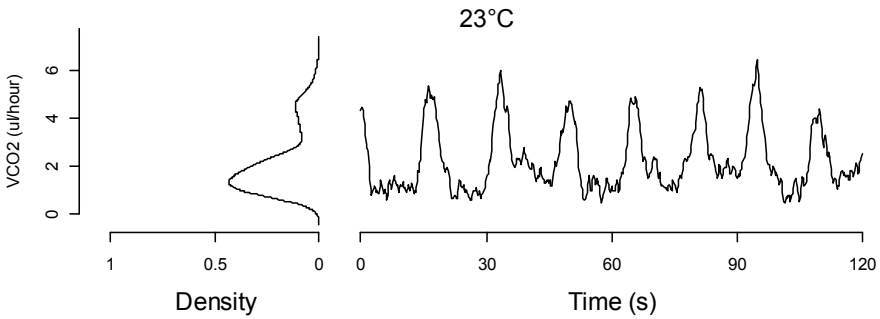


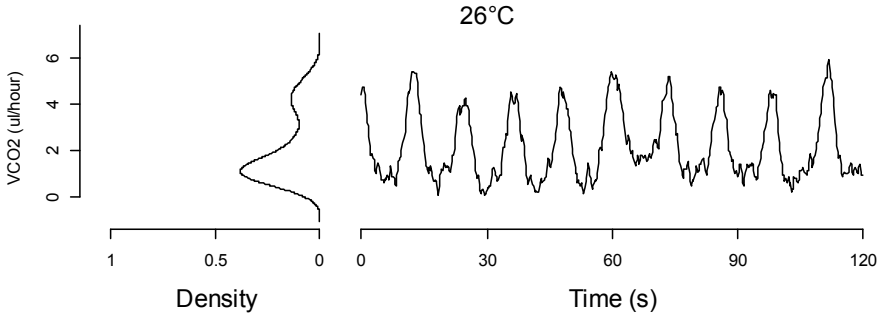


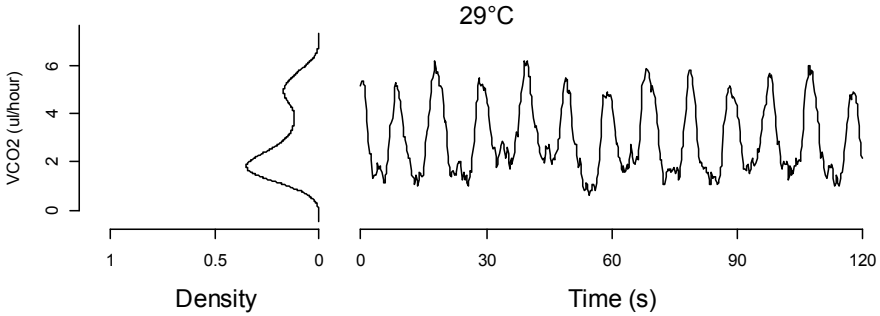


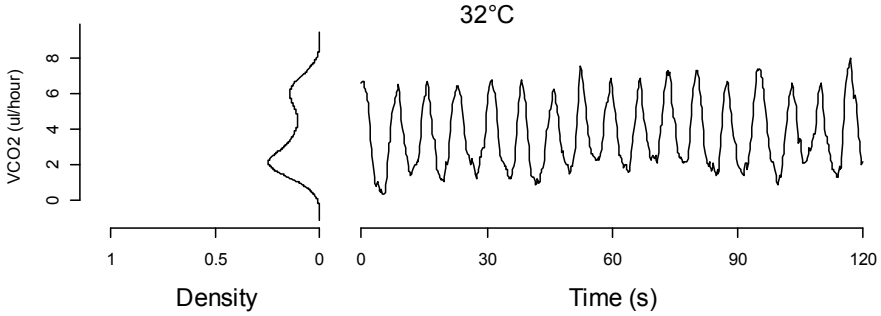
ID = u7
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.62
Living body mass (mg) = 1.852
Wing length (mm) = 3.06



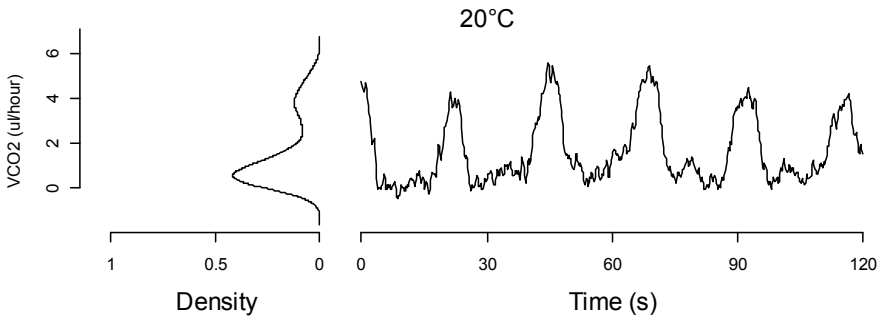


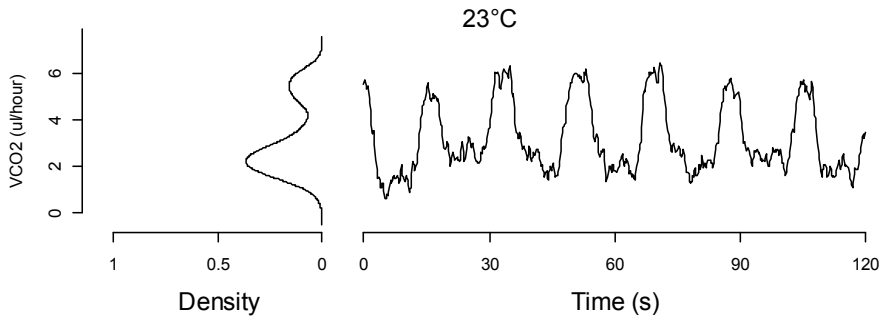


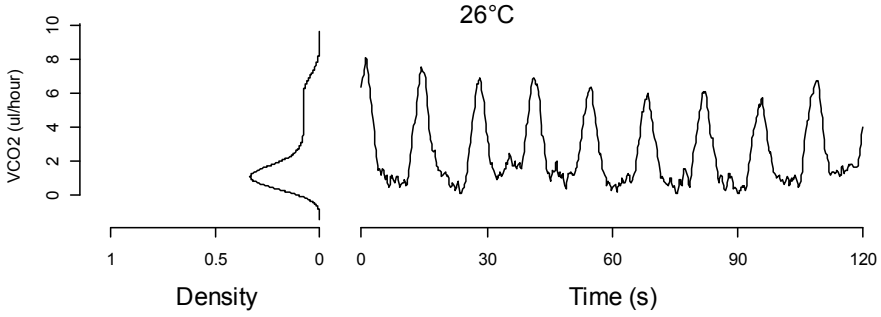


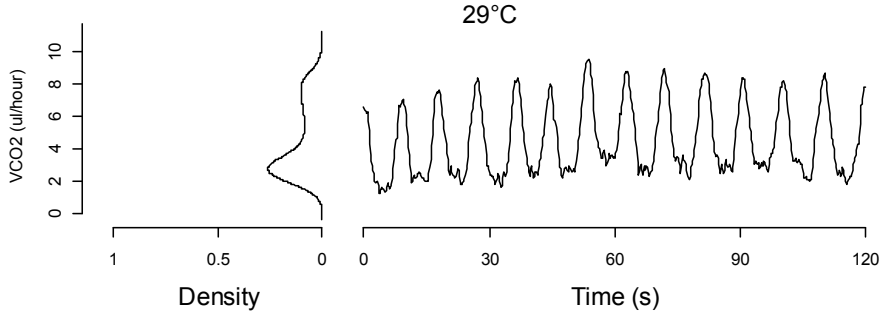


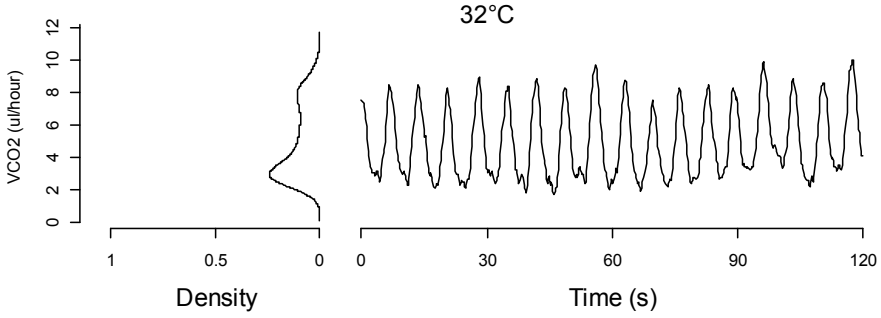
ID = u42
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.614
Living body mass (mg) = 1.625
Wing length (mm) = 3.0825



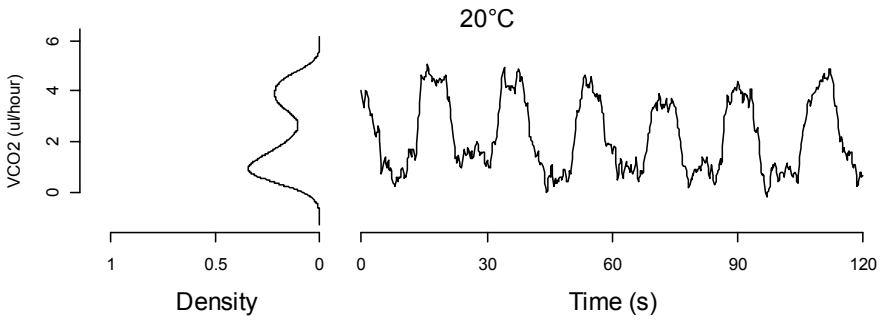


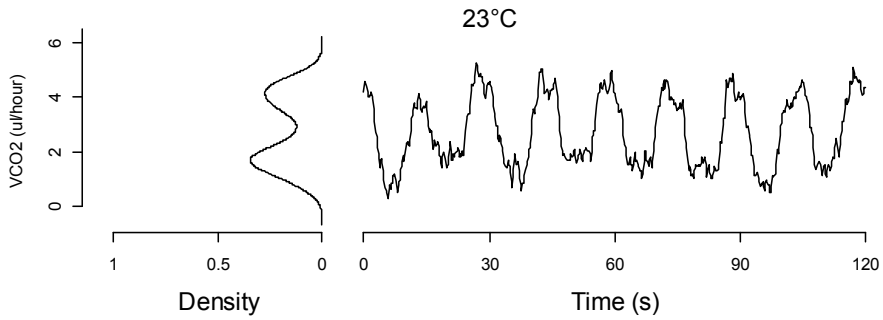


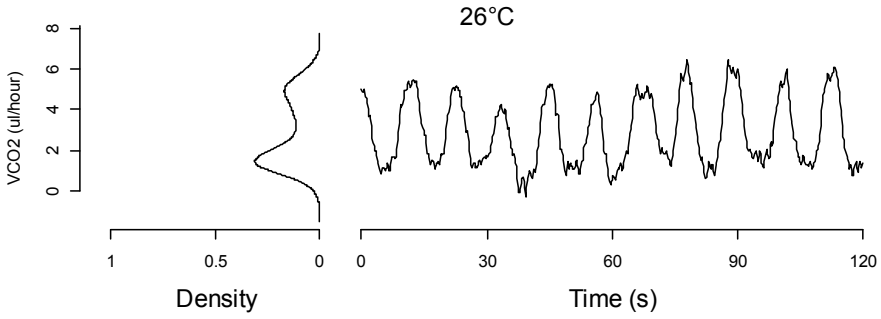


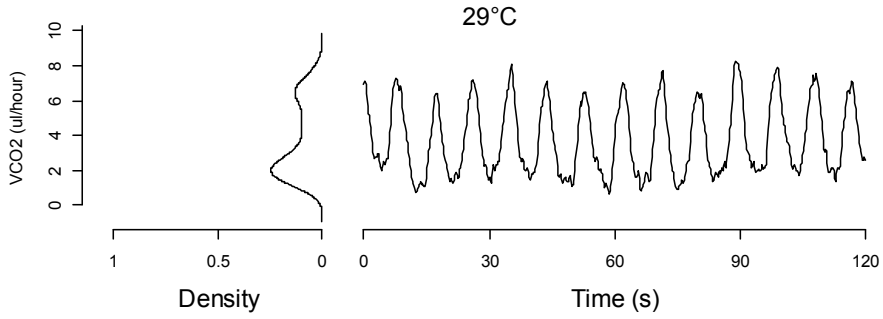


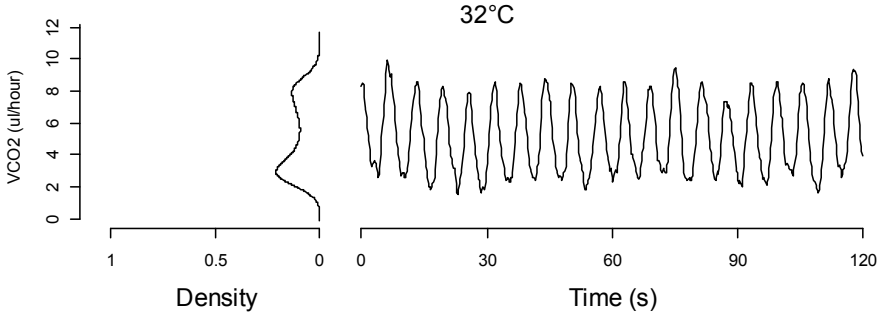
ID = u1
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.607
Living body mass (mg) = 1.7905
Wing length (mm) = 3.1275



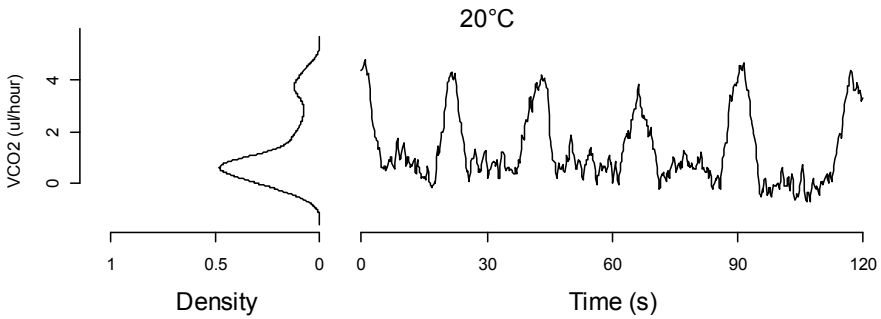


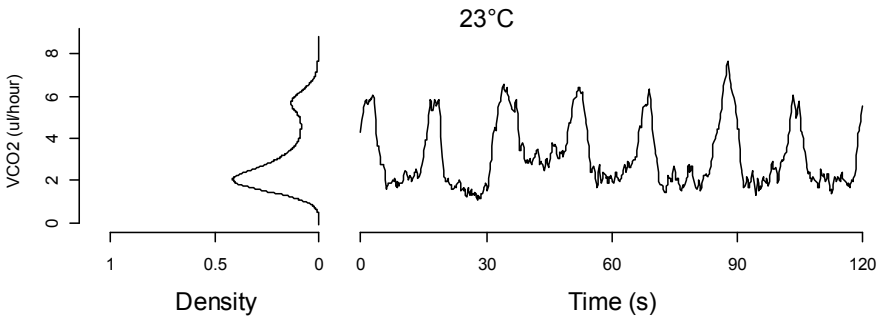


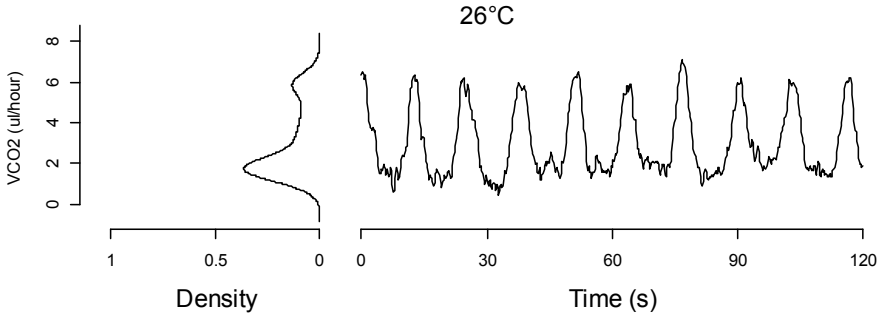


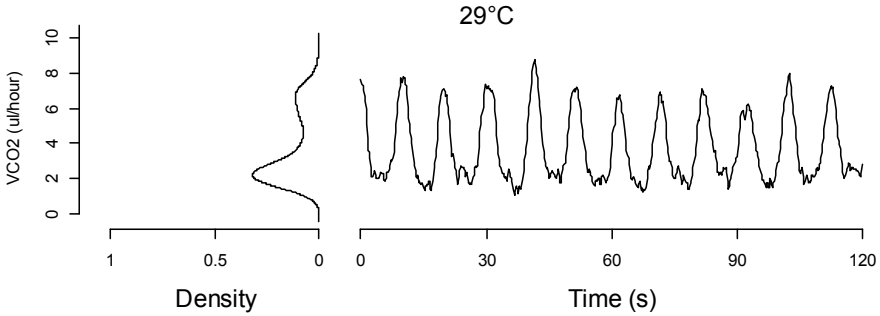


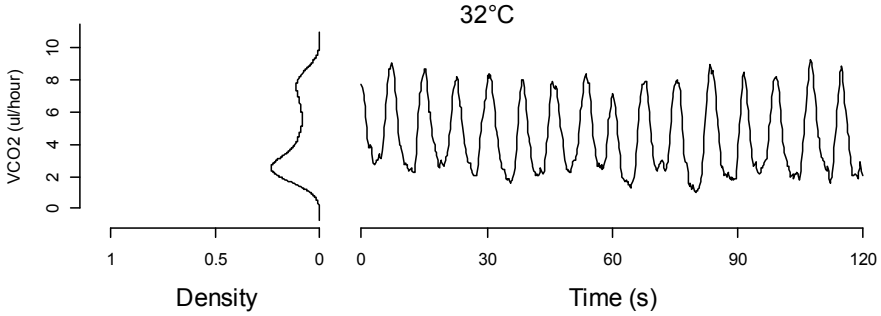
ID = u2
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.603
Living body mass (mg) = 1.7515
Wing length (mm) = 3.0375



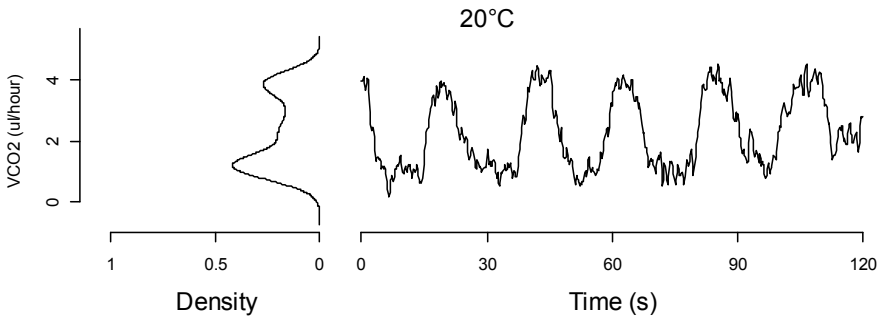


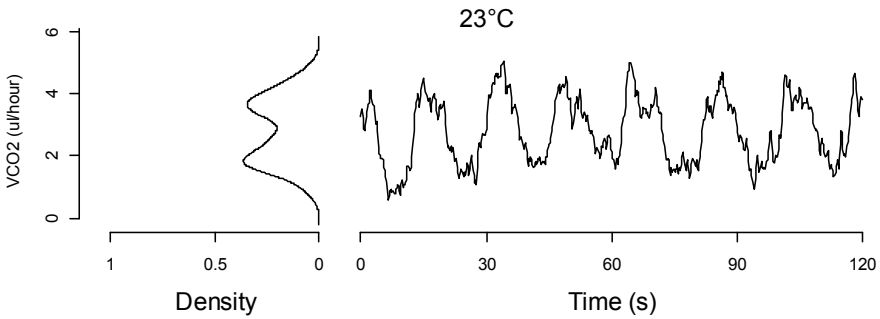


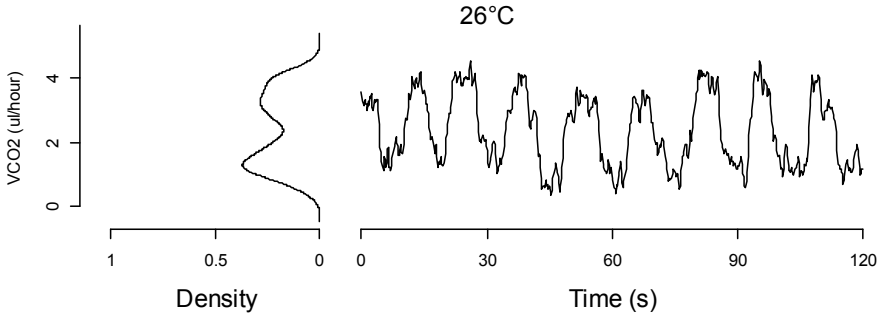


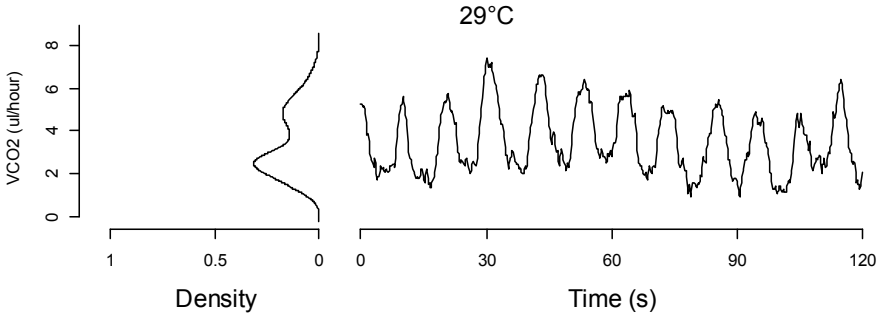


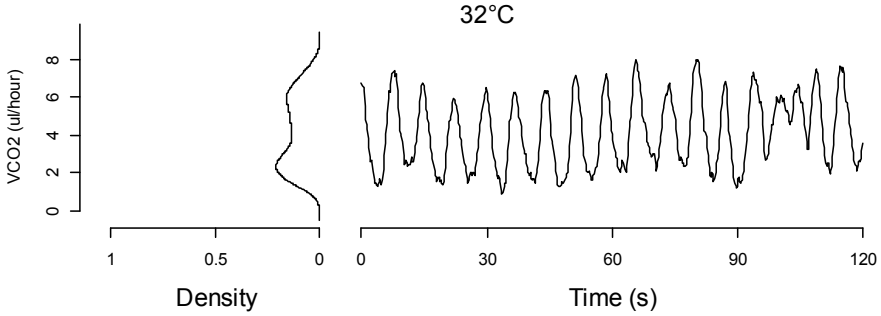
ID = d17
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.562
Living body mass (mg) = 1.6465
Wing length (mm) = 3.015



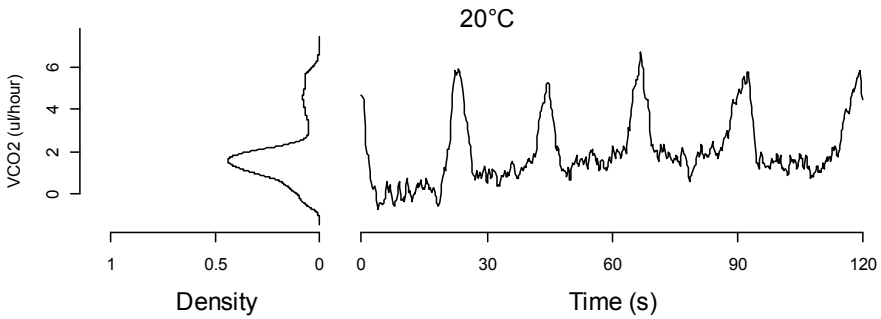


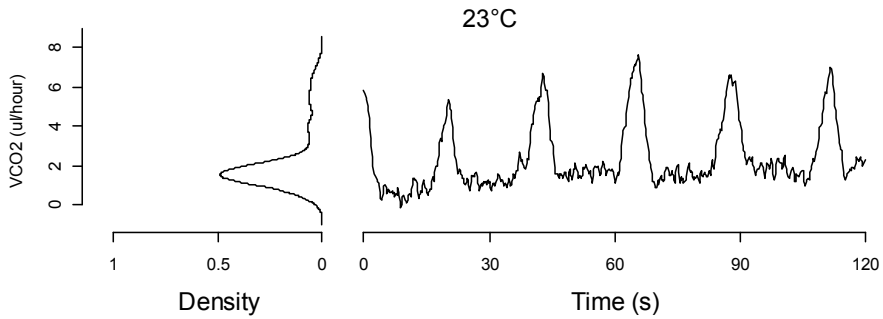


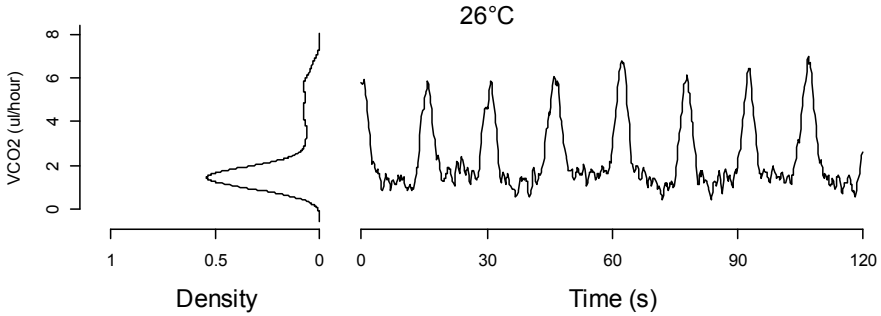


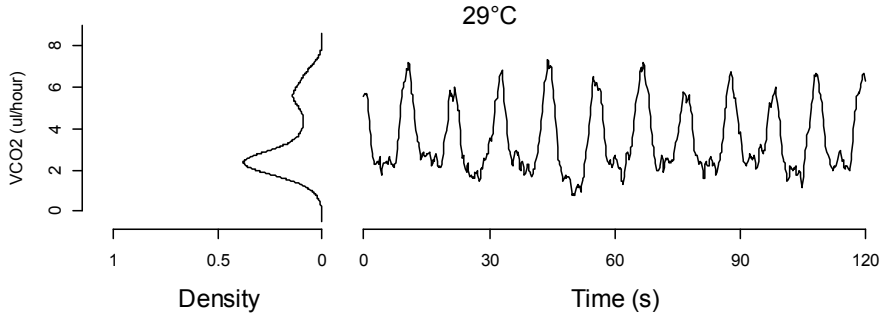


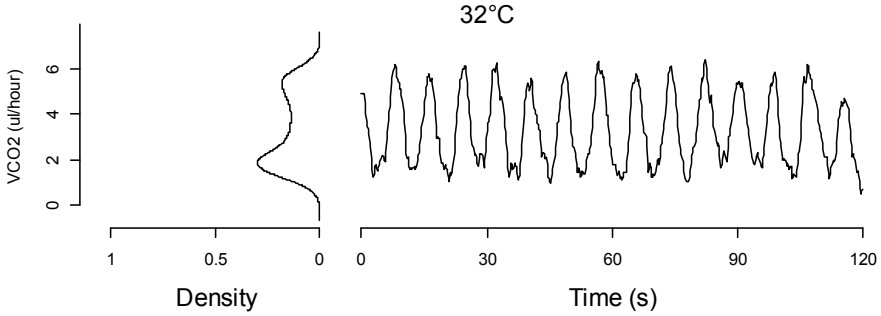
ID = u18
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.555
Living body mass (mg) = 1.7245
Wing length (mm) = 2.9925



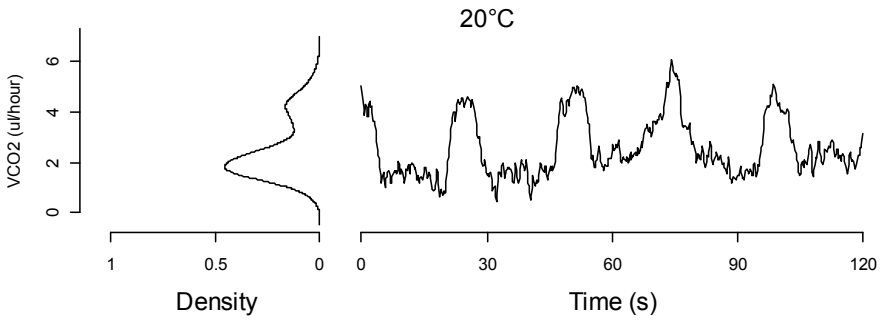


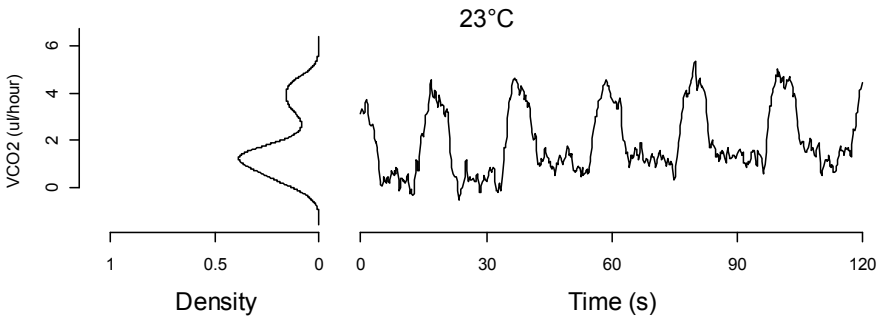


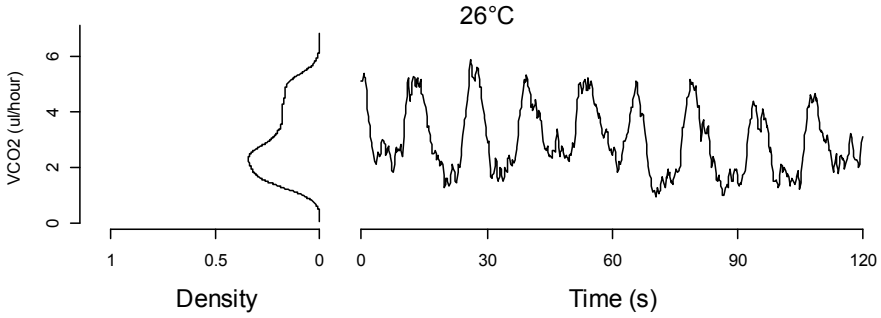


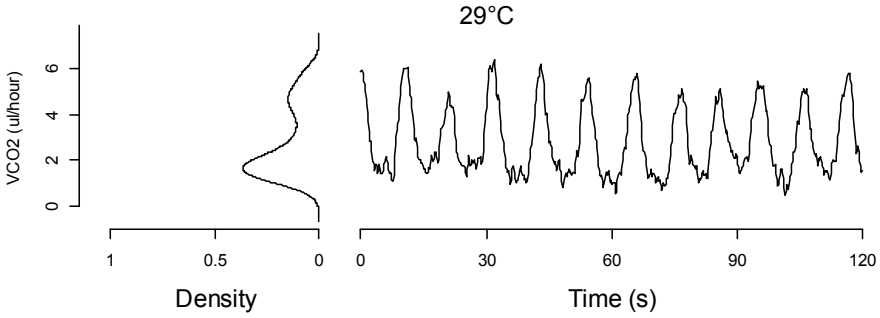


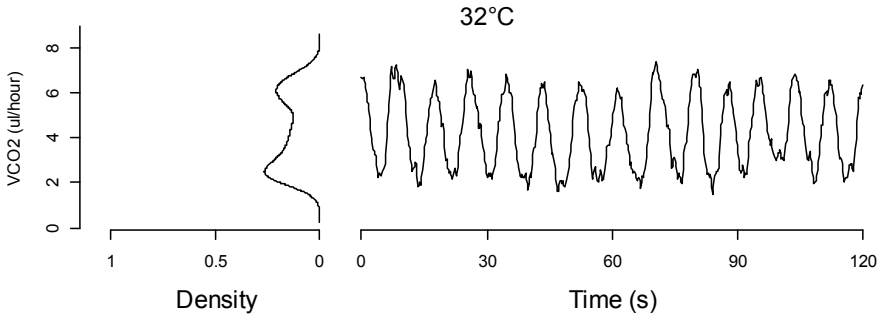
ID = d24
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.555
Living body mass (mg) = 1.685
Wing length (mm) = 3.06



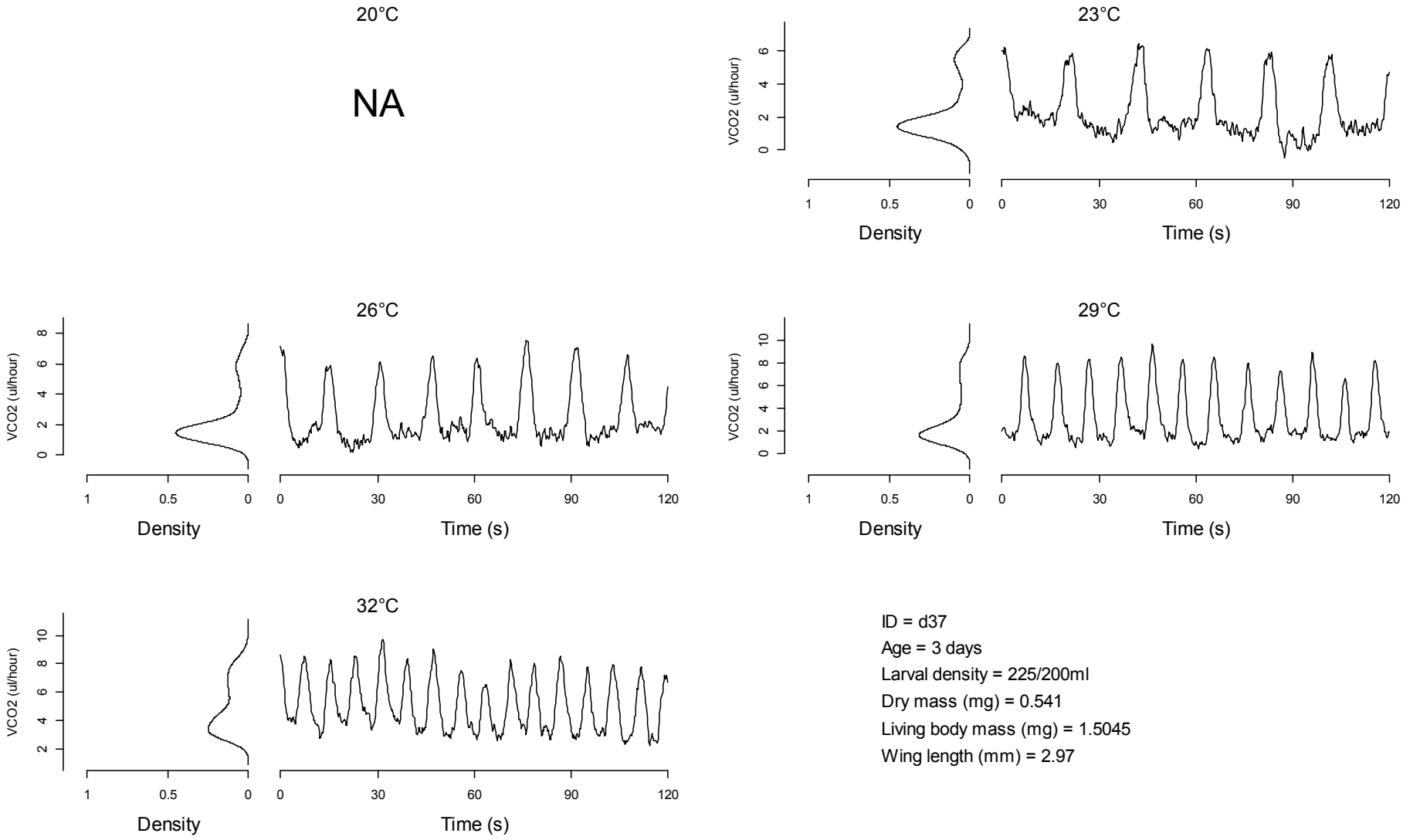






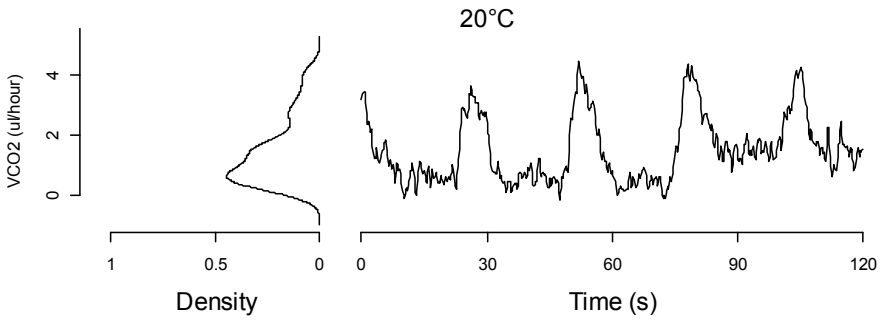


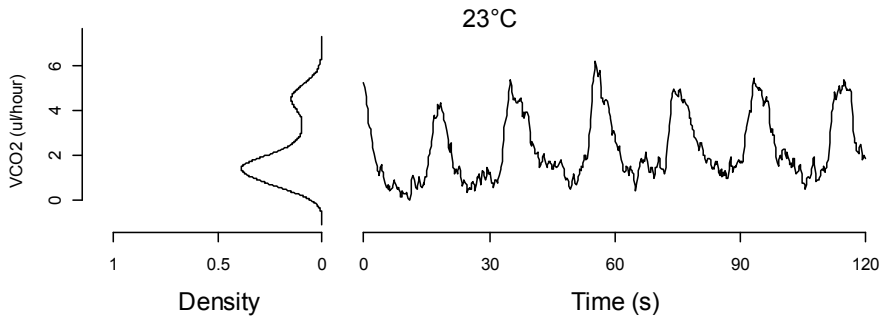
ID = d41
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.553
Living body mass (mg) = 1.566
Wing length (mm) = 3.195



ID = d37
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.541
Living body mass (mg) = 1.5045
Wing length (mm) = 2.97

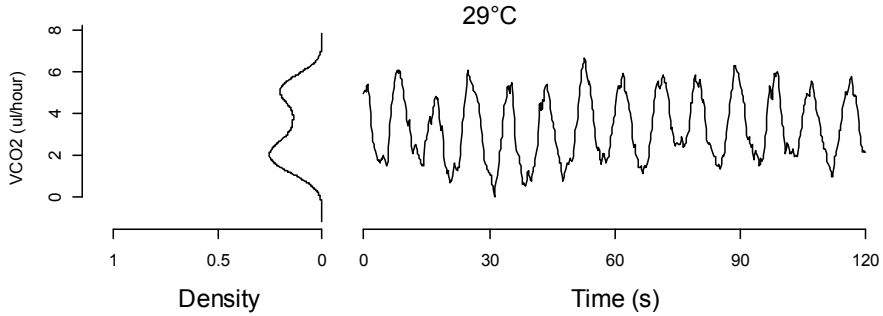
NA: data not available





26°C

NA

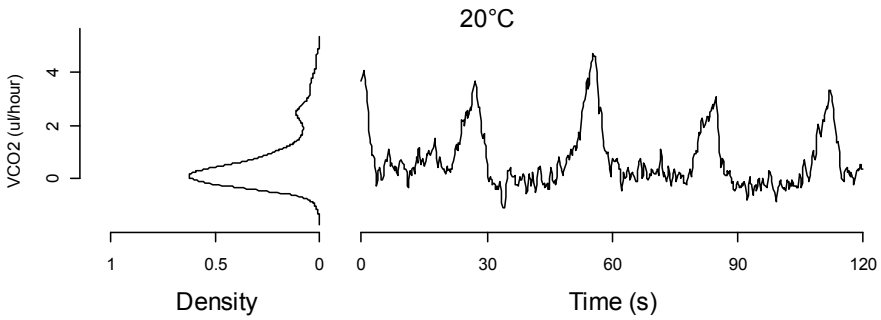


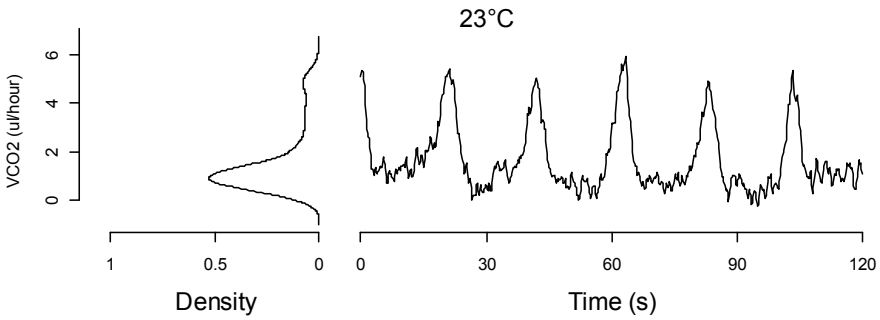
32°C

NA

ID = d43
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.532
Living body mass (mg) = 1.4595
Wing length (mm) = 3.24

NA: data not available



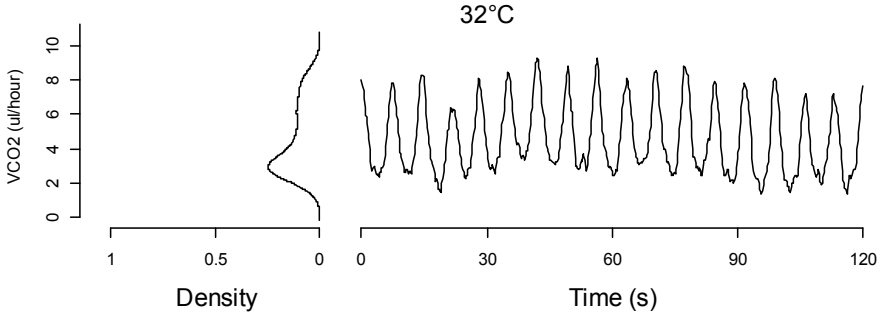


26°C

NA

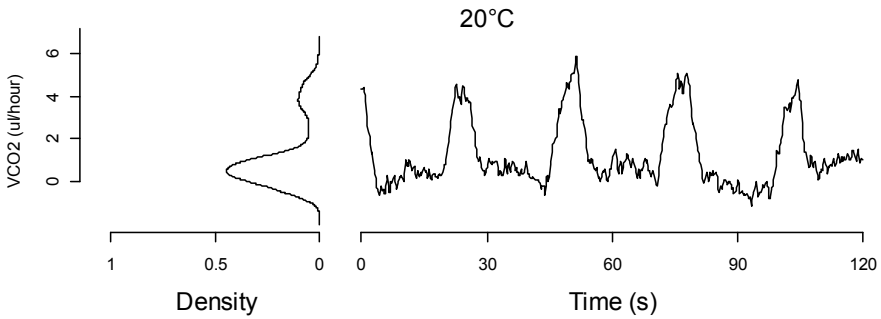
29°C

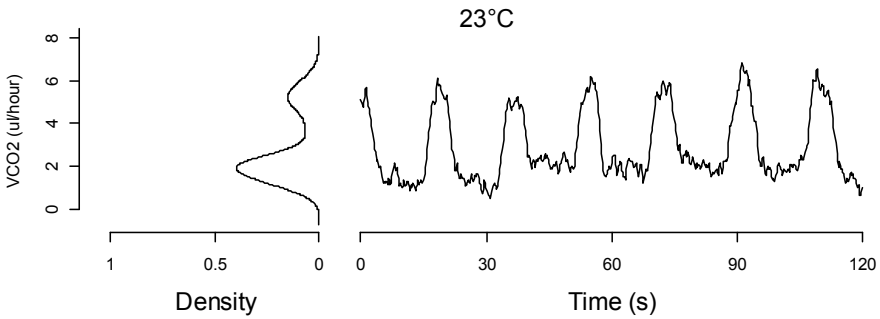
NA

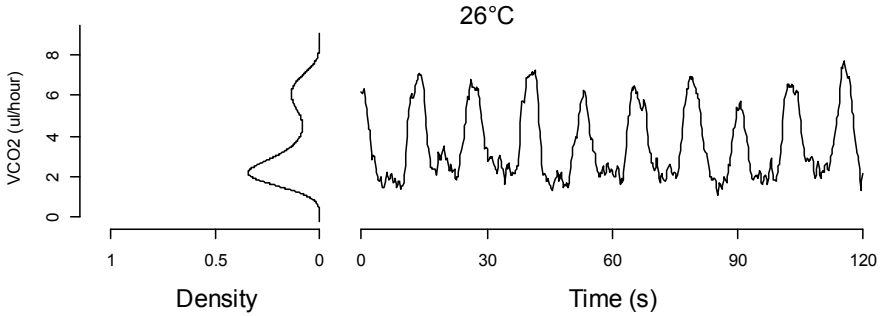


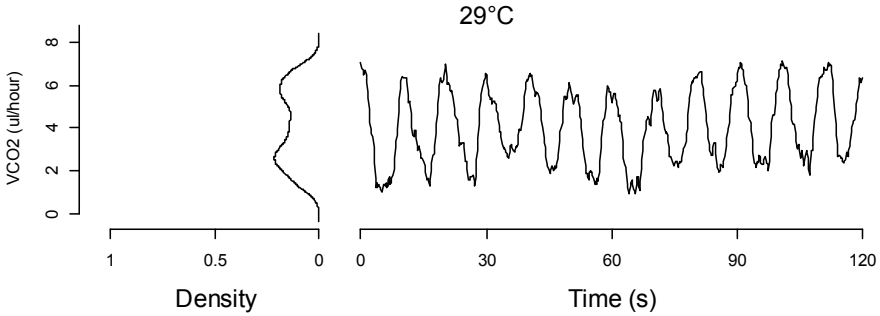
ID = d44
Age = 6 days
Larval density = 75/200ml
Dry mass (mg) = 0.525
Living body mass (mg) = 1.557
Wing length (mm) = 3.285

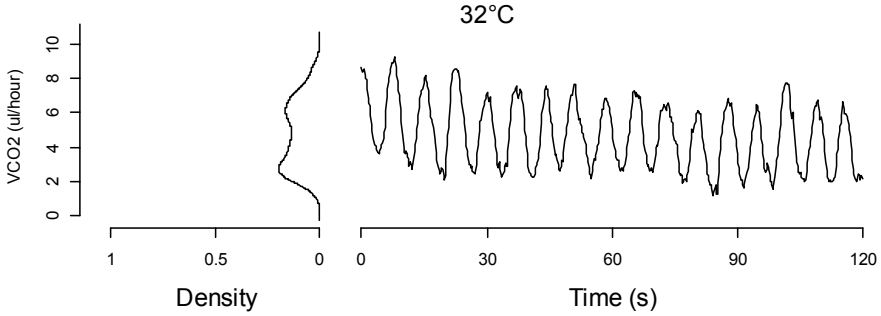
NA: data not available





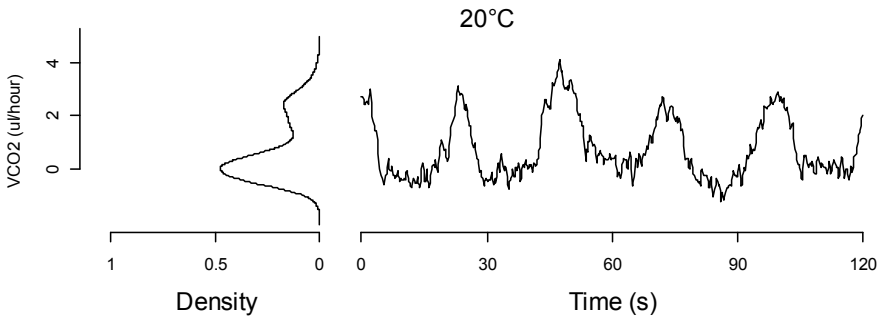


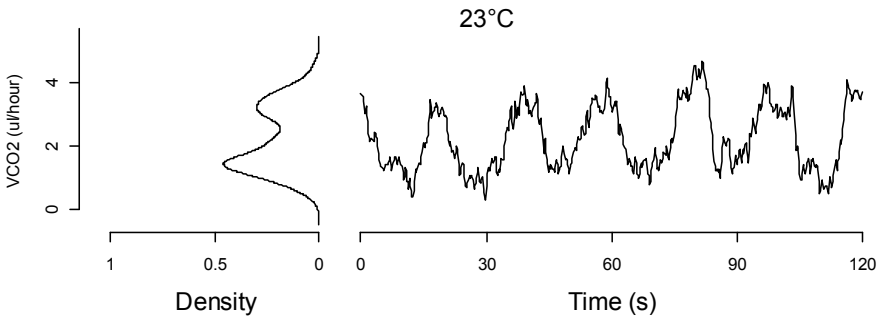


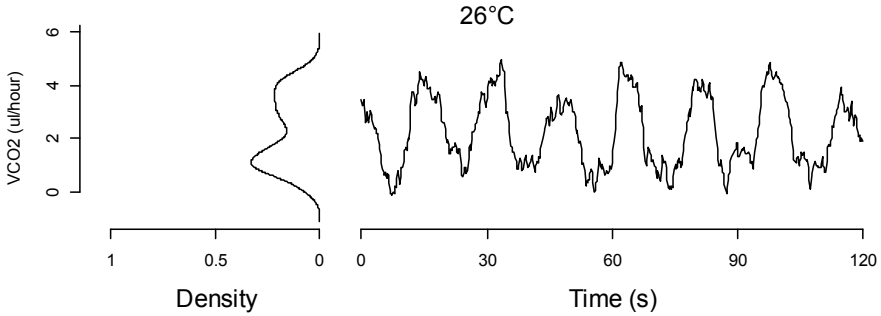


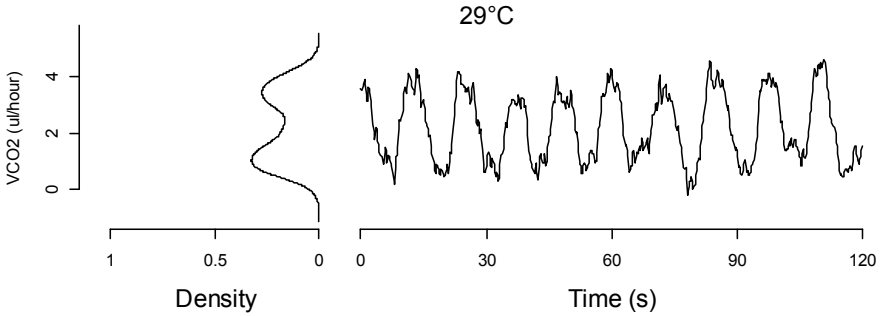
ID = d29
Age = 3 days
Larval density = 75/200ml
Dry mass (mg) = 0.523
Living body mass (mg) = 1.7

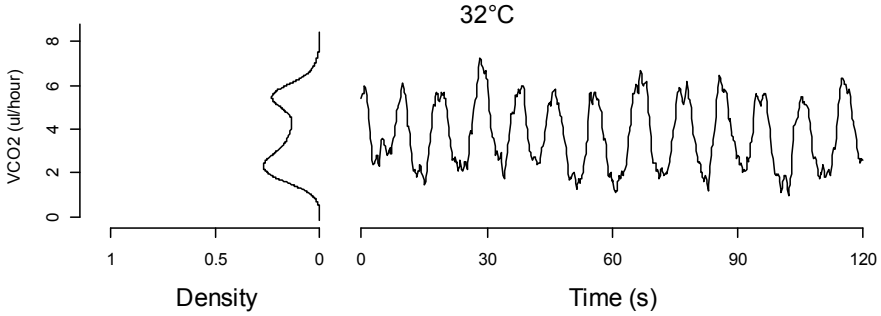
NA: data not available



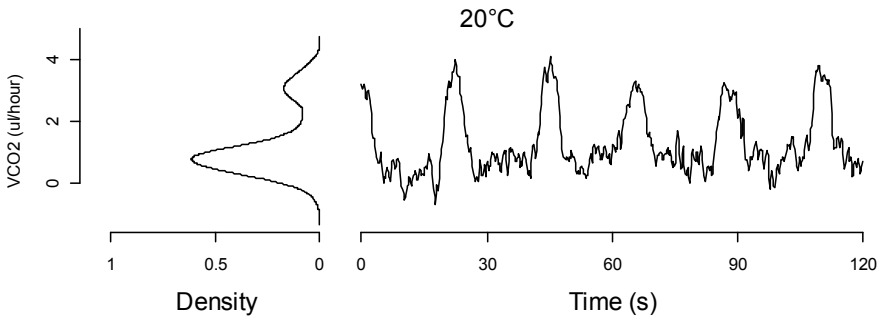


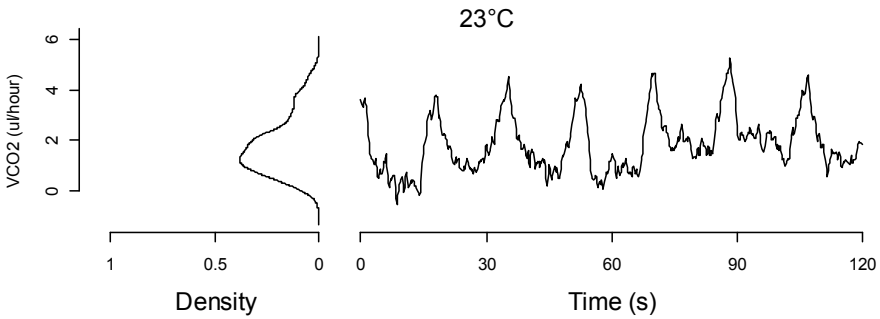


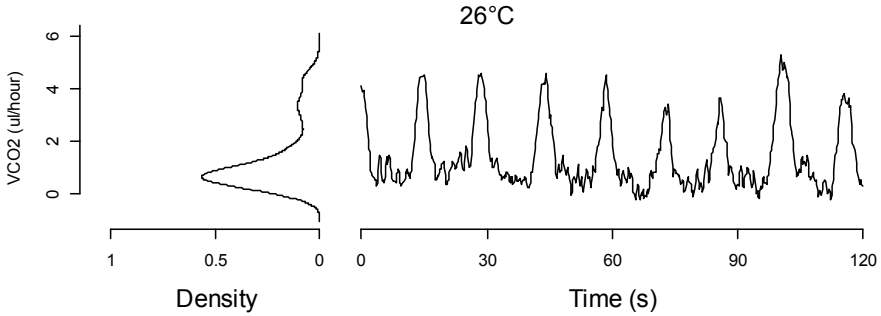


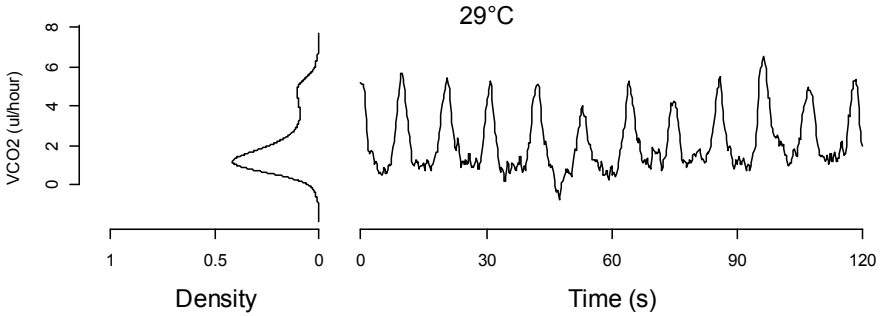


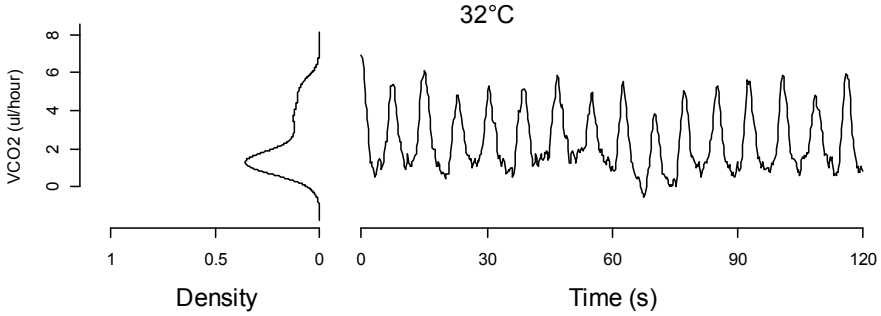
ID = u11
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.514
Living body mass (mg) = 1.4985
Wing length (mm) = 2.925



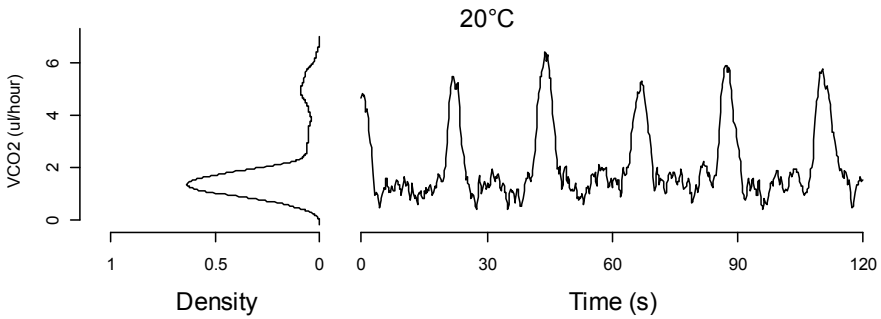


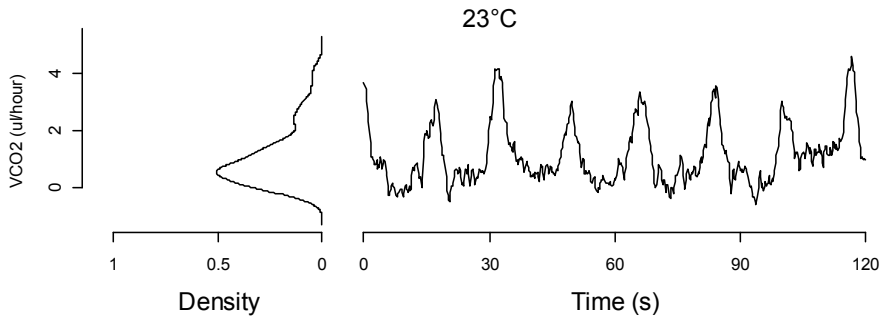


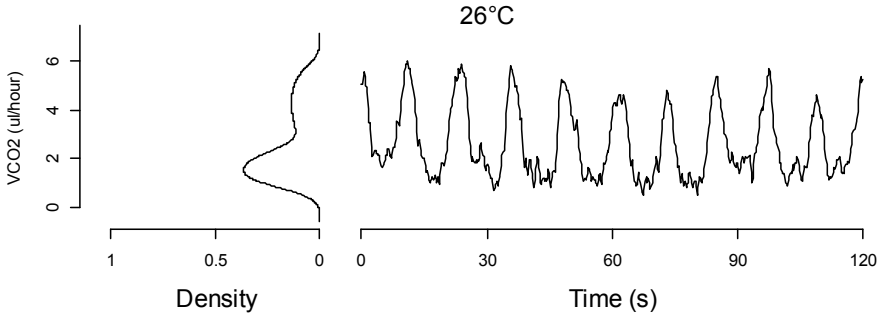


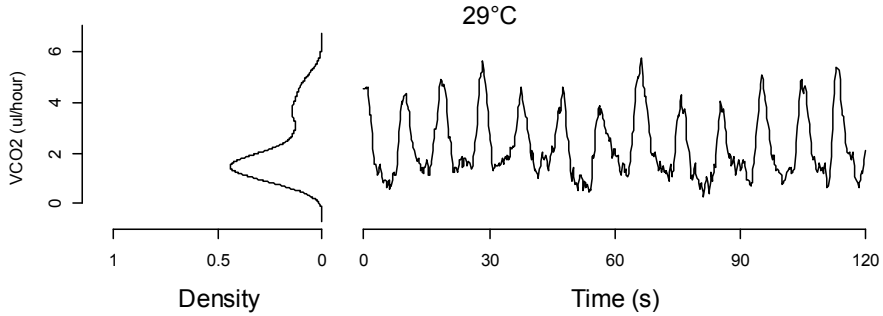


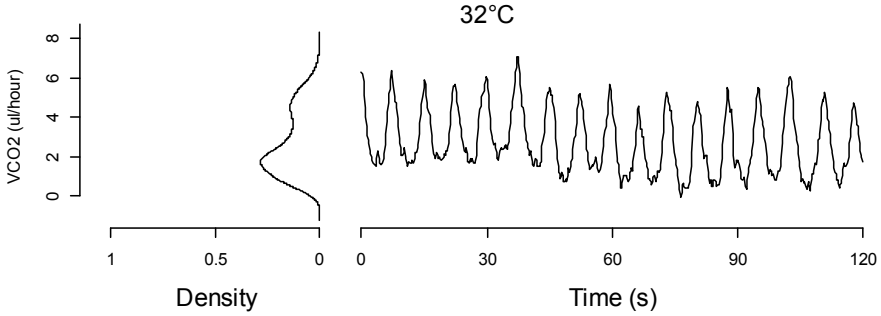
ID = u26
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.484
Living body mass (mg) = 1.3315
Wing length (mm) = 2.88



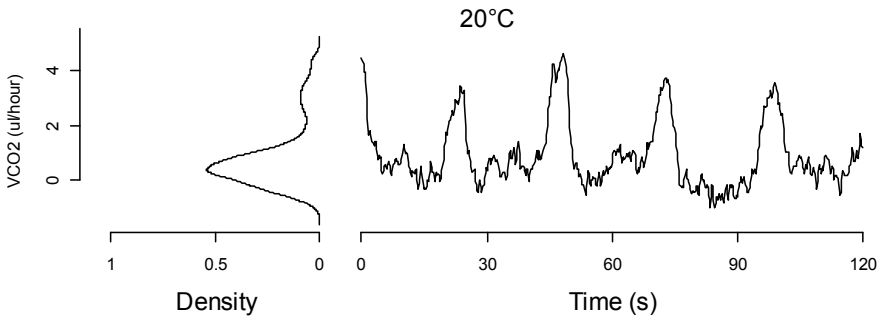


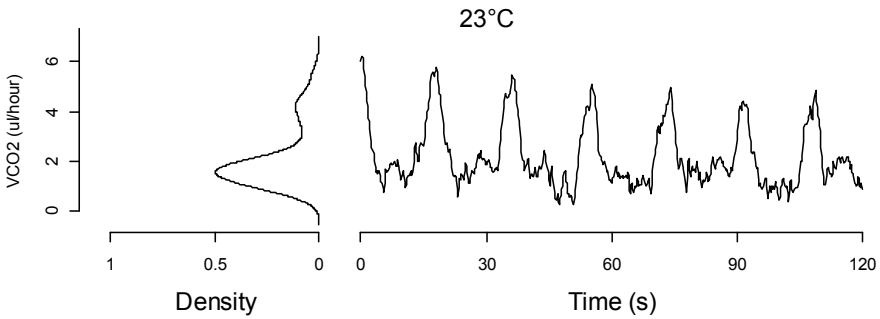


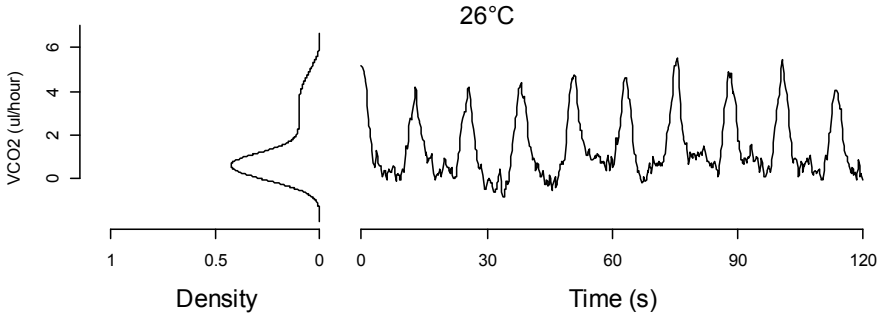


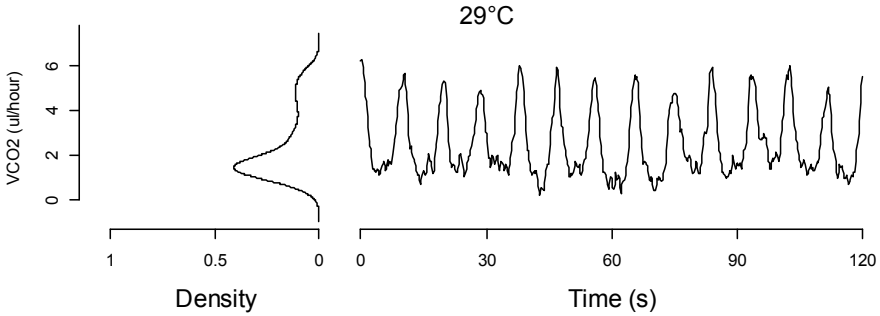


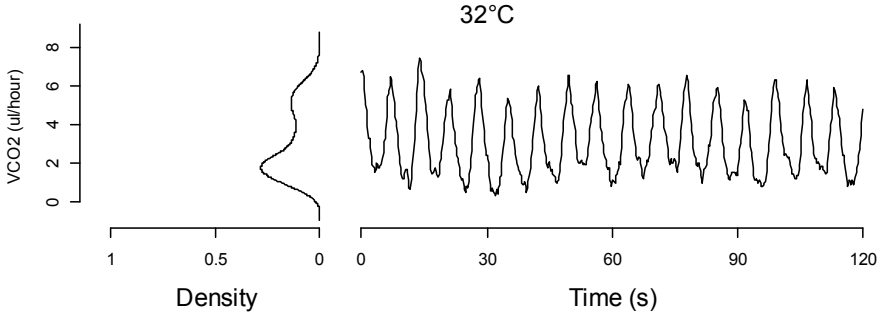
ID = u5
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.475
Living body mass (mg) = 1.4
Wing length (mm) = 2.7675



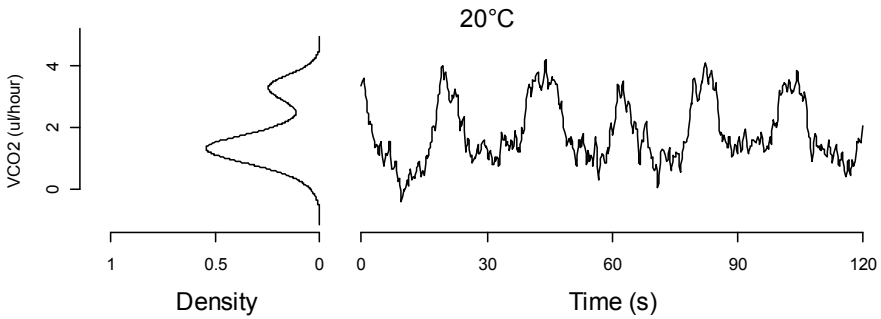


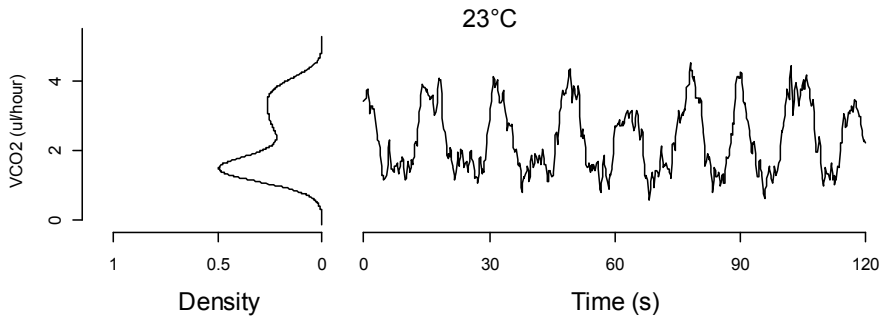






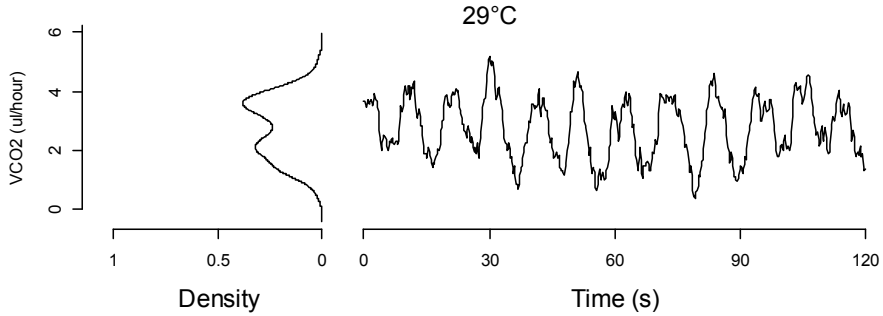
ID = d32
Age = 3 days
Larval density = 375/200ml
Dry mass (mg) = 0.454
Living body mass (mg) = 1.4115
Wing length (mm) = 2.835

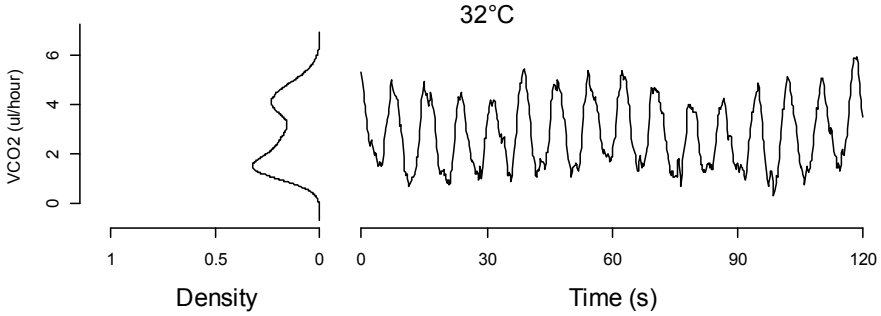




26°C

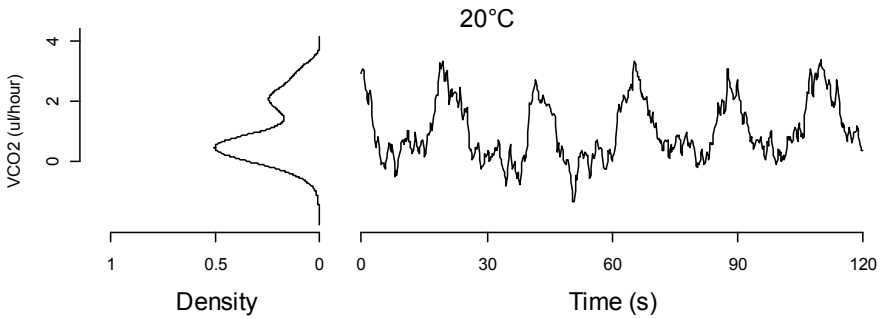
NA

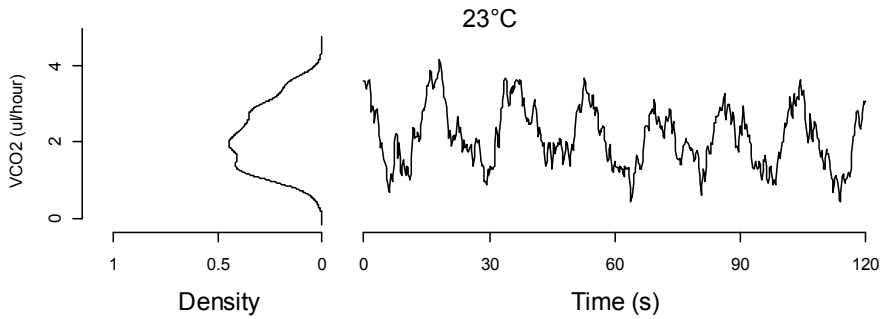


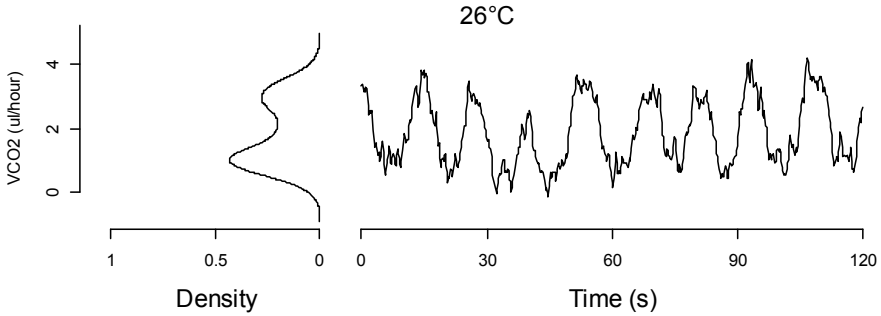


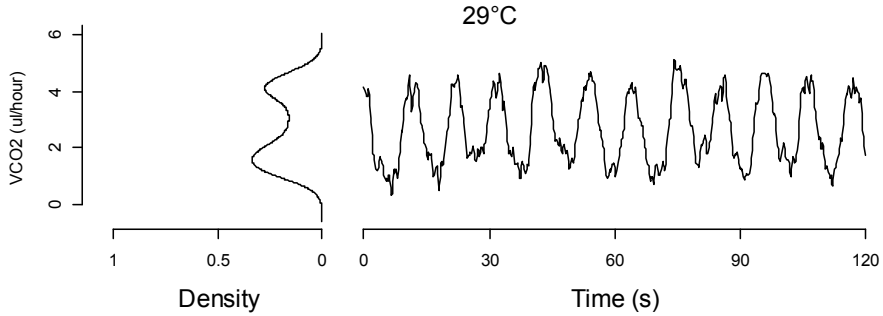
ID = u8
Age = 3 days
Larval density = 375/200ml
Dry mass (mg) = 0.442
Living body mass (mg) = 1.45
Wing length (mm) = 2.79

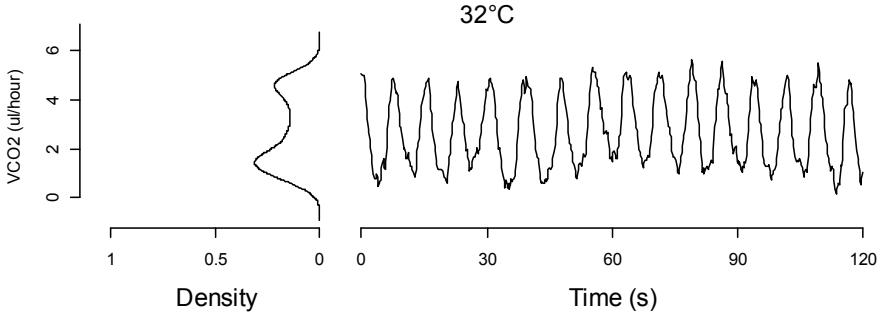
NA: data not available



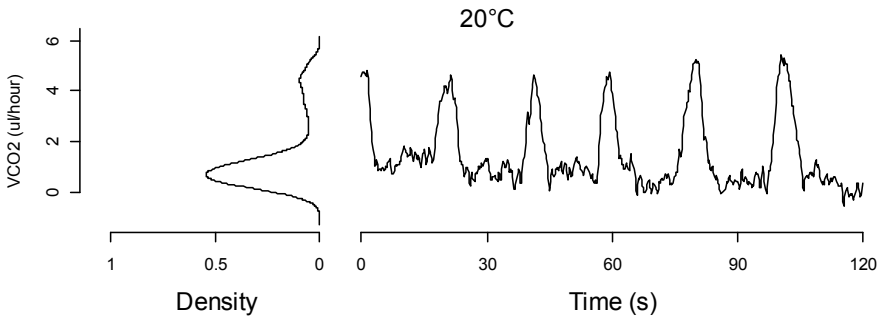


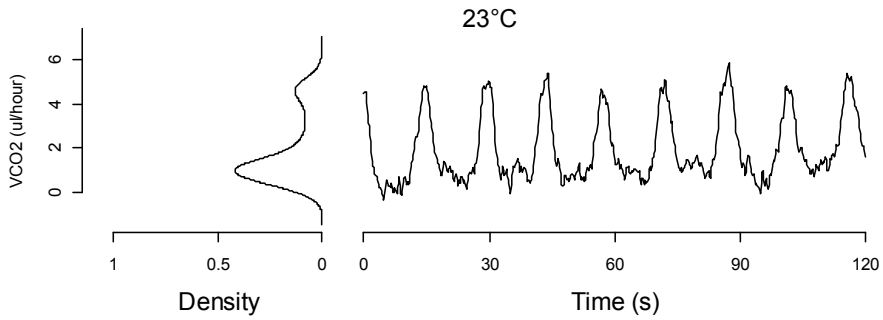


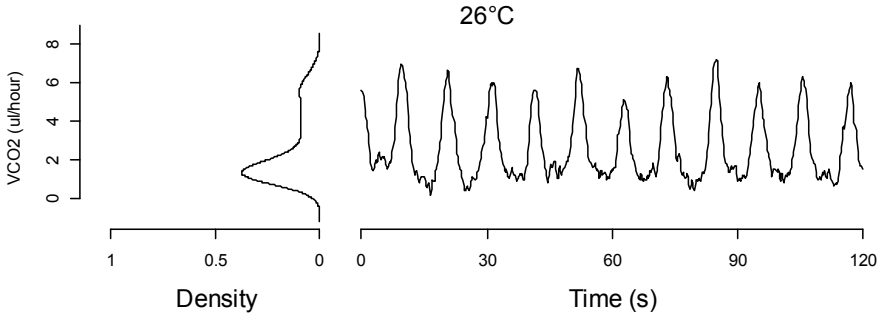


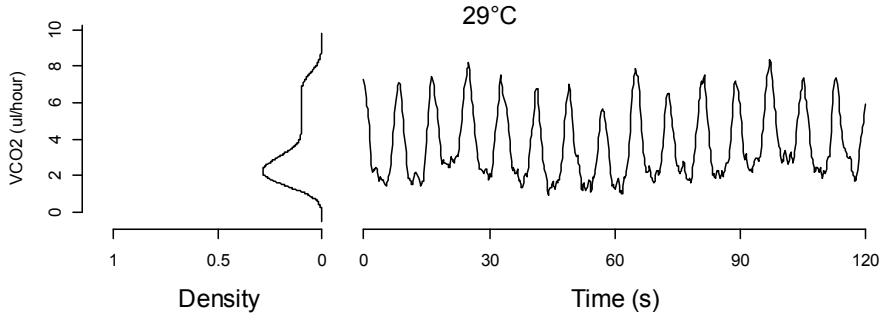


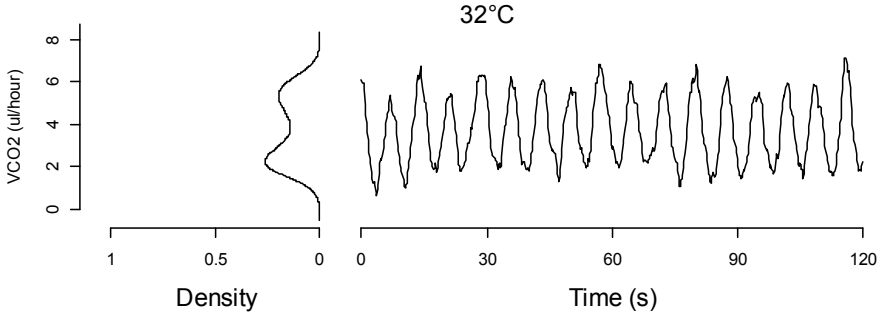
ID = u6
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.44
Living body mass (mg) = 1.3995
Wing length (mm) = 2.79



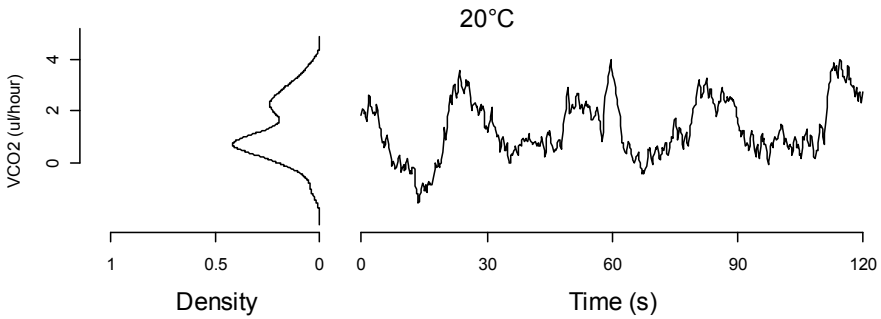


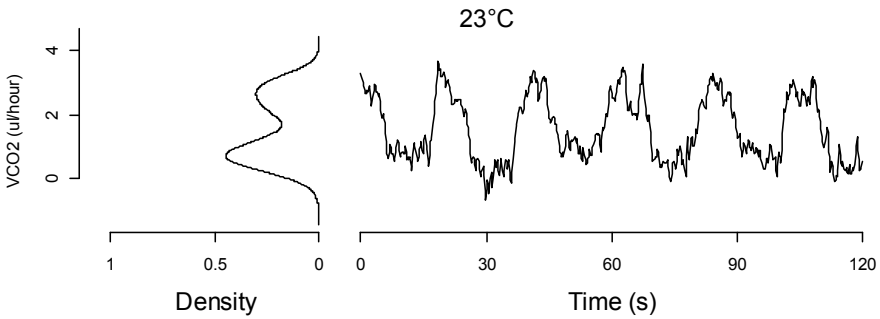


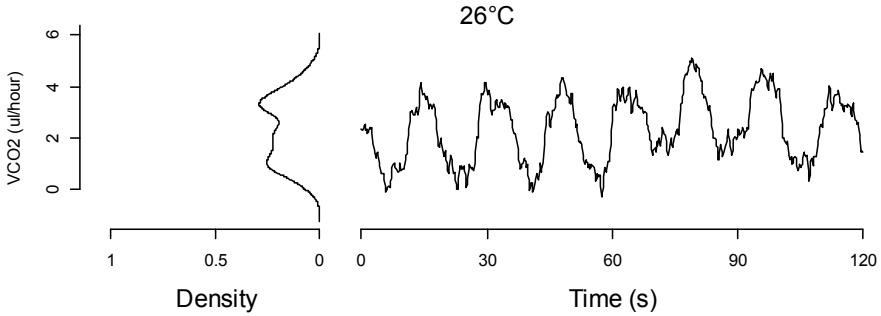


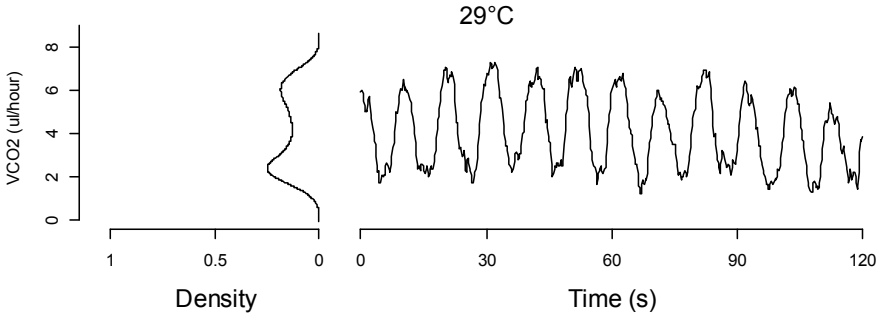


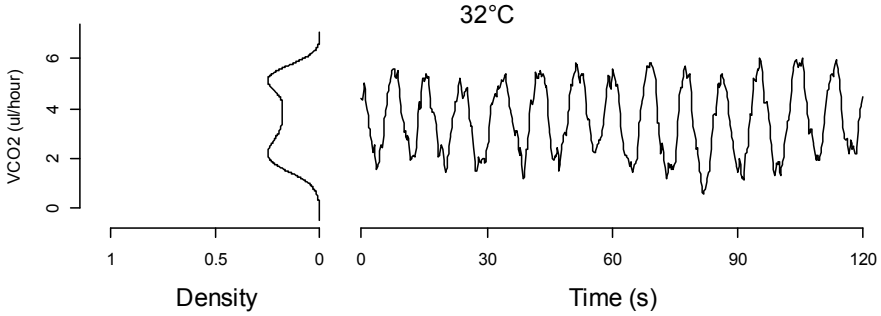
ID = d21
Age = 3 days
Larval density = 375/200ml
Dry mass (mg) = 0.433
Living body mass (mg) = 1.362
Wing length (mm) = 2.9025



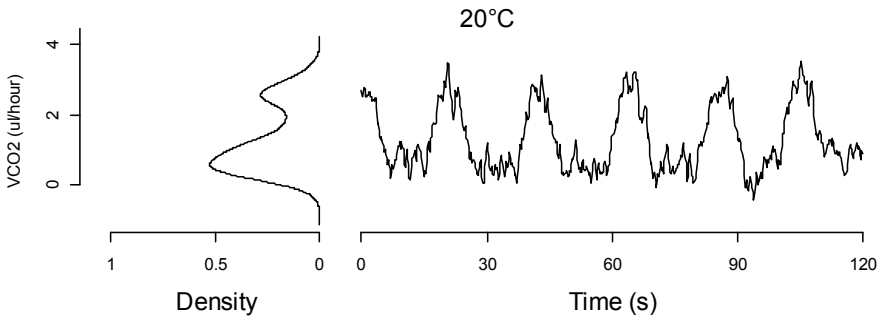


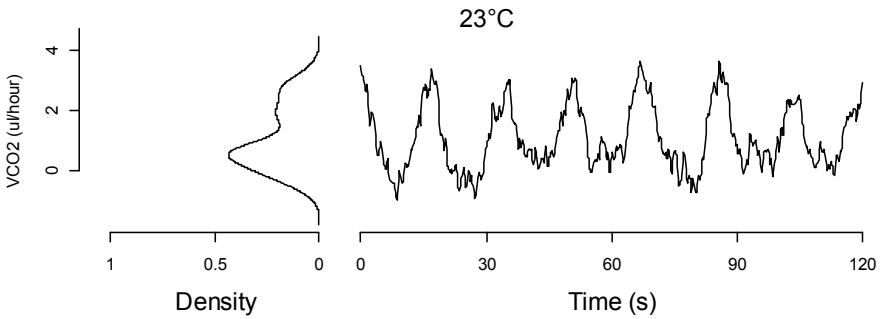


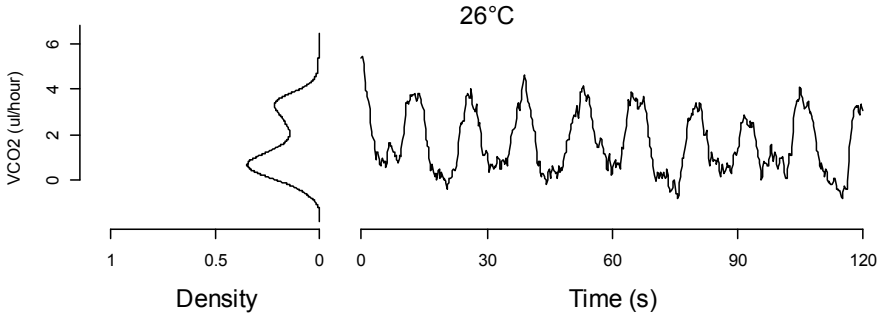


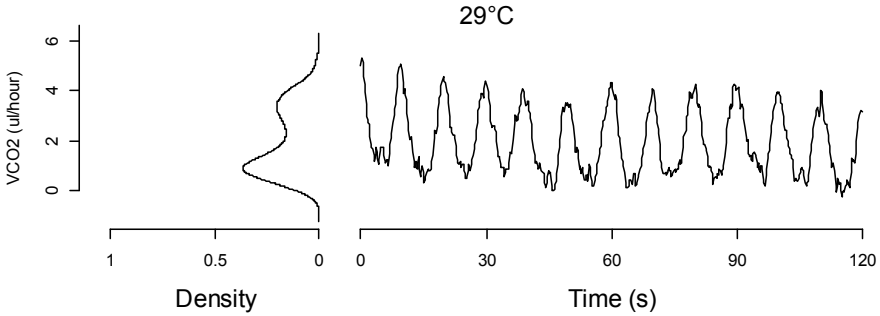


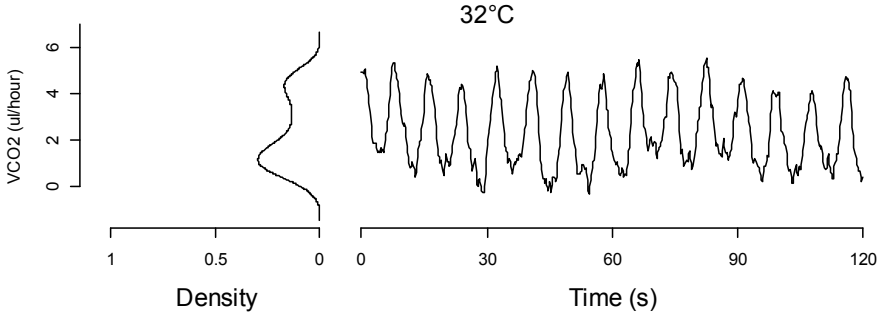
ID = d31
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.431
Living body mass (mg) = 1.331
Wing length (mm) = 2.835



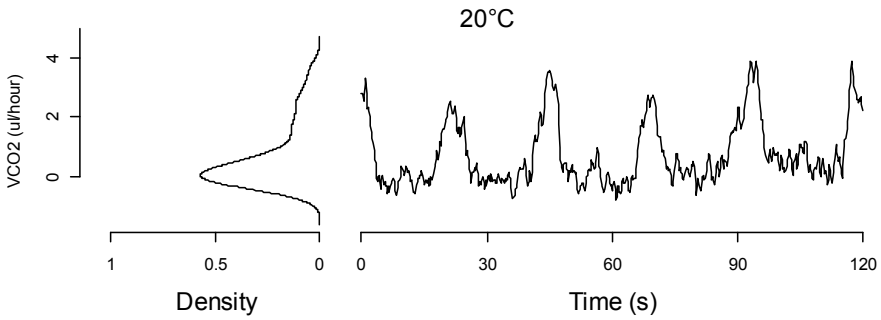


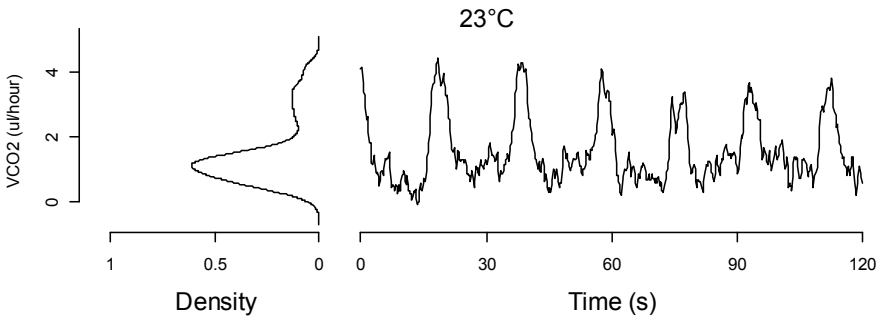


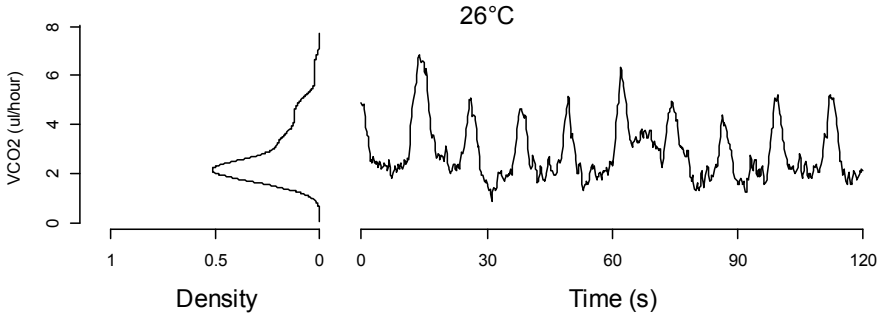


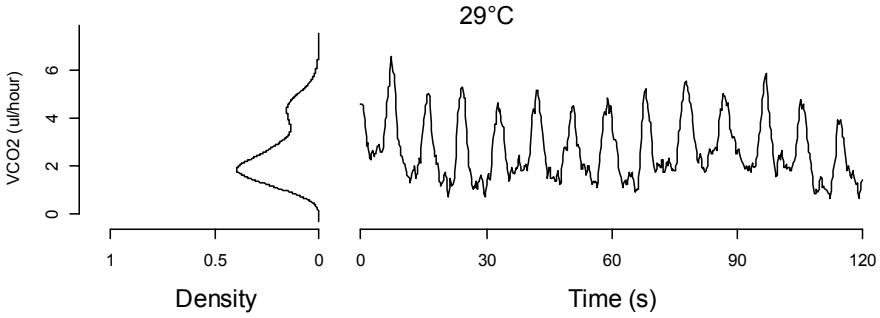


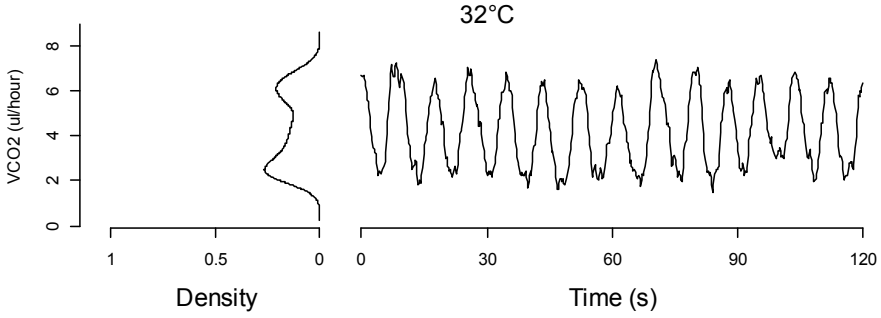
ID = u12
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.427
Living body mass (mg) = 1.3615
Wing length (mm) = 2.745



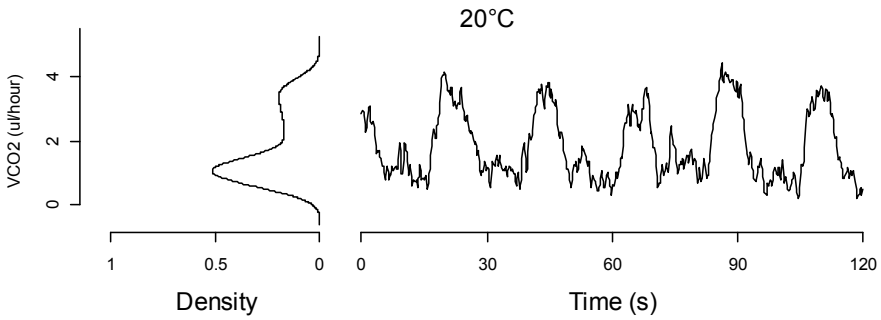


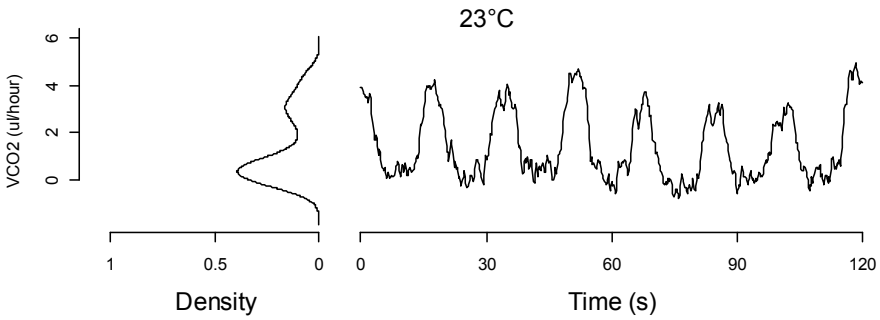


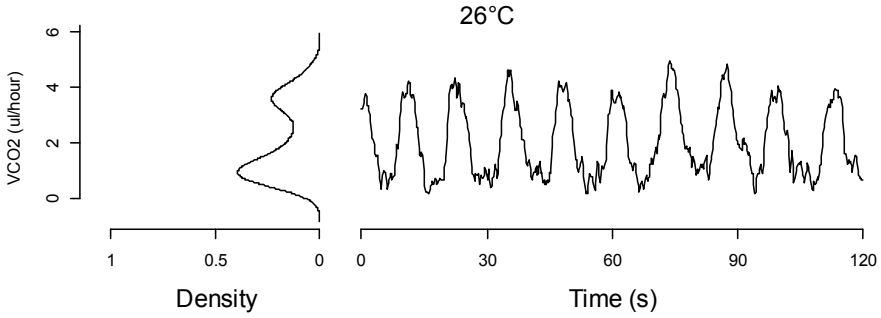


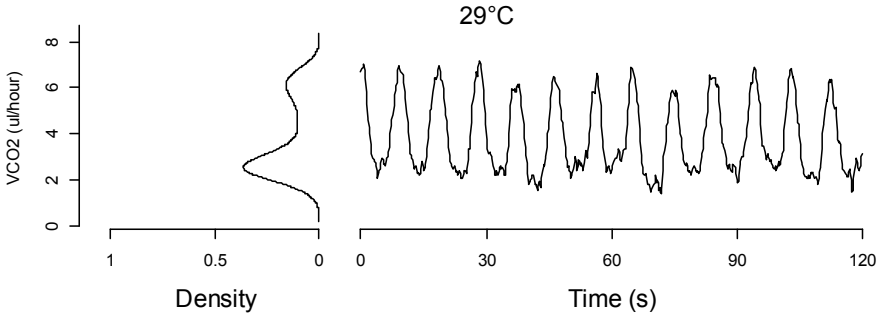


ID = d40
Age = 6 days
Larval density = 225/200ml
Dry mass (mg) = 0.422
Living body mass (mg) = 1.1635
Wing length (mm) = 2.88







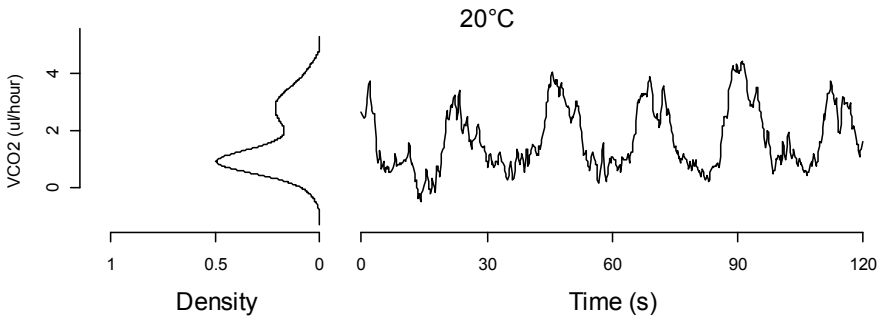


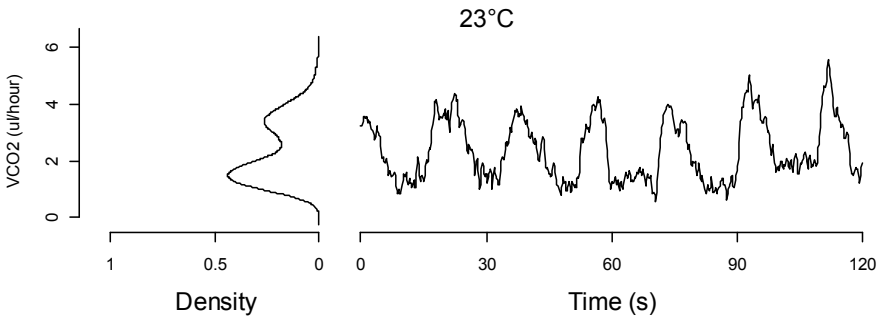
32°C

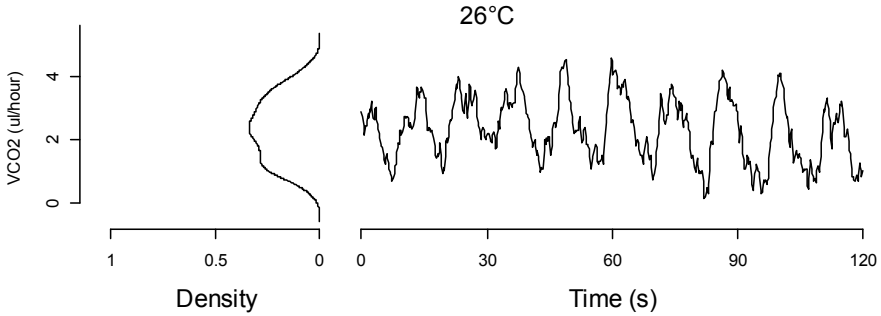
NA

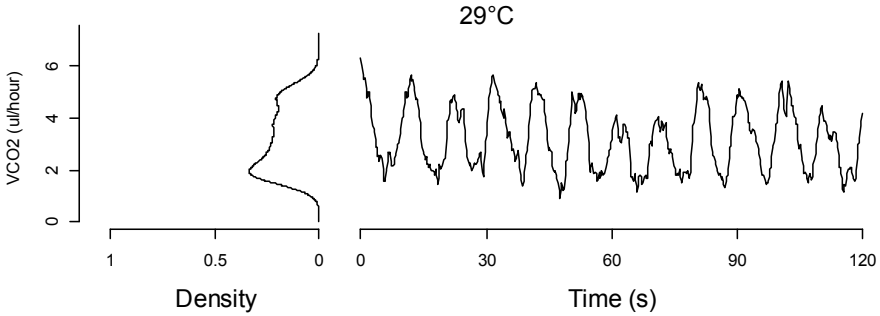
ID = u36
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.42
Living body mass (mg) = 1.232
Wing length (mm) = 2.925

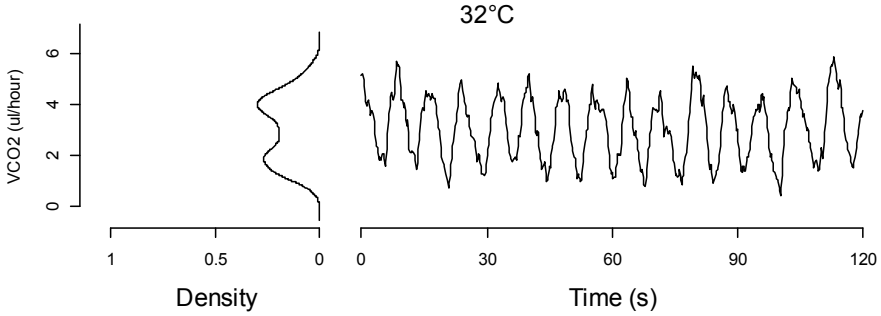
NA: data not available



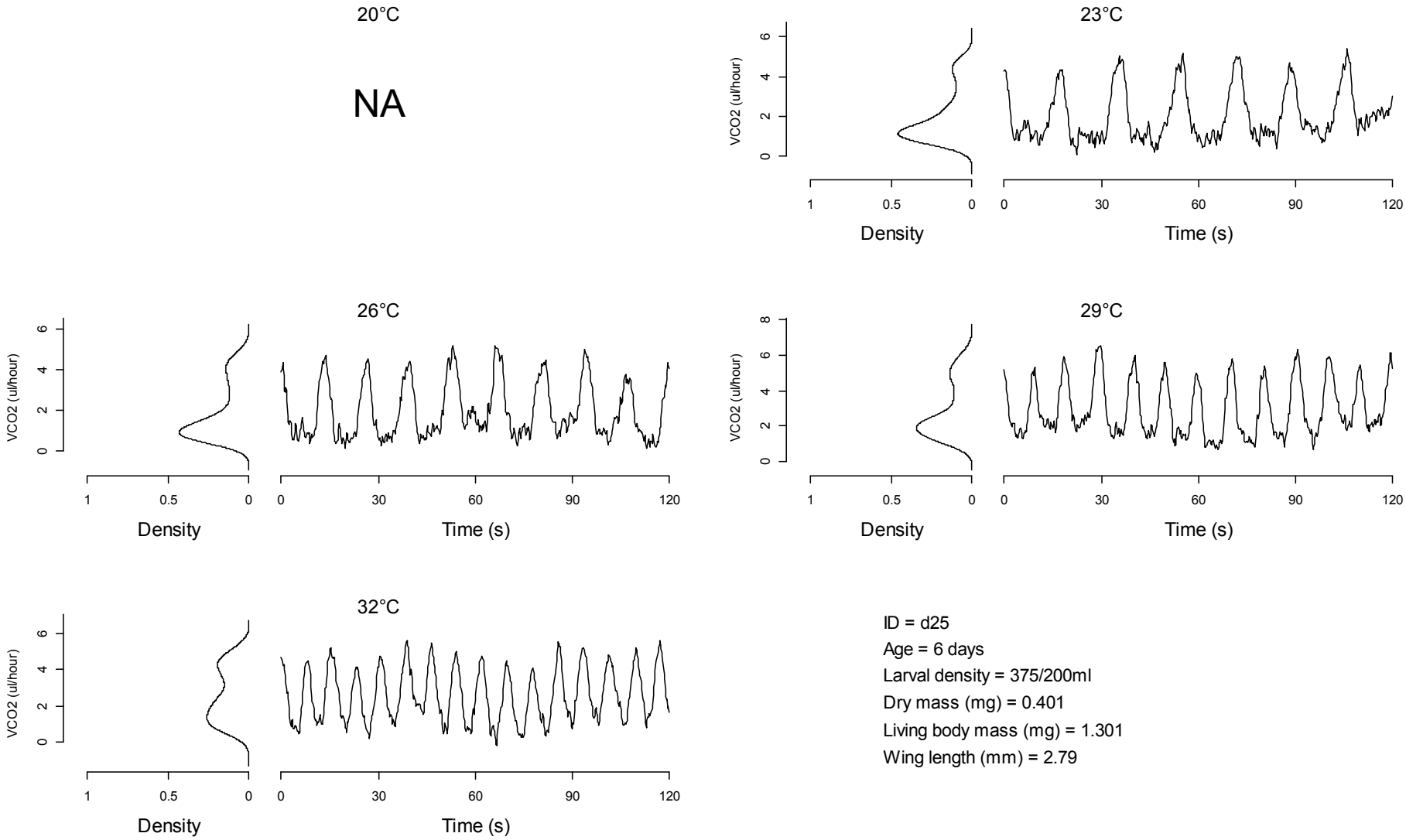






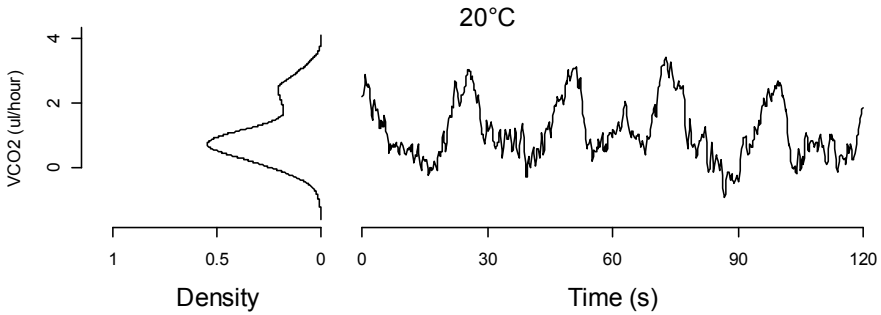


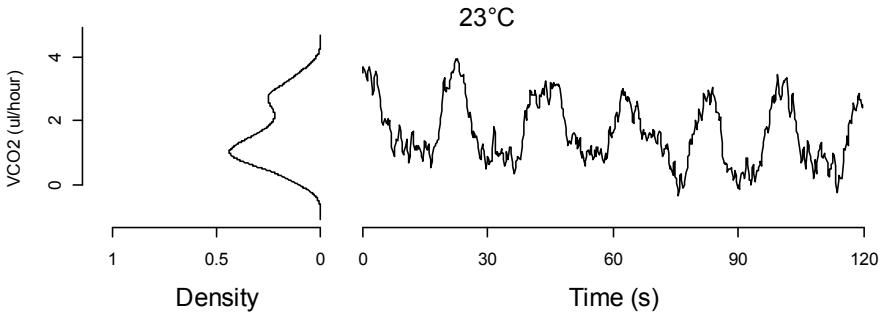
ID = d19
Age = 3 days
Larval density = 225/200ml
Dry mass (mg) = 0.411
Living body mass (mg) = 1.1835
Wing length (mm) = 2.925

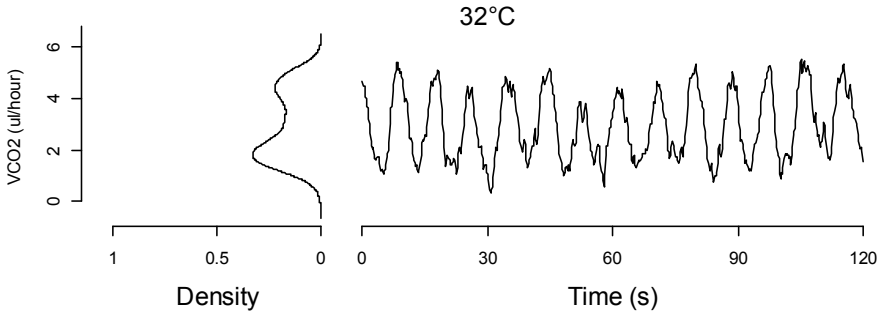


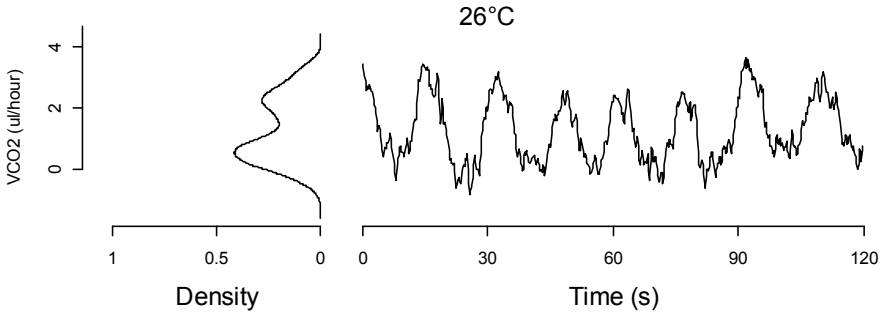
ID = d25
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.401
Living body mass (mg) = 1.301
Wing length (mm) = 2.79

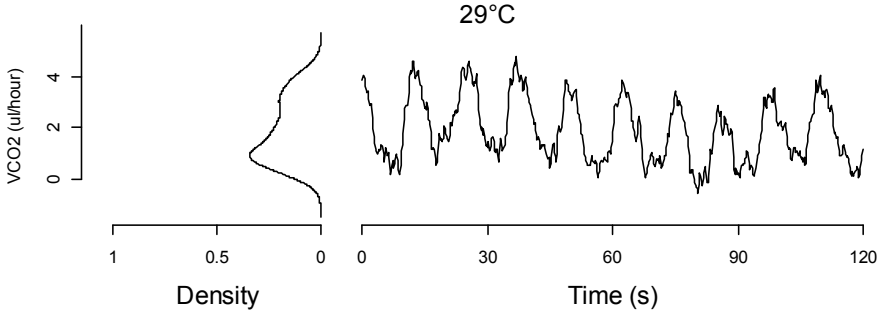
NA: data not available



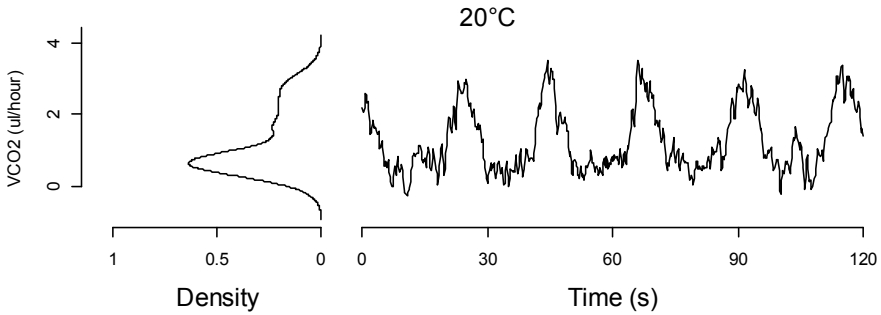


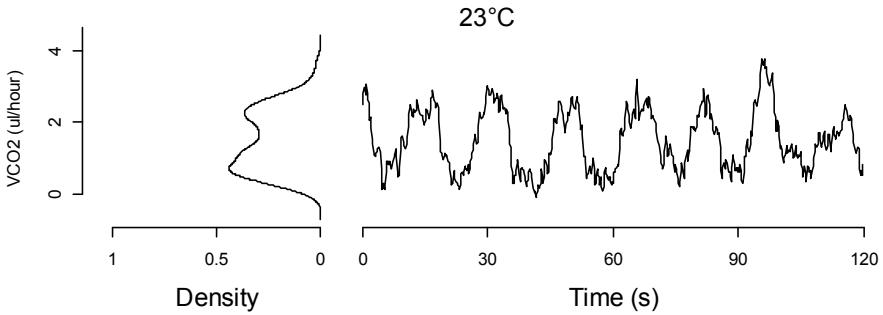


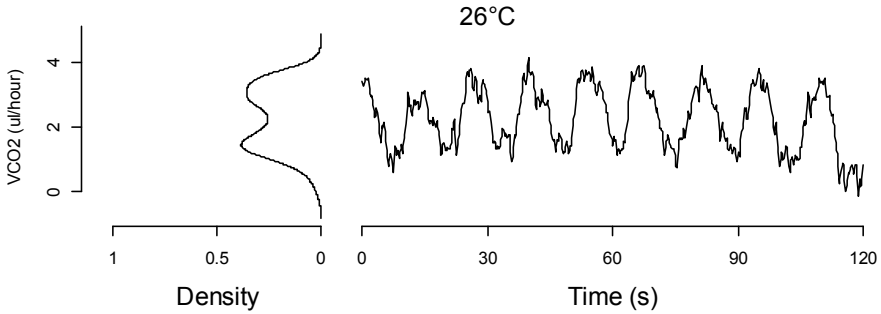


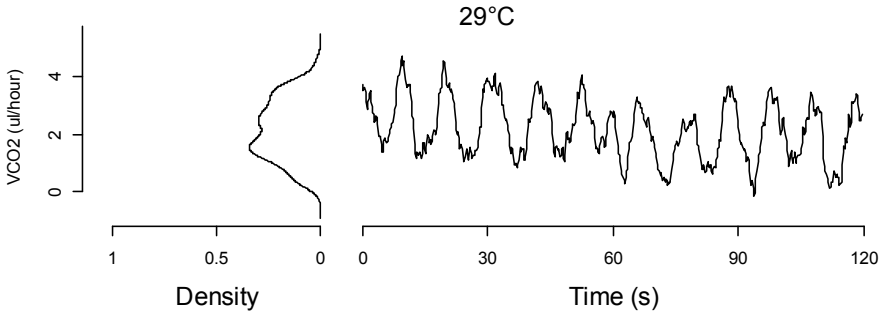


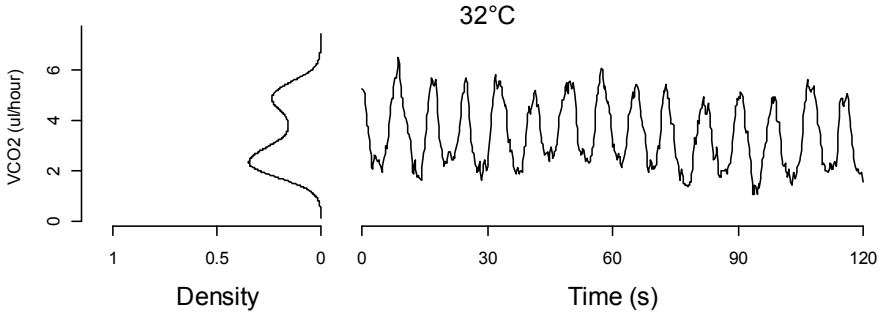
ID = u13
Age = 6 days
Larval density = 375/200ml
Dry mass (mg) = 0.392
Living body mass (mg) = 1.225
Wing length (mm) = 2.835



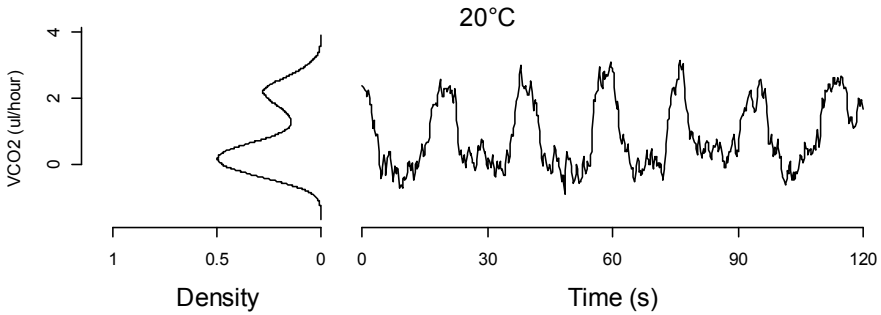


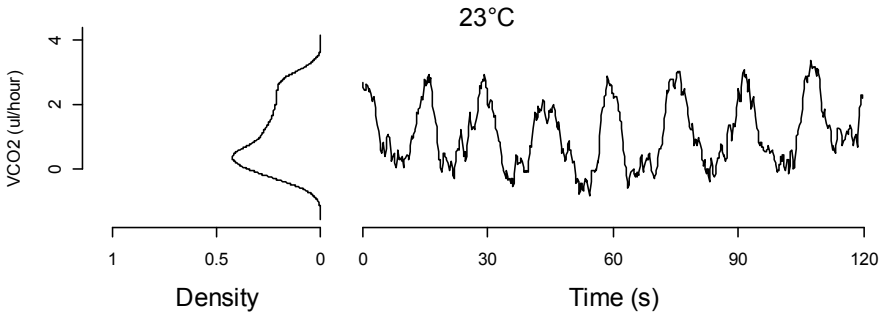


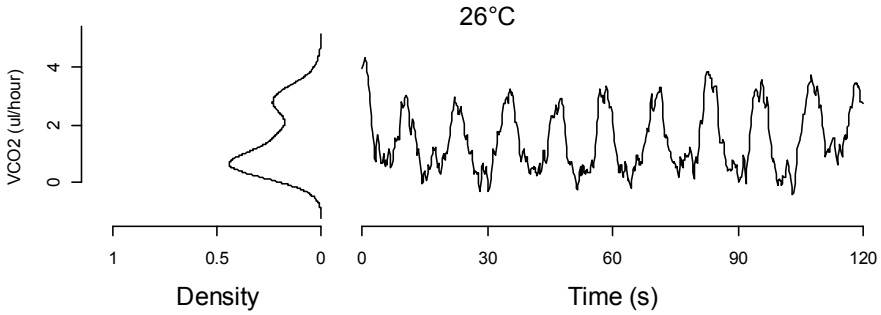


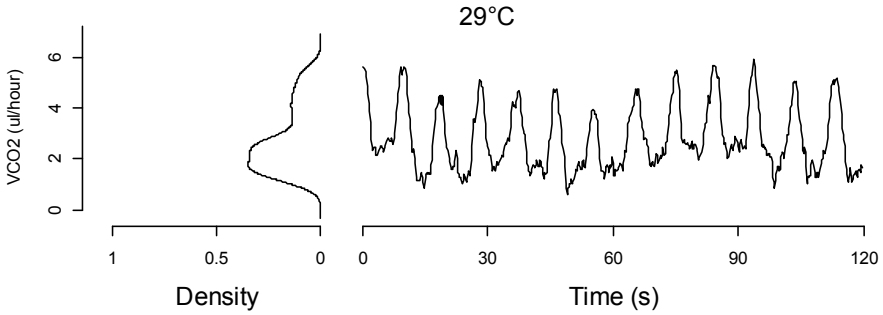


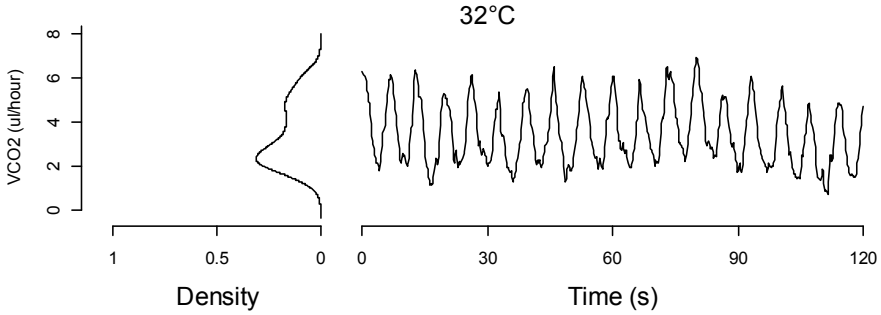
ID = u35
Age = 3 days
Larval density = 525/200ml
Dry mass (mg) = 0.387
Living body mass (mg) = 1.2275
Wing length (mm) = 2.745



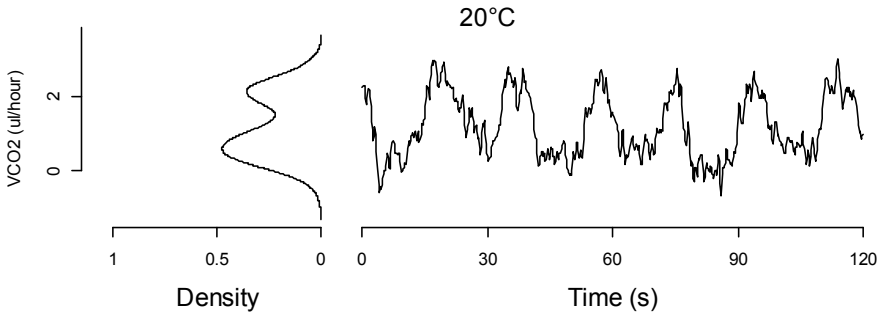


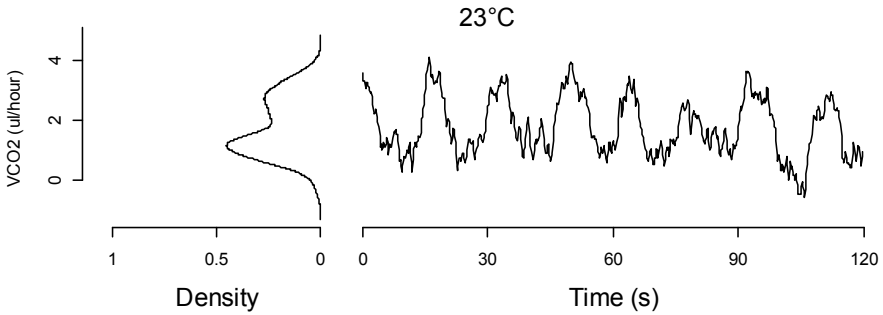


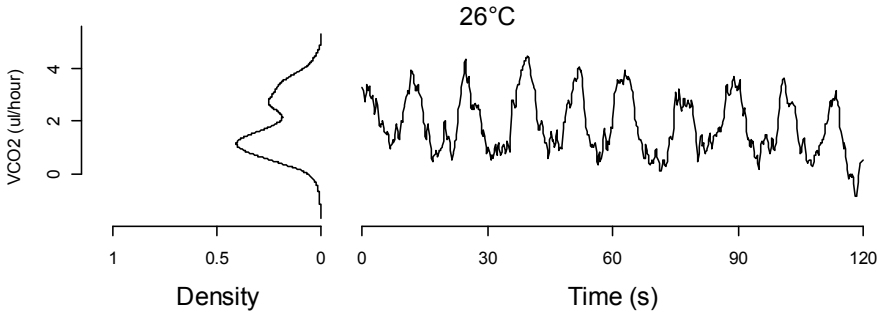


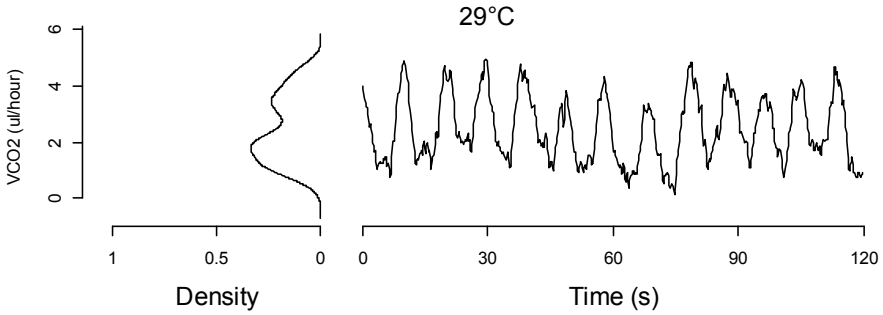


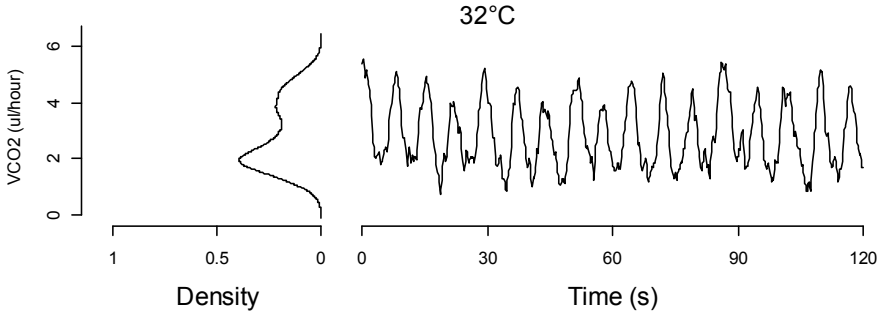
ID = u3
Age = 3 days
Larval density = 375/200ml
Dry mass (mg) = 0.365
Living body mass (mg) = 1.164
Wing length (mm) = 2.7225



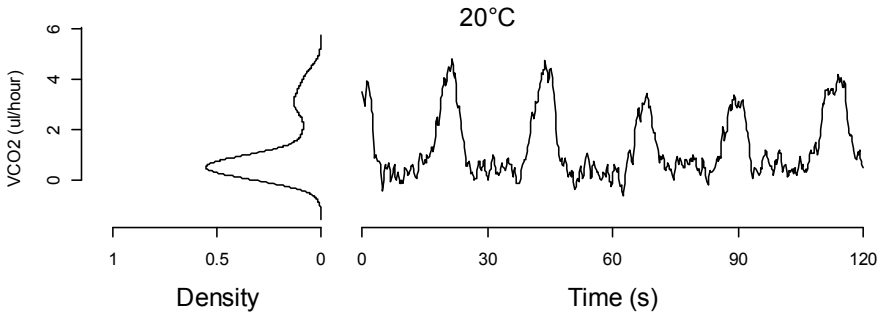


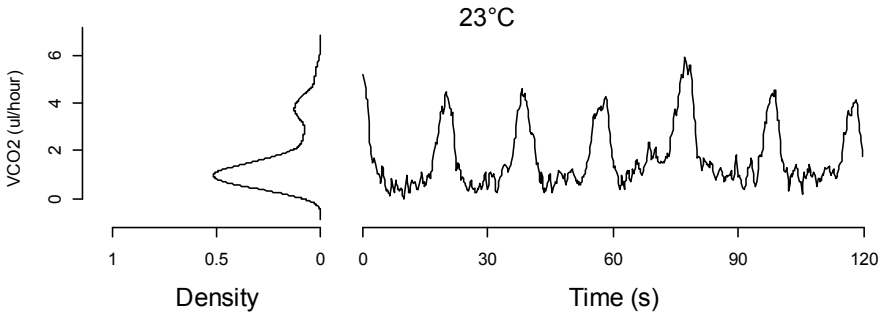


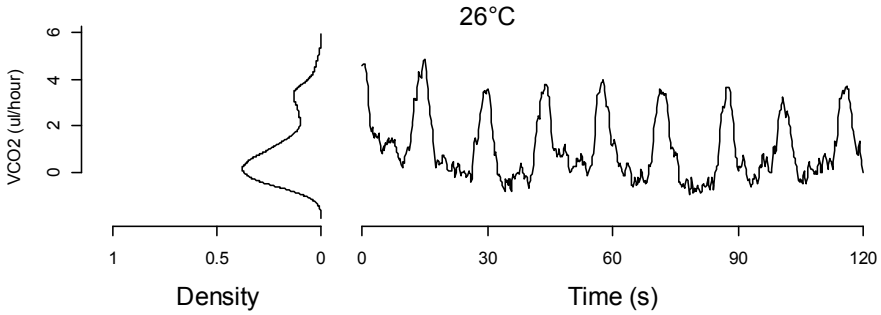


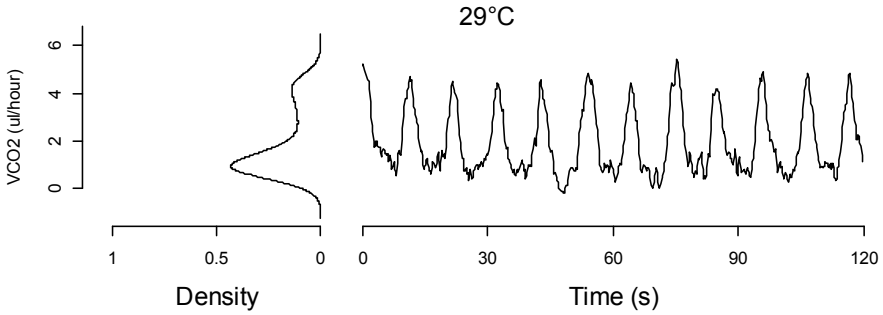


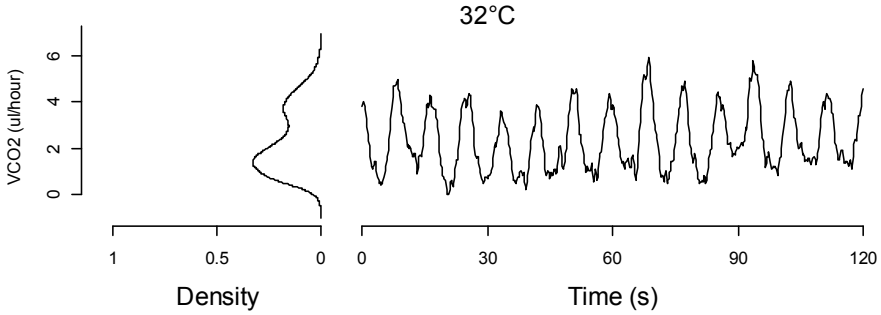
ID = u9
Age = 3 days
Larval density = 525/200ml
Dry mass (mg) = 0.358
Living body mass (mg) = 1.154
Wing length (mm) = 2.61



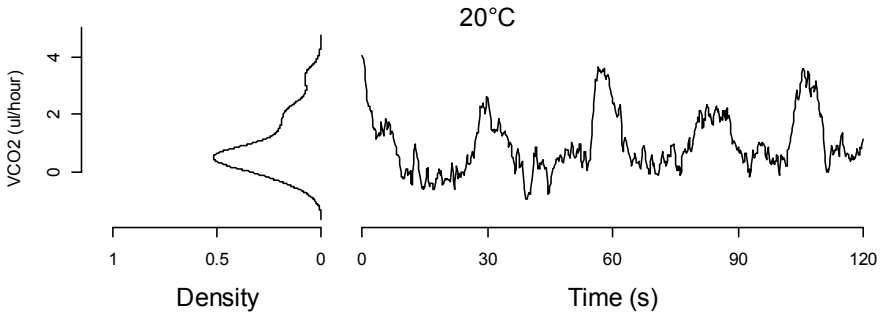


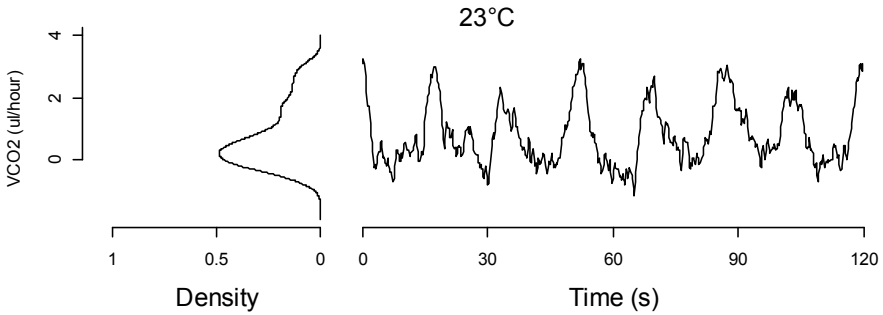


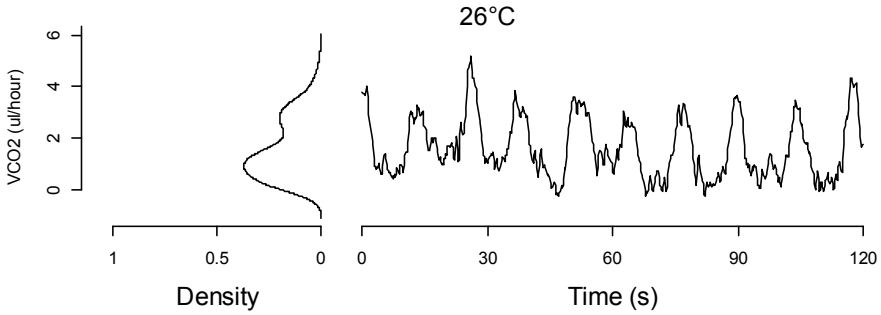


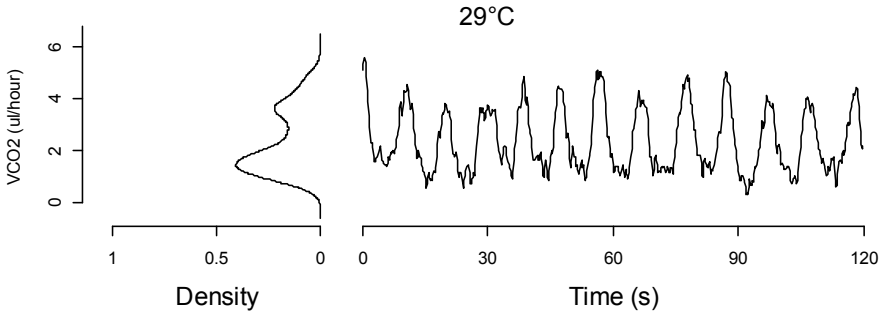


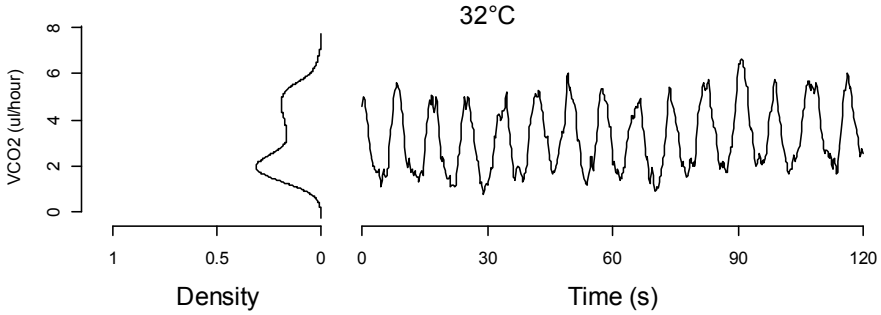
ID = d27
Age = 6 days
Larval density = 525/200ml
Dry mass (mg) = 0.345
Living body mass (mg) = 1.078
Wing length (mm) = 2.7



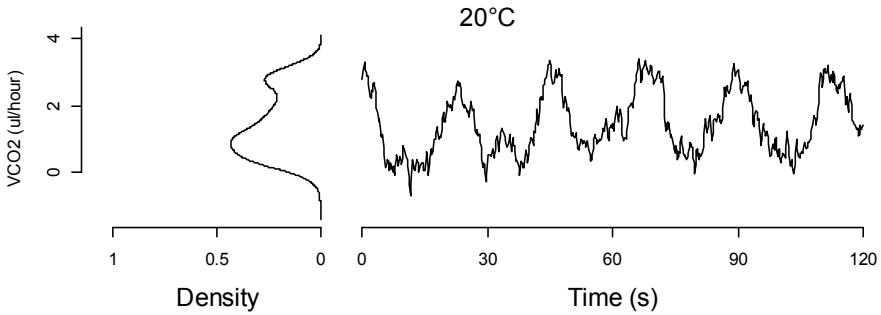






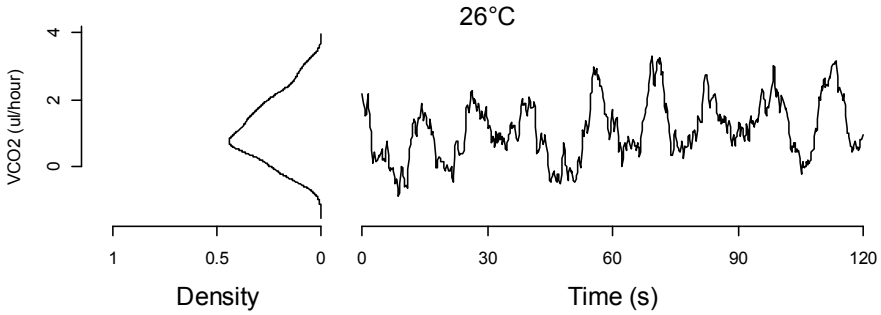


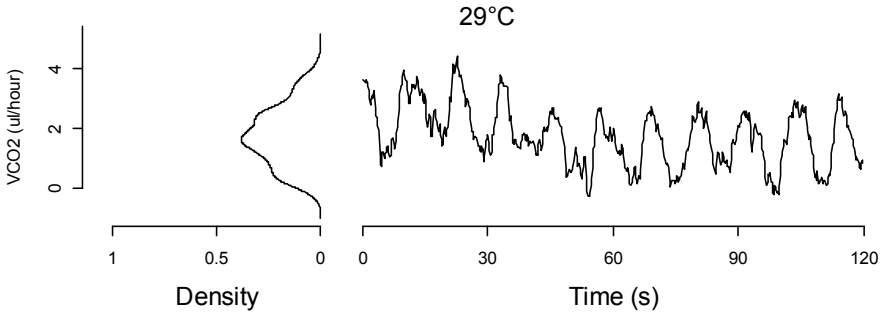
ID = d38
Age = 3 days
Larval density = 375/200ml
Dry mass (mg) = 0.336
Living body mass (mg) = 1.006
Wing length (mm) = 2.7675



23°C

NA



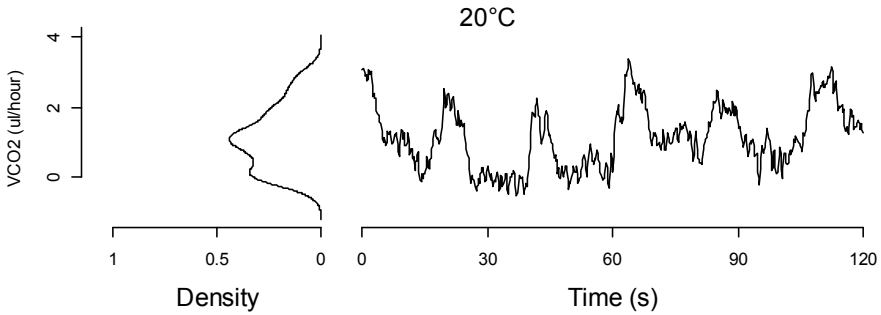


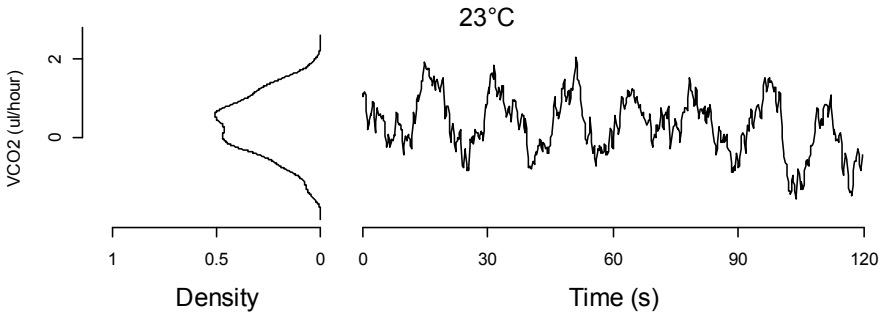
32°C

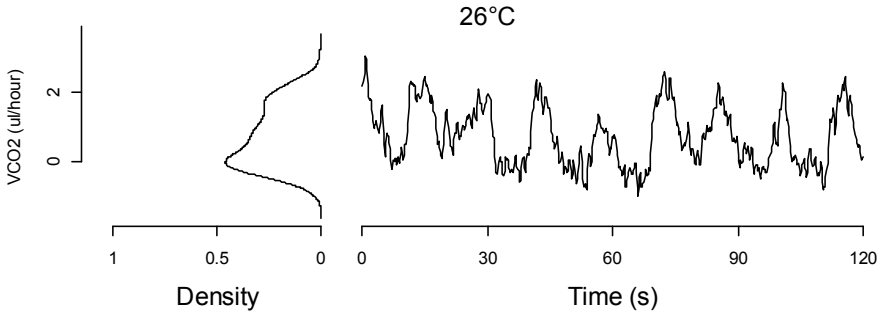
NA

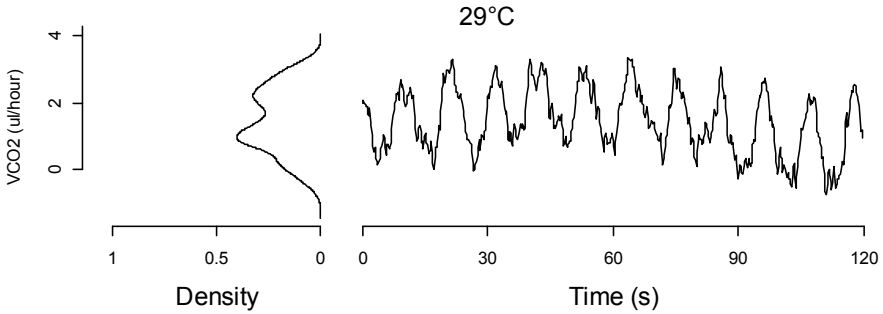
ID = u28
Age = 6 days
Larval density = 525/200ml
Dry mass (mg) = 0.334
Living body mass (mg) = 0.981
Wing length (mm) = 2.52

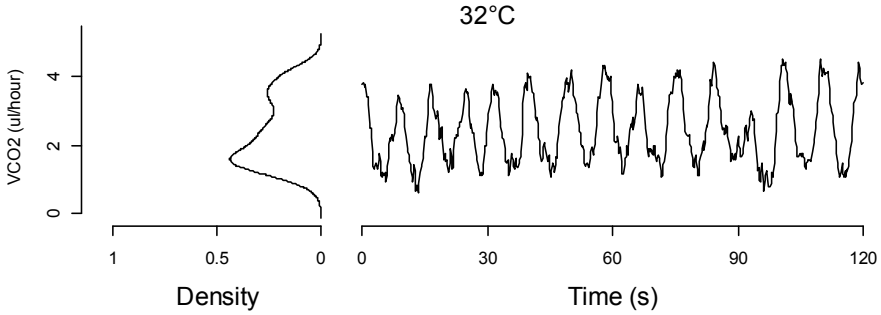
NA: data not available



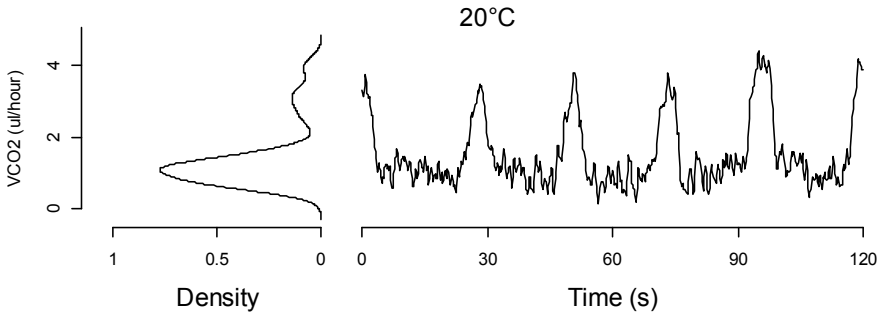


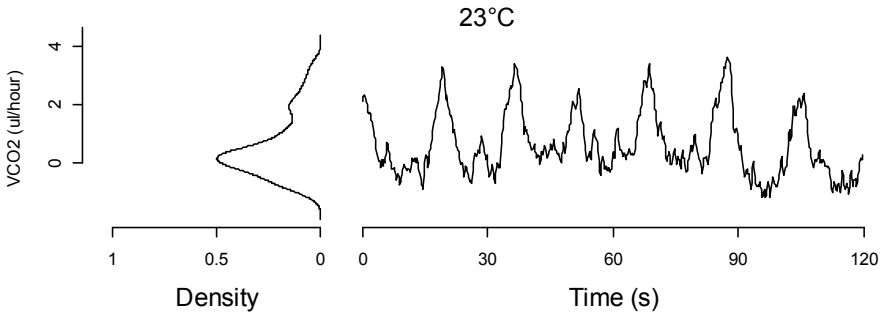


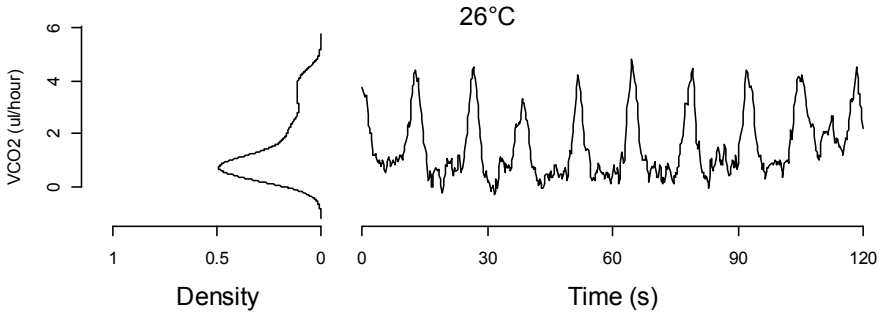


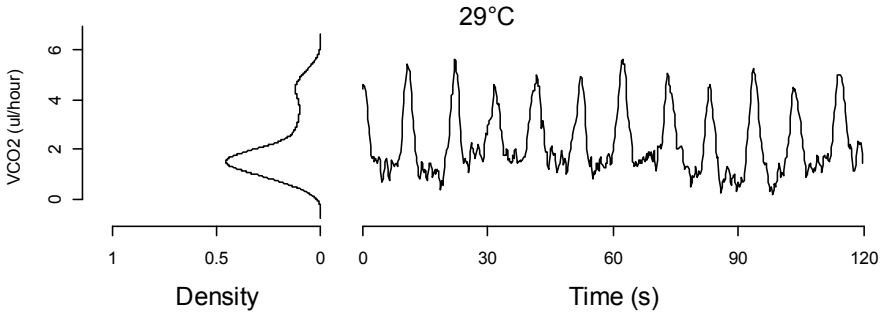


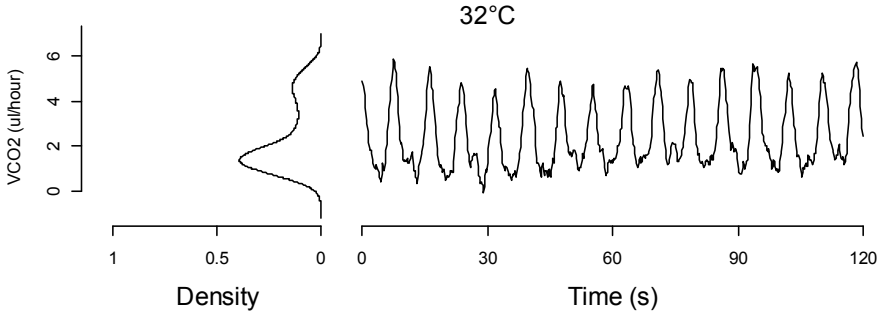
ID = u14
Age = 6 days
Larval density = 525/200ml
Dry mass (mg) = 0.315
Living body mass (mg) = 1.0035
Wing length (mm) = 2.475



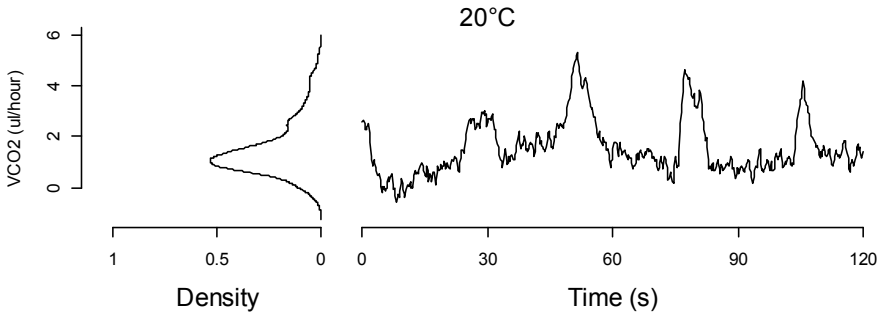


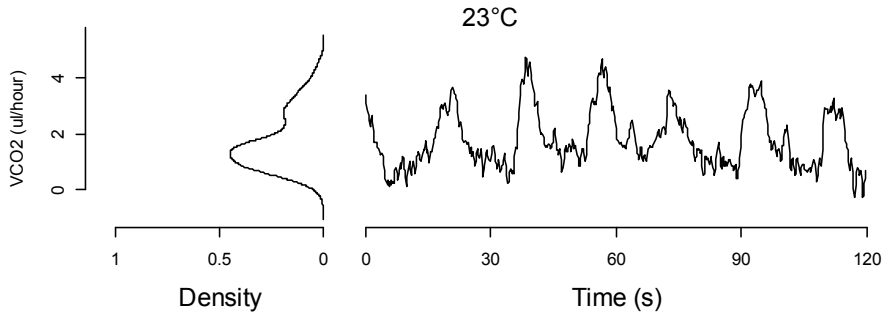


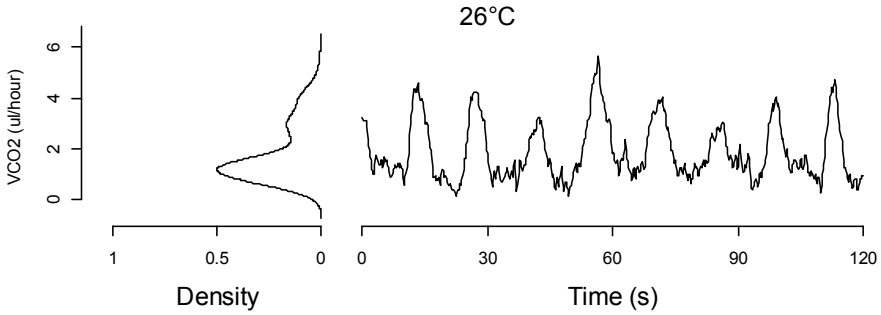


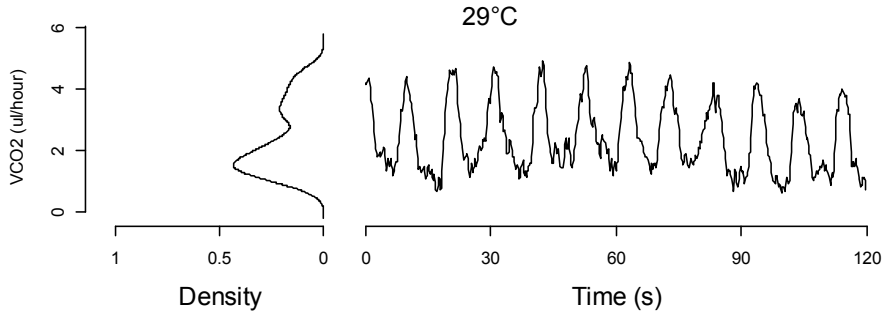


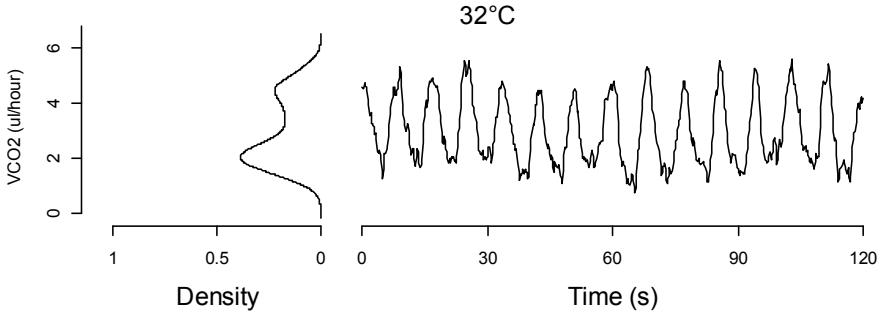
ID = d33
Age = 3 days
Larval density = 525/200ml
Dry mass (mg) = 0.309
Living body mass (mg) = 1.0025
Wing length (mm) = 2.61



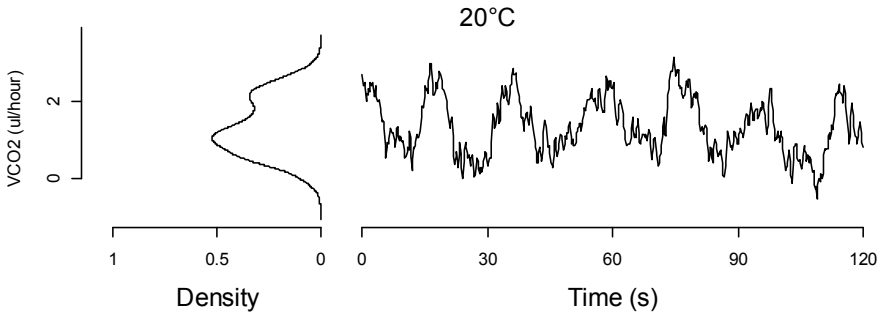


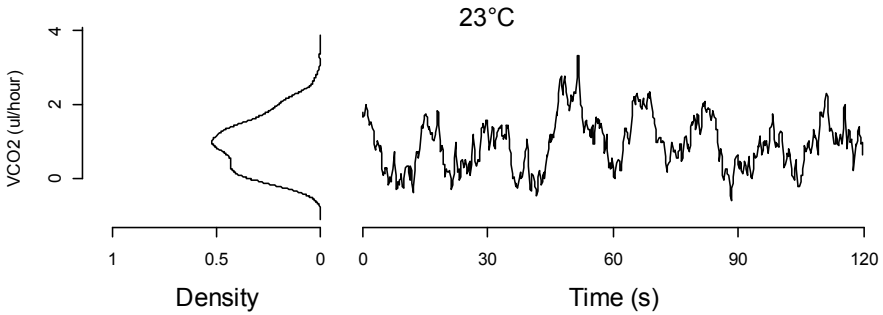


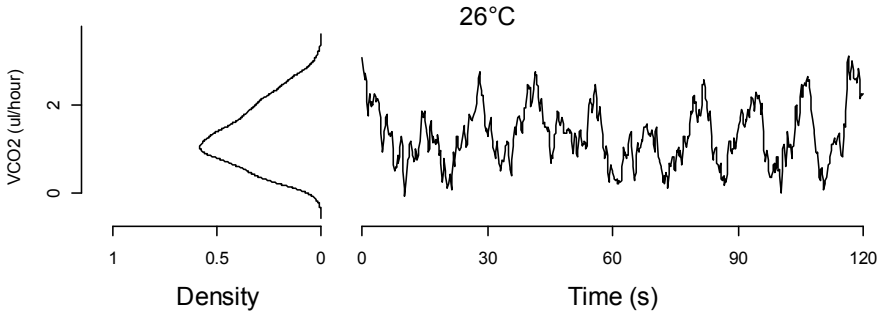


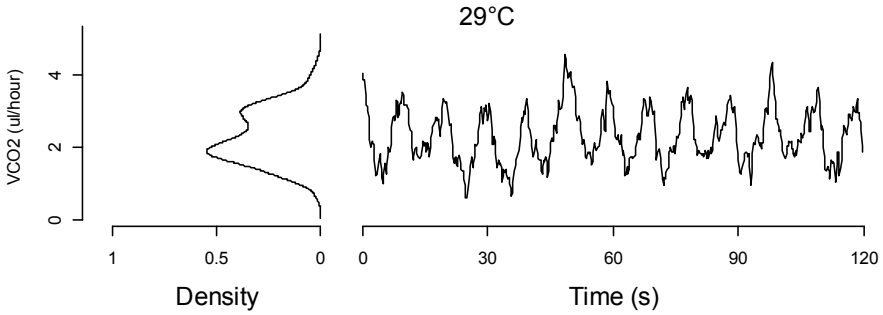


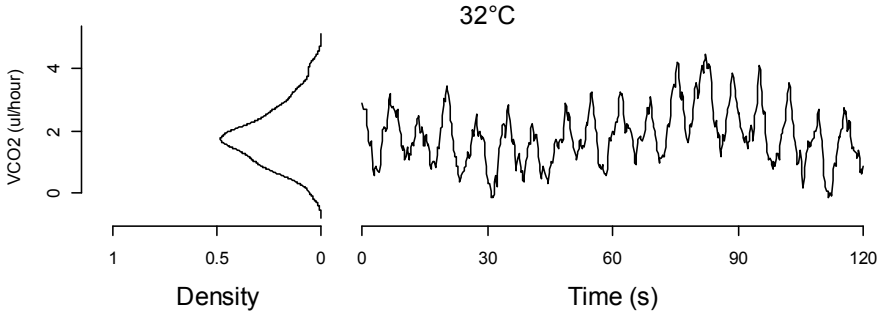
ID = d23
Age = 3 days
Larval density = 525/200ml
Dry mass (mg) = 0.293
Living body mass (mg) = 0.9145
Wing length (mm) = 2.61







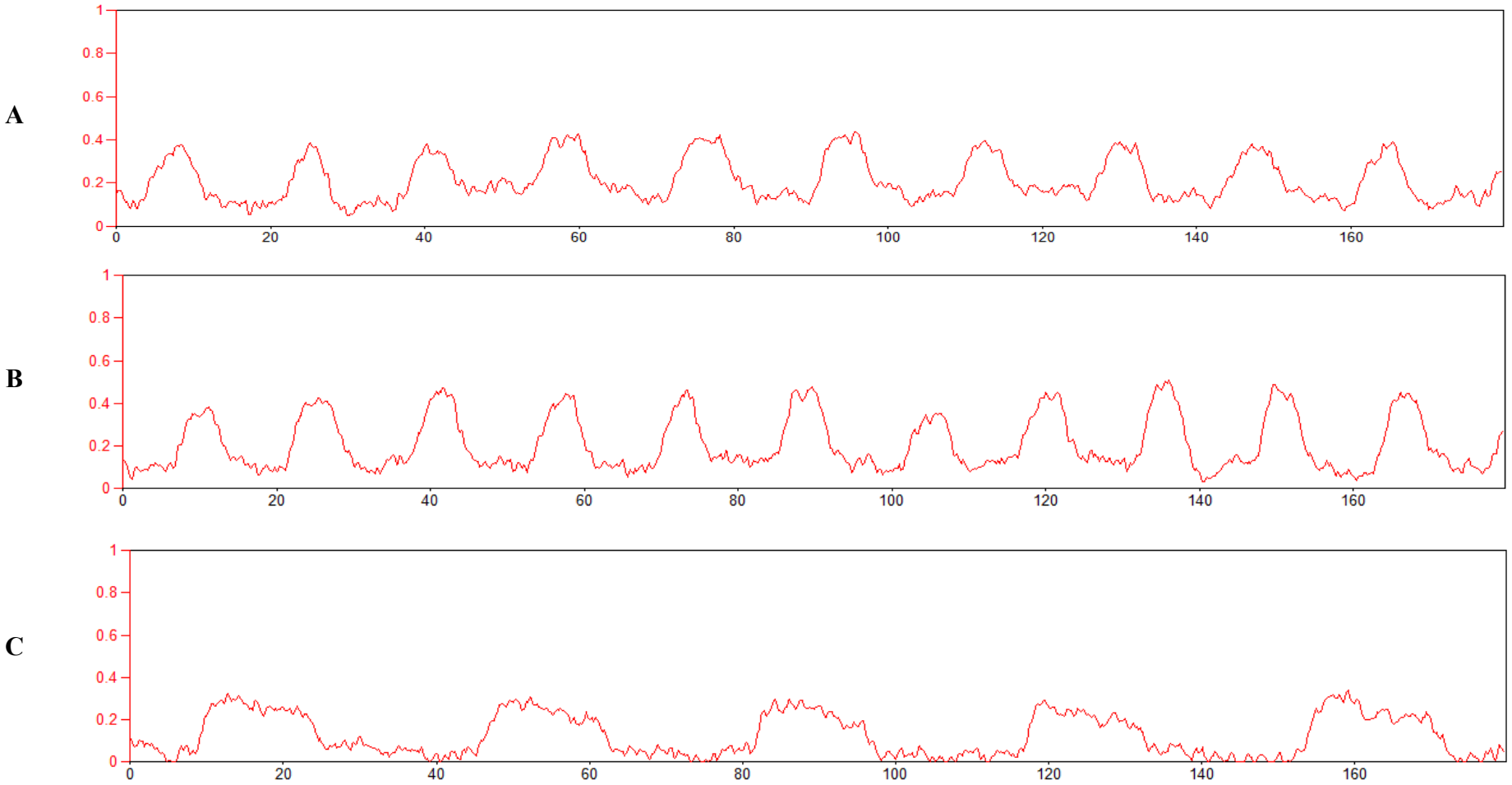




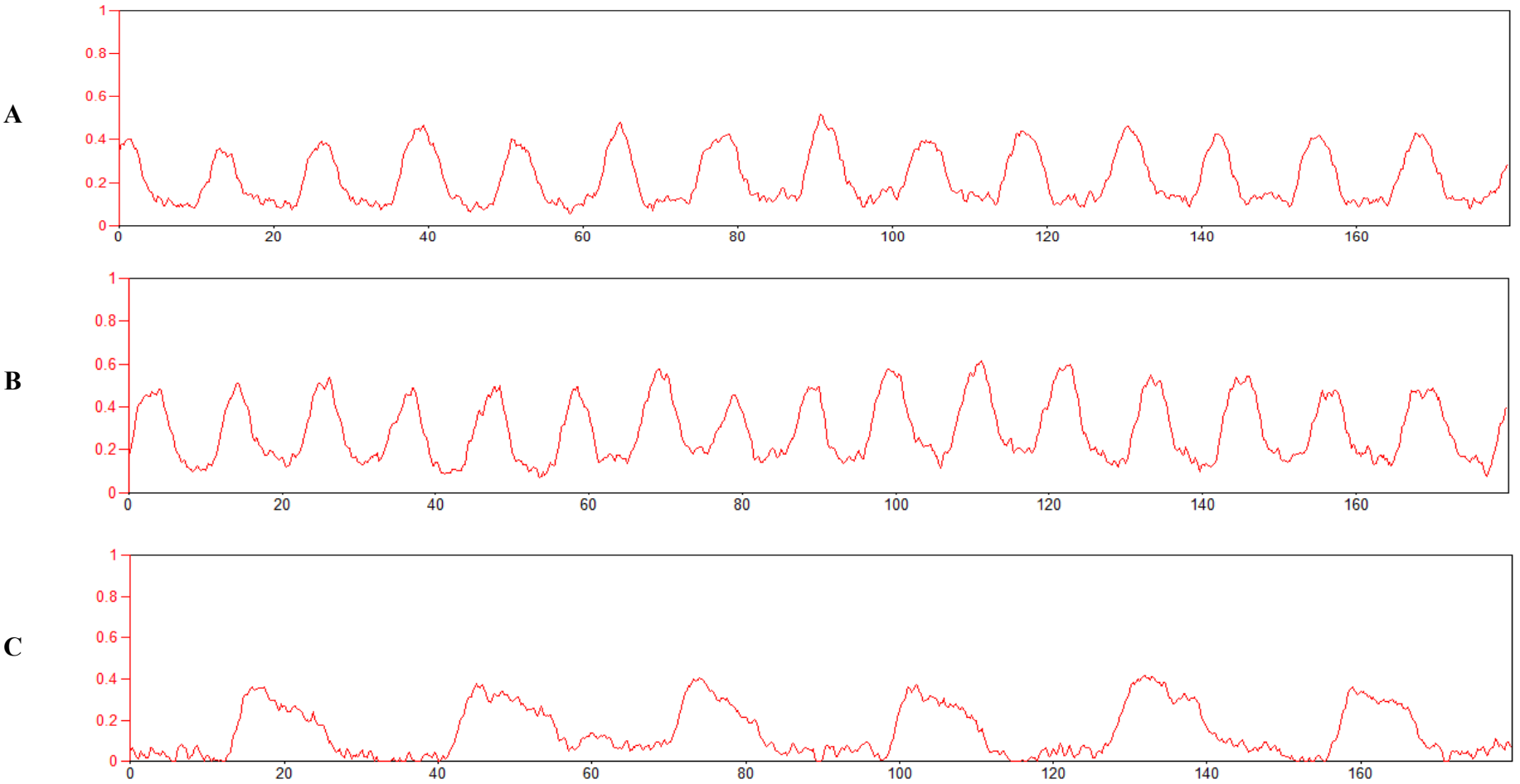
ID = u22
Age = 3 days
Larval density = 525/200ml
Dry mass (mg) = 0.279
Living body mass (mg) = 0.9455
Wing length (mm) = 2.475

8.2.2 Gas exchange pattern of *An. atroparvus*, *An. stefensi* and *An. gambiae* of similar body mass

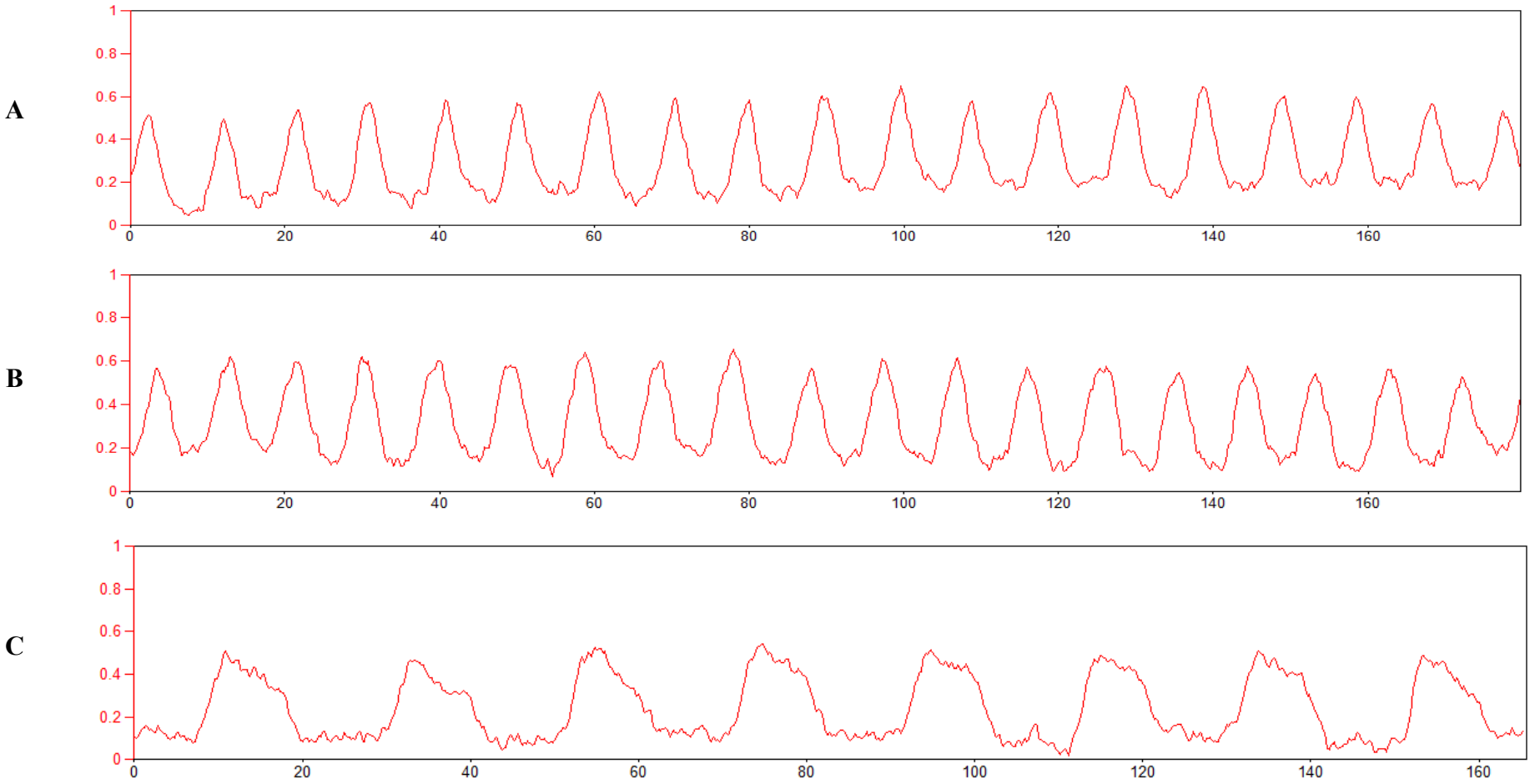
An. gambiae, *An. stephensi* and *An. atroparvus* at 23°C (A, B and C, respectively) with burst frequencies (F) ≈ 56, 61 and 28 mhz, respectively (time in seconds on abscissa and $s\dot{V}CO_2$ in ppm on ordinate, body mass ≈ 2.3 mg):



An. gambiae, *An. stephensi* and *An. atroparvus* at 26°C (A, B and C, respectively) with burst frequencies (F) ≈ 83, 89 and 34 mhz, respectively (time in seconds on abscissa and $s\dot{V}CO_2$ in ppm on ordinate, body mass ≈ 2.3 mg):

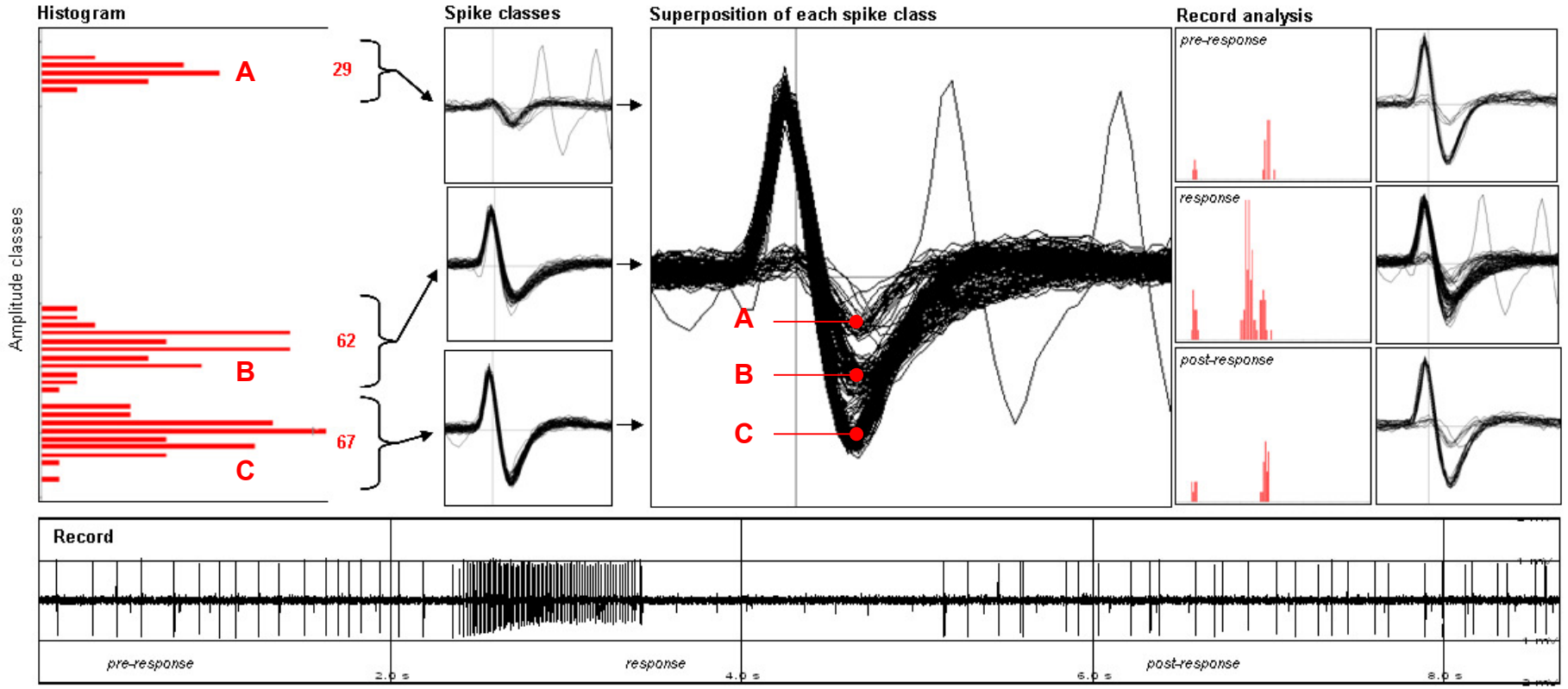


An. gambiae, *An. stephensi* and *An. atroparvus* at 29°C (A, B and C, respectively) with burst frequencies (F) ≈ 106, 106 and 44 mhz, respectively (time in seconds on abscissa and $s\dot{V}CO_2$ in ppm on ordinate, body mass ≈ 2.3 mg):



8.3 Chapters 4 and 5

8.3.1 Response of a multiporous capitated peg sensillum on the maxilla of *An. gambiae* stimulated with breath



Decomposition of the action potential signal (record at bottom) obtained with a tungsten microelectrode from a multiporous capitated peg sensillum stimulated with breath (see appendix 8.1.2). A histogram of spike amplitude classes (A, B and C with the number of spikes per amplitude indicated in red on left) can be computed for the whole recording with Autospike (Syntech, The Netherlands). Each spike amplitude class (A, B and C) corresponds to one of the 3 receptor neurons located in the sensillum. Furthermore, a histogram for the pre-response, response and post-response periods can be computed (panels with red bars on the right). Here the second spike class (B) which is absent from the pre and post response periods increases strongly in frequency during the response period clearly indicating that the neuron associated with this class is very sensitive to breath. This neuron class is probably a receptor neuron for carbon dioxide, the major stimulating constituent of breath.