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Distinct Physiological, biochemical and morphometric adjustments in the malaria vectors *Anopheles gambiae* and *An. coluzzii* as means to survive to dry season conditions in Burkina Faso

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Summary

Aestivation and dispersive migration are the two strategies evoked in literature to explain the way by which malaria vectors, *Anopheles coluzzii* and *An. gambiae*, survive the harsh climatic conditions of the dry season in sub-Saharan Africa. However, the physiological mechanisms regulating these two strategies are unknown. Here, mosquito species were submitted to controlled environmental parameters mimicking the rainy and dry seasons conditions of south-western Burkina Faso. Survival strategies were studied through morphometric (wing length), ecophysiological (respiratory gas exchanges), biochemical (cuticular hydrocarbons composition) and molecular (AKH mRNA expression levels) parameters of which variations are classically considered as hallmarks of aestivation and dispersion mechanisms in various insects. Our results showed that ecophysiological and morphometric adjustments are put in place in both species to prevent water losses during the dry season. However, the classical metabolic rate modifications expected as signatures of aestivation and migration were not evidenced here, highlighting specific and original physiological mechanisms sustaining survival in malaria mosquitoes during the dry season. Differences in epicuticular hydrocarbons composition and AKH levels of expression were found between the permanent and temporary *An. coluzzii* populations, illustrating the great phenotypic plasticity of this mosquito species. Altogether, our work underlines the diverse and complex pattern of changes occurring in the two mosquito species and at the population level to cope with the dry season and highlights potential targets of future control tools.

Key-words

Aestivation; migration; adipokinetic hormone (AKH); cuticular hydrocarbons; metabolic rate

List of abbreviations

AKH= Adipokinetic hormone; ODS = Onset of the dry season; RS = Rainy season; VCO_2= Rate of carbon dioxide release; VH_2O = Rate of water loss; WL = Wing length.

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1. Introduction

In Western Africa, malaria is mainly transmitted by the infectious bite of two anthropophilic female mosquito species, *Anopheles coluzzii* and *An. gambiae*. Both species can occupy a large range of ecological niches, but their densities follow the pace of the availability of their larval breeding-sites, *i.e.* standing water. In dry savannah areas, these larval habitats dry-up at the onset of the dry season, leading to the fall of mosquito populations (Dao et al., 2014; Mamai et al., 2015). Only the mosquito *An. coluzzii* can locally persist all year long, as it can also exploit permanent larval breeding-sites like dams, rice fields or river edges, which do not entirely disappear during the dry season (Baldet et al., 2003; Costantini et al., 2009; Diabate et al., 2002, 2004). The wider range of habitats exploited by *An. coluzzii* for larval breeding (temporary drying-up at the onset of the dry season, or permanent) may be explained by its aptitude to express different adaptive phenotypes related to the local breeding-site characteristics (Gimonneau et al., 2012a, 2012b, Hidalgo et al., 2015a, 2016). Recent works suggested that *An. coluzzii* mosquitoes collected from temporary larval breeding-sites might be "strong aestivators" programmed to enter a dormant state following specific (yet unknown) stimuli at the onset of the dry season (Hidalgo et al., 2016; Yaro et al., 2012). However, when *An. coluzzii* mosquitoes exploit permanent breeding-sites, they are considered as "weak aestivators" entering a facultative dormant state depending, at least in part, on the breeding abilities of the areas they exploit. By contrast, the mosquito *An. gambiae* exploits temporary larval breeding-sites only. Using population dynamics studies, it has been proposed that these mosquitoes did not enter a dormant state, but rather display high dispersal abilities to rapidly colonize new favourable habitats at the onset of the dry season (Dao et al., 2014; Huestis and Lehmann, 2014; Mamai et al., 2015). Consequently, physiological traits, in particular those associated with desiccation resistance or dispersal abilities, may differ between the two species at the onset of the dry season (Arcaz et al., 2016; Hidalgo et al., 2014; Mamai et al., 2016).

Aestivation is a well-known survival process in insects inhabiting arid areas, but the physiological mechanisms and regulative metabolism controlling this process remain controversial (Denlinger and Armbruster, 2014). As suggested for insect overwintering diapause, metabolic rate depression should be one of the most significant physiological traits characterising aestivation (Benoit, 2010; Storey and Storey, 1990). Decrease of the metabolic rate has already been observed to prevent water losses and increase fitness of several insect species inhabiting arid environments (Chown, 2002; Hadley, 1994; Rourke, 2000). Yet, this pattern has not been observed in field-sampled *An. coluzzii* (Huestis et al., 2011, 2012), even in

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populations assumed to be "strong aestivators". When field-sampled during the rainy season and at the onset of the dry season, the high inter-individual variability of mosquitoes' phenotypes, resulting from uncontrolled age, trophic and reproductive states, may have prevented the drawing of a clear seasonal phenotypic pattern (Nespolo et al., 2003; Rogowitz and Chappell, 2000).

In addition to changes in metabolic rate, decreased permeability of the cuticle *via* changes in hydrocarbon composition for instance, have already been linked to desiccation resistance in several arthropod species (Blomquist and Bagnères, 2010; Gibbs and Rajpurohit, 2010; Stinziano et al., 2015). In particular, amounts of long-chain hydrocarbons correlate to increased cuticle hydrophobicity, and we expect that such adjustments would occur in anopheline species at the onset of the dry season. In parallel, the catabolism of specific organic compounds, like stored glycogen which generates significantly higher amounts of bounded water than any other energetic substrates (Schmidt-Nielsen, 1997), can contribute to reduce body water losses. The catabolism of glycogen by the glycogen phosphorylase is under the control of the adipokinetic hormones (AKH) (Wilps and Gäde 1990; Gäde 2004; Ziegler et al. 2011), whose variations may signal changes in metabolism activity at the entry of the winter for dormant insects (Hahn and Denlinger 2011). Similarly, variations of AKH levels may depict changes in desiccation resistance over the year in anopheline species, and increased amounts of this peptide can be expected in *An. coluzzii* at the onset of the dry season (Hidalgo et al., 2016). AKH is also particularly important for flight maintenance, as it supplies the energetic substrates needed for striated muscle contraction in insects (Arrese and Soulages, 2010; Van der Horst, 2003). Thus, an increased expression of AKH gene expression levels in female *An. gambiae* at the onset of the dry season could be part of the "dispersive" phenotype.

In the present study, we aimed to experimentally trigger and characterize the specific phenotypes associated with current assumptions about survival strategies of *An. coluzzii* and *An. gambiae*: (1) females from *An. gambiae* species are expressing a dispersive behaviour to escape the harsh conditions of the dry season, whereas *An. coluzzii* females only virtually disappear and aestivate, and (2) the intensity of the aestivation phenotype in *An. coluzzii* females depends on the larval breeding-site characteristics (permanent to temporary pattern).

To address these assumptions, female mosquitoes were experimentally exposed to the environmental conditions they experience during the rainy (RS) and the onset of the dry season (ODS) in Burkina-Faso (West Africa). Variations in their metabolic rates, water contents, allometric measures (*i.e.* body size and dry mass), AKH peptides mRNA expression levels

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(Anoga-AKH-I, Anoga-AKH-II and their putative receptor Anoga-AKH-R), and cuticular hydrocarbon fingerprints were measured and compared (i) between RS and ODS conditions for each mosquito population, and (ii) among populations. We assumed that (i) *An. coluzzii*, females would have enhanced abilities to prevent body water losses at ODS, (ii) a decreased metabolic rate (which would also help preventing body water losses) should be observed at ODS in the aestivating females of *An. coluzzii*, but not in *An. gambiae*, where we expected to measure an increase in CO_2 release, (iii) differential AKH gene expression levels between populations and experimental conditions that would reveal different metabolic mobilisation and, hence, different survival strategies (aestivating *vs.* dispersive), and (iv) seasonal changes in hydrocarbon composition of the cuticle should be observed in both species, notably in aestivating *An. coluzzii* specimens. Finally, we assumed that distinct ecophysiological phenotypes between populations of *An. coluzzii* should be observed according to their site of collection (*i.e.* temporary or permanent breeding-sites areas).

2. Materials and methods

2.1. Mosquito populations and rearing conditions

2.1.1. Mosquito populations

Experiments were conducted using two mosquito populations of *An. coluzzii*, and one population of *An. gambiae*. The two populations of *An. coluzzii* were established in September-October 2012 from 50 gravid females collected from two localities of Burkina Faso (Bama and Soumousso). At Bama [11°23'N, 04°24'W], *An. coluzzii* is the prevailing mosquito species (Gimonneau et al., 2012a), and it is breeding all year long in rice fields (*i.e.* permanent population). By contrast, at Soumousso [11°01N, 04°02'W], population dynamics of *An. coluzzii* match the seasonal variations of local climatic conditions (Dabiré et al., 2007). The population of *An. gambiae* used in this study was established at the IRSS insectary in 2009 from females of temporary populations collected in the village of Soumousso. This population thrives in sympatry with populations of *An. coluzzii*.

The three populations were maintained under the same insectary conditions (27 ºC, 70% humidity, light/dark cycles of 12/12h) prior to being used for the analysis (approximatively 6 generations). The three populations were maintained in separate rearing rooms to avoid any crossbreeding.

2.1.2. Experimental rearing conditions

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The three mosquito populations were reared from eggs to adults into two climatic chambers (Binder KBF720 VWR international S.A.S), programmed to reproduce the natural daily fluctuations of rearing conditions experienced by mosquitoes during the rainy season (RS; *N*=1 climatic chamber) and at the onset of the dry season (ODS; *N*=1 climatic chamber). Programmed daily temperature and relative humidity conditions mimicked field conditions, as earlier used by (Hidalgo et al., 2015b, 2014, 2016; Mamai et al., 2014). Moreover, variation of day length was also accounted by using hourly-recorded light intensity data during the RS and ODS conditions with a weather monitoring station (Weatherlink; Davis Instruments, Hayward, CA, U.S.A.) (**Supplementary data 1**). Water temperature in rearing trays was similar to air temperature (Hidalgo et al., 2014, 2016).

For each mosquito population, three independent batches of eggs were synchronously collected from more than 50 different caged females, and reared in RS or ODS conditions. The climatic conditions inside climatic chambers were switched between each batch of eggs to account for any potential "chamber effect". Upon emergence, only females were maintained within the climatic chambers in small cages at a density of 30 females per cage, until they reached four to six-day old. Emergent females were fed *ad libitum* with glucose (10% w/v) and water. Twenty-four hours before the experiments (*i.e.* when females were three to five days old), females were food- and water-deprived in order to avoid any bias in carbon dioxide ($VC0_2$) and water loss rate (VH_2O) measurements. The first batch corresponded to mosquitoes used for VCO_2, VH_2O and allometric measurements. The second batch was used for analysing the mRNA expression of the AKH peptides. The third batch corresponded to mosquitoes used for cuticular hydrocarbons analyses.

2.2. Gas exchange, flight and allometric measurements

2.2.1. Metabolic (VCO_2) and water loss (VH_2O) rates

The VCO_2 and VH_2O rates of females from the three populations were measured using a flow through respirometry and a CO_2-H_2O gas analyzer (Sable System, Li-7000 CO_2/H_2O infrared gas analyzer, Li-Cor-Biosciences, Lincoln, NE). For each population and experimental condition (*i.e.* RS; ODS), six to nine runs were performed on pools of four randomized females (so that the minimum CO_2 identification threshold within the gas analyzer system was reached). Four to six-day old females were placed into a 200 x 400 mm (L x l) glass chamber. The chamber was connected to the gas analyzer, and flushed with a constant flow of air at a rate of 200 mL/min. The temperature and relative humidity of the air entering the chamber were modulated using a Peltier effect temperature controller and a Dew point generator/relative

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humidity controller system, respectively. The programmed conditions corresponded to the hottest and driest period of the day for each season during which females are resting (from noon to four p.m.). Hence, the relative humidity and temperature of the flowing air were respectively 18% and 34 ºC for the ODS conditions, and 70% and 29 ºC for the RS conditions.

Each run lasted 100 min during which both the rates of CO_2 and H_2O releases (VCO_2 and VH_2O) were recorded. Following an acclimation period of 25 min, measurements of VCO_2 and VH_2O were performed for 15 min. The measurement sequence was repeated three times. Control measures were performed in an identical parallel system without mosquitoes for 10 min before each measurement sequence (*i.e.* 3 x 10 min). Data were analyzed using the Expedata software (Sable System International V.1.0.1). Amounts of mosquitoes' VCO_2 and VH_2O were calculated by subtracting the control values to those measured with mosquitoes in the chamber. After each run, viability of the females was checked before they were snap-frozen and dried for 3 days at 60 ºC, and weighed (Sartorius, 0.1mg accuracy). For each run, VCO_2 and VH_2O were averaged per 15 min period and expressed in µl/min/mg of mosquito dry mass to assess for mass variability between species and conditions (Gray and Bradley, 2006; Huestis et al., 2011, 2012).

2.2.2. Flight activity

The flight activity, which might influence the rate of CO_2 and H_2O released by females, was recorded during the VCO_2 and VH_2O analyzes. Females were recorded using a HD Camera (Sanyo, model). A total of 44 batches of four pooled female mosquitoes (*i.e.* 176 females – representing from six to nine batches by experimental condition and population) were analyzed using the ImageJ1.41.0 software.

2.2.3. Allometric measures

The females' body size was estimated using their wing length (WL). Immediately after VCO_2 and VH_2O running sequences, females were snap-frozen. The right wing was removed before females were dried and weighed. Wings were mounted on a microscope slide, and pictures were taken under a stereomicroscope (x 20, Leica DFC425). Wing length was measured from the allula to the wing tip as described by (Charlwood, 1996). The measures were performed using ImageJ1.41.0 software (Wayne Rasband, National Institute of Health, Rockville, MD, U.S.A.) to an accuracy of ±0.001 mm. Damaged wings were discarded from the analysis. WL was divided by the dry mass of mosquitoes to estimate the surface to volume

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ratio of the specimens. A total of 158 wings was measured (*i.e.* from 24 to 36 per experimental condition and mosquito population).

2.3. *AKH*-related genes expression variations

2.3.1. RNA extraction and cDNA synthesis

For each population and experimental condition (RS; ODS), three to four samples of ten randomly pooled four day-old female mosquitoes were analysed. Collected mosquitoes were immediately snap-frozen in liquid nitrogen, and conserved at -80 °C with RNA*later* solution (Ambion, USA) until further processing.

Before RNA extraction, the RNA*later* solution was removed, and the total RNAs of each sample were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) coupled with the RNeasy Kit (QIAGEN). Samples were thereafter treated with DNase I (Ambion, USA) following the manufacturer's instructions. RNA amounts were quantified by spectrophotometry at 260 nm (Nanodrop2000, Thermo Scientific). Single-stranded cDNAs were then synthesized with Superscript II reverse transcriptase (Gibco BRL, Invitrogen) as described in Bigot et al. (2012) and according to the manufacturer's instructions.

2.3.2. Real-time quantitative PCRs

All real-time quantitative PCRs were conducted as described in Bigot et al. (2012). Ten genes (*Actine*, *Rps13*, *Rps7*, *Rpl5*, *h3a*, *Cytp450*, *Tubulin*, *hsp83*, *EGFR*, and *18s*) were tested as putative housekeeping genes, following a BestKeeper analysis (Pfaffl et al., 2004). The *Rps13* was selected as the reference gene since its expression was stable in all samples, whatever the experimental condition or the anopheline population tested. Further, mRNA of the two AKHs genes *Anoga-AKH-I* and *Anoga-AKH-II*, and the AKH receptor gene *Anoga-AKH-Receptor* (Kaufmann and Brown, 2006) were examined. Specific primers (reverse and forward) for both housekeeping genes and target genes were designed using the Eprimer3 software (http://emboss.bioinformatics.nl/cgi-bin/emboss/eprimer3; **Table 1**).

Each PCR reaction was technically triplicated and consisted of 6 µL of absolute Blue SYBR Green Fluor (ROCHE Molecular Systems Inc., USA), 2 µL of cDNA (25 ng.µl^{-1}), 0.5 µL of each reverse and forward primers (10 µM), and 3 µL of RNA free water. The qPCR program and conditions have been described in Bigot et al. (2012). The cycle threshold values (Ct-values) for both reference and target genes were determined using the Light-Cycler® 480 software (Roche, FRANCE). The average Ct value of each technical triplicate was used to

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normalise candidate gene expression levels to the geometric mean of the reference gene level using the Q-Gene software (Simon, 2003).

2.4. Cuticular hydrocarbon fingerprints

For each mosquito population exposed to RS and ODS conditions, the external cuticle (*i.e.* epicuticular) hydrocarbons were extracted from six to eight samples of four randomly pooled four day-old females, so that the minimum samples' mass reached 1 mg. Samples were washed for 10 min three times in 100 µL of dichloromethane. The three elution solutions were pooled and dried up under nitrogen. Dried extracts were conserved at -20 °C until further processing. Upon analysis, samples were re-suspended in 50 µL of dichloromethane containing 60 ng of *n*-tetradecane (internal standard). Samples were analyzed by gas-chromatography (GC) on a HP 6850A chromatograph equipped with a HP-5MS fused silica column (30m x 0.25mm; film thickness 0.25mm; Chrompack, Les Ulis, France) and an ionization flame detector (FID). We used the procedure described by (Trabalon et al., 1996). Briefly, the oven temperature increased from 100 °C to 200 °C at a pace of 5 °C/min, and from 200 °C to 320 °C at a pace of 3 °C/min. The composition of the total hydrocarbon extract of each sample was identified by GC-MS on a HP 1890A equipped with a HP-5MS column. Only clearly defined peaks were used for profile characterization and quantitative analyzes (surface greater than 0.1% of the total amount of chemical compounds detected for each sample). The compounds were identified by comparing their mass spectra and GC retention times with those of pure standards. The results were expressed as mass/µL extract injected (ng/µL). Quantification was done by GC-FID analysis using internal and external standards:fatty acids (C16:0, C18:0, C18:1 and C18:2), methyl esters (palmitate, stearate) and n-alkanes (C13 to C34), Sigma®.

2.5. Data analysis

All statistical procedures were conducted using the *R* 3.1.1 statistical software (R Development Core Team, 2008). Before analysis, the normal data distribution and homoscedasticity of variables were verified using Shapiro-Wilk and Bartlett's tests, respectively. Accordingly, analyzes were further processed using parametric or non-parametric tests. For all analyzes, main effects and all relevant first and second order interactions were tested in full models. Model simplification used stepwise removal of terms, where the significance of terms was estimated using the difference in Akaike's information criterion (AIC). When needed, a Tukey HSD procedure was used to perform *post-hoc* comparisons among the levels of significant factors.

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A two-way ANOVA was first performed to test whether allometric measures (dry mass, wing length and surface to volume ratio) and the flight activity of females varied according to the rearing conditions (RS, ODS), or among their population origins.

Generalised linear models (GLM, quasi-poisson error and logit function) were used to investigate the variations of metabolic rate (VCO_2; expressed as µL/min/mg of dry mass), including the rearing conditions, the anopheline populations, the flight activity level, the VH_2O, the wing size and the surface to volume ratio as explanatory variables. A second GLM was used to investigate the amount of water loss in females (VH_2O, expressed as µL/min/mg of dry mass) with the same above-mentioned variables (except that the VH_2O was replaced by VCO_2). Because of a significant effect of the surface to volume ratio on VH_2O (ddl=1, χ^2=91.04, P<0.001), a linear regression model was plotted to observe the direction and amplitude of this correlation. This relationship significantly differed according to the rearing conditions (RS or ODS) the mosquitoes were exposed to (ddl=2, χ^2=5.91, P<0.05), but was similar among their population origins (ddl=1, χ^2=1.0, P=0.59). Values of the three populations were then concatenated, and linear regressions between surface to volume ratio and VH_2O were plotted distinctly for RS and ODS conditions.

The expression levels of the genes *Anoga-AKH-I*, *Anoga-AKH-II* and *Anoga-AKH-R* were analyzed using two way ANOVA procedures, where the experimental condition and the mosquito population were used as explanatory variables.

The cuticular hydrocarbon concentrations were first log-transformed ($x = \log_{10}[X + 1]$) to meet the assumption of normally distributed residuals. An ANOVA was performed to test for the effect of mosquito population and experimental conditions on cuticular hydrocarbon fingerprints. In addition, a MANOVA was performed to examine qualitative differences in cuticular hydrocarbon profiles among anopheline populations and experimental conditions. Two-way ANOVAs (where the experimental conditions and population of origin were considered as explanatory variables) were conducted for each individual hydrocarbon. A linear discriminant analysis (LDA) was performed to further assess qualitative and quantitative differences among the mosquito populations and rearing conditions. The between- and within-group(s) degrees of freedom together with the F-value were reported for each LDA axis. The distribution's significance of the LDA was assessed using a Monte Carlo test with 10,000 permutations ($P < 0.001$).

3. Results

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3.1. Gas exchange, flight and allometric measurements

3.1.1. Metabolic (VCO_2) and water loss (VH_2O) rates

Overall, the three populations displayed equivalent VCO_2 (GLM, *ddl*=2, χ^2= 0.15, *P*=0.46). However, this parameter was significantly influenced by the rearing conditions (GLM, *ddl*=1, χ^2= 7.45, *P*<0.01; **Fig. 1**). The difference was particularly striking for *An. coluzzii* mosquitoes from the temporary population exposed to ODS conditions: females were characterized by an important increase of their VCO_2 (**Fig. 1**). A similar trend, yet non-significant, was observed for the permanent population of *An. coluzzii* (GLM, *ddl*=2, χ^2= 5.11 *P*=0.07).

Rates of VH_2O significantly differed among the three populations, and according to the environmental conditions (**Table 2**). VH_2O of all populations significantly decreased when mosquitoes were exposed to ODS conditions (**Table 2; Fig. 1**). The decrease of VH_2O from RS to ODS was higher in females of *An. gambiae* (**Fig. 1**).

3.1.2. Flight activity

Flight activity was similar among the three populations (ANOVA, *ddl*=2, F=0.33, *P*=0.72). An increase of the flight activity was observed for the three populations subjected to ODS conditions, but this trend was not significant (ANOVA, *ddl*=1, F=2.79, *P*=0.11, **Fig. 2**).

3.1.3. Allometric measures

Analyses showed that both wing size and female's dry mass significantly differed among the three populations (ANOVA, *ddl*=2, F_{size}=12.91, F_{mass}=16.02, *P*<0.001; **Fig. 3A-B**). Only dry mass was significantly influenced by rearing conditions (ANOVA, *ddl*=1, F=5.4, *P*<0.05; **Fig. 3B**), and this effect was mainly due to the significant increase of the dry mass of *An. gambiae* females reared in ODS conditions compared to those reared in RS ones (**Fig. 3B**). Overall, the surface to volume ratio of females differed among populations (ANOVA, *ddl*=1, F=13.0, *P*<0.001, **Fig. 3C**) but not between rearing conditions (ANOVA, *ddl*=1, F=0.26, *P*=0.61).

A significant interaction between environmental conditions and populations was observed (ANOVA, *ddl*=2, F=4.8, *P*<0.05), suggesting that the impact of the environmental conditions on the surface to volume ratio differed among the populations. Indeed, only females of *An. gambiae* exhibited significant phenotypic modifications, with the ones reared under ODS conditions exhibiting a 1.8 fold lower surface to volume ratio (**Fig. 3C**). This decrease is due to the significant increase of dry mass in the corresponding females (see above, **Fig. 3B**).

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3.1.4. Can flight activity and allometry influence females' VCO_2 and VH_2O?

VCO_2 was not influenced by wing size, flight activity, nor the surface to volume ratio (GLM, *ddl*=1, χ^2= 1.62, *P*=0.20; χ^2= 0.47, *P*=0.49; χ^2= 0.08, *P*=0.49, respectively).

VH_2O was influenced by the variation of the surface to volume ratio of the mosquitoes, but not by the variation of their wing size and flight activity (**Table 2**). For all populations and for both rearing conditions, VH_2O increased linearly with the surface to volume ratio of the females (**Fig. 4**). This linear relationship was different in mosquitoes exposed to RS than in those exposed to ODS (**Fig. 4**), as supported by the significant interaction terms between surface to volume ratio and rearing conditions (**Table 2**).

3.2. *AKH*-related genes expression variations

Expression levels of the *Anoga-AKH-R* and *Anoga-AKH-I* genes differed among the populations (ANOVA, *ddl*=2, $F_{AKH\text{-}R}$=5.95, $F_{AKH\text{-}I}$=2.80, *P*<0.05), but not those of *Anoga-AKH-II* (ANOVA, *ddl*=2, F=2.11, *P*=0.16). Expression levels of the three genes were not significantly influenced by the rearing conditions, while significant interaction term between rearing conditions and population was found (ANOVA, *ddl*=2, *Anoga-AKH-Receptor*: F=15.76, *P*<0.001; *Anoga-AKH-I*: F=8.33, *P*<0.01; *Anoga-AKH-II*: F=5.53, *P*<0.05).

Hence, *Anoga-AKH-R* and *Anoga-AKH-I* genes displayed similar patterns of expression, with a significant increase of their mRNA amounts between RS and ODS conditions for the permanent population of *An. coluzzii*. Conversely, a decrease was observed for *An. coluzzii* from temporary sites (**Fig. 5A-B**). A distinct pattern was reported for the *Anoga-AKH-II* gene in which mRNA levels significantly increased for both populations from temporary sites, whereas a decrease (although marginally significant) was observed for *An. coluzzii* from permanent breeding sites (**Fig. 5C**).

3.3. Cuticular hydrocarbon fingerprints

Thirteen compounds were identified as mosquito cuticular hydrocarbons. Total amount of cuticular hydrocarbons significantly differed among the three mosquito populations (ANOVA, *ddl*=2, F=31.81, *P*<0.001), but there were no significant effects of the rearing conditions (ANOVA, *ddl*=1, F=0.78, *P*=0.38; **Fig. 6**). In particular, females *An. gambiae* expressed 8.75- to 15.30-fold more cuticular hydrocarbon amounts than *An. coluzzii* populations under RS and ODS conditions, respectively. No difference between the two *An. coluzzii* populations was observed (**Fig. 6**).

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Distinct cuticular hydrocarbon profiles were measured among the three populations (MANOVA, $F_{2,26}$=6.65, P<0.001), the two rearing conditions (MANOVA, $F_{1,13}$=17.43, P<0.01), and the interaction between these two terms (MANOVA, $F_{2,26}$=3.80, P<0.01). In the LDA analysis, the first axis (LD1) accounted for 25.99% of the total inertia, and the variation among groups was 19.31 times higher than the variation within groups (**Fig. 7**). LD1 mainly separated female *An. coluzzii* from permanent breeding sites reared under ODS conditions from all other groups. This clear cut-off was mainly characterized by the increased amounts of 11-tetracosane and 12-tetracosane in ODS-reared females of this permanent *An. coluzzii* population, and 11-methyltricosane, 13-methylhexacosane, 13-methylpentacosane, 3-methyltricosane, 3-methylpentacosane, cholesterol, *n*-hentriacontane, *n*-pentacosane, *n*-tetracosane and *n*-tricosane in the other groups (**Fig. 7** and **Supplementary data 2**). The second axis (LD2) accounted for 22.43% of the total inertia, and the variation among groups was 5.06 times higher than the variation within groups. LD2 mainly separated female *An. coluzzii* from temporary breeding sites reared under ODS conditions from all other groups (**Fig.9**). Increased amounts of *n*-nonacosane were measured in ODS-reared temporary population of *An. coluzzii*, and *n*-heptacosane in RS-reared temporary and permanent *An. coluzzii* (**Fig. 7** and **Supplementary data 2**).

4. Discussion

The present work aimed at characterizing and comparing the influence of environmental conditions found in sub-Saharan Africa at the onset of the dry season (ODS), and at the rainy season (RS), on phenotypic adjustments displayed by females of *An. gambiae* and *An. coluzzii* sampled from localities where larval breeding-sites are permanent or temporary. Our results showed that all measured phenotypic traits displayed significant changes depending on the environmental climatic conditions (*i.e.* temperature, RH and photoperiod) experienced by the mosquitoes during their development. Phenotypic adjustments also varied according to the considered population, and the larval breeding-site dynamics (temporary *vs.* permanent).

4.1. Seasonal metabolic rate variations are not consistent with the idea of an aestivation strategy in *An. coluzzii*

According to studies lead on dormant insect species, and more particularly on those that overwinter as diapausing organisms (Hahn and Denlinger, 2007; Kambule et al., 2011), we predicted that metabolic rate would have been lowered in *An. coluzzii* reared under ODS

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conditions, as these mosquitoes are assumed to enter a dormant state named aestivation. Conversely, metabolic rate should have been increased in female *An. gambiae*, as they are supposed to exhibit a dispersive flight strategy. Surprisingly, VCO_2 emitted by *An. gambiae* did not show any significant variation from RS to ODS conditions, and significantly increased, or tended to do so, in temporary and permanent populations of *An. coluzzii*, respectively. The physiological coercions that insects have to face during aestivation may partly differ from those faced during overwintering diapause, as revealed by the unexpected VCO_2 fingerprints we are reporting with this work. Denlinger and Armbruster (2014) pointed out that resting metabolic rate of malarial mosquitoes is not lower, but rather exhibits a very complex pattern of change during the course of the dry season, depending on the temperature and on the female's reproductive status. The high temperatures encountered during aestivation may impose unique physiological constraints (Denlinger and Armbruster, 2014), and the sensitivity of aerobic metabolism to temperature changes has been evidenced in earlier studies (Clarke, 1993; Huestis et al., 2012; Terblanche et al., 2005), including in field-sampled mosquitoes (Huestis et al., 2011, 2012). The extreme temperatures and low humidity levels encountered during the dry season seem to promote an increased metabolic activity of female mosquitoes, maybe underlining the increased demand of energetic substrates (*i.e.* ATP) to flee the desiccating conditions (migration) or resist locally (aestivation). The increase of flight activity - only marginally significant – of the *An. coluzzii* females at ODS may have also contributed to increase VCO_2 values.

By contrast, female *An. gambiae* did not show significant variation of their metabolic rate from RS to ODS conditions, underlying that they express lower plasticity levels than *An. coluzzii* and put in place distinct physiological mechanisms to cope with hot and dry conditions, as we initially suggested.

4.2. Mosquitoes exhibit different ways to prevent body water loss at ODS

The increase in VCO_2 observed at ODS in *An. coluzzii* females should participate to increase its desiccation level, as gas exchanges are supposed to increase water losses in insects. Yet, our results did not support this assumption, as VCO_2 variations observed in all mosquitoes at both RS and ODS conditions did not influence their rates of water losses (VH_2O). Previous studies showed that insect's metabolic rate fluctuations do not necessarily lead to significant changes in water losses (Bradley et al., 1999; Chown, 2002; Djawdan et al., 1997; Rourke, 2000; Williams et al., 1998; Williams and Bradley, 1998). In particular, the control of water losses is mainly achieved by adjustments of cuticular permeability, as cuticular transpiration is

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the principle way through which insects experience desiccation (Benoit, 2010). Yet, mosquito's water losses significantly decreased in all populations at ODS, suggesting that mechanisms other than adjustments of the metabolic rate participate to reduce anopheline transpiration under such climatic conditions.

The reduction of the surface to volume ratio is described in several species as one of the mechanisms preventing body water losses (Benoit and Denlinger, 2007; Hadley, 1994). Overall, our data showed a clear linear relationship between the rate of VH_2O in female *An. gambiae* and their surface to volume ratio. Such seasonal allometric change in *An. gambiae* is due to an increase of females' dry mass at ODS, highlighting potential differences in their abilities to seasonally harvest, store and/or use their metabolic resources, as shown for *Aedes albopictus* (Reiskind and Zarrabi, 2012).

Variation levels of adipokinetic peptides (AKH) may also help to enhance desiccation resistance in anopheline mosquitoes, as shown in overwintering dormant insects (Hahn and Denlinger 2011). Here, we measured the expression levels of two AKH peptide genes (*Anoga-AKH-I* and *Anoga-AKH-II*) and their putative receptor (*Anoga-AKH-Receptor*). Overall, we highlighted that both *Anoga-AKH-I* and *Anoga-AKH-Receptor* genes showed very similar patterns of expression, suggesting that their expression levels are regulated in parallel, as expected for receptor/peptide couples. By contrast, *Anoga-AKH-Receptor* expression levels did not match with those of the *Anoga-AKH-II*. Few years ago, *Anoga-AKH-II* was described to code for an intermediary AKH and corazonin protein named AKH/corazonin-related (ACP) (Kaufmann and Brown, 2006). Functional roles of corazonin remain obscure in insects, even if they seem to have cardio-acceleratory effects, and should thus increase metabolic rate, as observed in the American cockroach (Sláma et al., 2006). The protein corazonin is involved in migratory processes of gregarious locusts (Tawfik et al., 1999), and is released under nutritional stress (Veenstra, 2009), thus corroborating the hypothesis of a dispersal strategy at ODS in *An. gambiae*. In addition, variation of *ACP* mRNA levels correlates to those observed for *glycogen phosphorylase* mRNA in *Anopheles* species (Hidalgo et al., 2016), suggesting a possible physiological interaction between these two actors in *An. gambiae*. In particular, the increase in ACP expression in *An. gambiae* at ODS could help degrading large amounts of stored carbohydrates, thus providing non-negligible amounts of metabolic water and energetic substrates to sustain an increased dispersal (Huestis and Lehmann, 2014; Mamai et al., 2015).

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By contrast, under ODS conditions, the AKH-I/AKH-Receptor couple was over-expressed in the permanent population of *An. coluzzii*, whereas it was under-expressed in the temporary one, and did not show any seasonal variation in *An. gambiae*. Expression levels of AKH-I mRNA characterized the distinct molecular response of permanent *An. coluzzii* populations, which continue to be active and reproduce at ODS. Oddly, these results are in contrast to those we previously obtained using 7-day-old females, in which no seasonal differences were observed with the same *An. coluzzii* populations (Hidalgo et al., 2016). Such a difference may be explained by the younger age of our mosquitoes, in addition to taking in account photoperiod fluctuations in our experimental rearing. AKH peptides are suspected to mediate physiological responses to desiccation, as they allow the release and transport, from the fat body to the hemolymph, of organic compounds with osmoprotectant functions (*i.e.* trehalose, proline), notably in overwintering insects (Gäde, 2004; Hahn and Denlinger, 2011; Isabel et al., 2005; Wilps and Gäde, 1990; Ziegler et al., 2011). Further studies are required to confirm this hypothesis, but *An. coluzzii* populations may require increased levels of osmoprotectants to deal with the desiccating conditions of the dry season while remaining reproductively active. Overall, seasonal variations of AKH-I and ACP expressions may represent valuable markers of the survival strategies and/or stress response mechanisms elicited in *An. coluzzii* and *An. gambiae* populations.

4.3. Seasonal changes in cuticular hydrocarbon fingerprints suggest distinct desiccation resistance strategies in anopheline mosquitoes

Changes in cuticular hydrocarbon composition are expected in anopheline mosquitoes reared under ODS conditions as cuticular transpiration is the main way of water loss in insects (Chown, 2002; Chown and Nicolson, 2004; Hadley, 1994; Johnson and Gibbs, 2004). Using gas chromatography techniques, we showed quantitative differences in the total amount of cuticular hydrocarbons between the two sibling species, with *An. gambiae* females displaying the highest amounts, whatever the environmental conditions, while no difference was evidenced between the two *An. coluzzii* populations. Interestingly, total amount of cuticular hydrocarbons did not vary in mosquitoes according to the rearing conditions. In insects, desiccation resistance is not related to the total amount of cuticular hydrocarbons a species exhibits (Arcaz et al., 2016; Gibbs et al., 1997; Kwan and Rundle, 2010), but rather to their relative composition (Arcaz et al., 2016; Gibbs et al., 1997; Gibbs and Rajpurohit, 2010; Nelson and Lee Jr., 2004).

We highlighted qualitative hydrocarbon differences between the two *An. coluzzii* populations on one hand, and *An. gambiae* on the other, whatever the season we considered.

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The profile of *An. gambiae* females is mainly characterized by higher amounts of (1) cholesterol, a precursor of insects' steroids, and (2) methyl-branched alkanes, known as important semiochemical cues for social and sexual insect recognition (Howard and Blomquist, 2005). Such qualitative differences might be part of the biological basis leading to assortative mating between the species and, to a lesser extent, to distinct innate biology and ecology (Niang et al., 2015).

Our data underlined distinct cuticular hydrocarbon adjustments between the two species in response to seasonal changes. While cuticular hydrocarbons did not change with environmental conditions in *An. gambiae* females, seasonal differences were observed in *An. coluzzii*. This finding gives credit to our earlier observations, which suggested the occurrence of seasonal changes in cuticular composition (*i.e.* aromatic amino acids and RR2-proteins) at ODS in these females but not in *An. gambiae* (Hidalgo et al., 2014).

Lastly, distinct seasonal adjustments of cuticular hydrocarbons were observed between *An. coluzzii* exploiting temporary or permanent larval breeding-sites. Temporary population of *An. coluzzii* displayed an increased amount of *n*-nonacosane ($C_{29}H_{60}$) at ODS, a compound already observed to increase under desiccating winter in the scorpion *Centruroides sculpturatus* (Toolson and Hadley, 1979). Conversely, permanent population of this species showed increased amounts of the 11- and 12-tetracosane ($C_{24}H_{50}$). The three compounds melt at hot temperatures, but *n*-nonacosane has a higher melting point (62 to 66 °C) than *n*-tetracosane (48 to 54 °C). Insect literature suggests that diapausing species over-express long chain hydrocarbons (Benoit, 2010; Kankare et al., 2016), because it improves the cuticle impermeability avoiding desiccation (Chung and Carroll, 2015; Gibbs, 2011). Thus, the increased amount of longer hydrocarbon chains in *An. coluzzii* females collected from temporary breeding-sites (*n*-nonacosane) could support the "strong" aestivation strategy suggested for these mosquitoes during the dry season. Interestingly, *n*-tetracosane accumulated in temporary population of *An. coluzzii* at ODS is known to increase during the reproductive period of Dipterans (Jurenka et al., 1998) supporting the expectation of a maintained reproductive activity in these mosquitoes at ODS (Baldet et al., 2003; Costantini et al., 2009; Diabate et al., 2002, 2004). Further studies are still needed to tear apart the exact roles of these candidate hydrocarbons, but, at least, their distinct profiles between conditions and populations consolidate the expectation of distinct survival strategies in *An. coluzzii* as a function of the ecology of their larval breeding-sites.

5. Conclusion

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Understanding the survival strategies put in place by malaria mosquitoes and other tropical insects to cope with the dry season conditions is a major challenge (Denlinger and Armbruster, 2014; Yaro et al., 2012). Our study underlined distinct ecophysiological adjustments in *An. coluzzii* and *An. gambiae* females to prevent water losses under ODS conditions. These adjustments were also driven by the type of breeding site exploited by the mosquito population (*i.e.* permanent or temporary larval ecotype). In addition, specific changes in epicuticle hydrocarbons and AKH mRNA expression levels between mosquitoes highlights the high phenotypic diversity in *An. gambiae s.l.* species complex. These results are in line with the hypothesis of distinct survival strategies among these species (Yaro et al., 2012). Further investigations are required to examine the exact roles of these markers in the mosquito metabolism and biology, but we believe they could constitute relevant markers to highlight specific dry season strategies developed by "permanent" and "temporary" anopheline populations during the dry season. Altogether, our results bring better understanding of mosquito's biology during the dry season, opening the way for alternative method of malaria control in sub-Saharan Africa.

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Figure captions

Fig.1 Mean ± s.e.m. rate of CO_2 release (in µl/min/mg of mosquito dry mass) (A) and H_2O loss (in µl/min/mg of mosquito dry mass) (B) by the three anopheline populations under the rainy season (RS, *white bar*) and the onset of the dry season (ODS, *grey bar*) environmental conditions. Different letters represent significant differences between experimental modalities at *P* value<0.05 (GLM). n= 8, 6 and 9 samples of four pooled females for RS, and n= 7, 8 and 6 samples of four pooled females for ODS in, respectively, the permanent and temporary populations of *An. coluzzii*, and *An. gambiae*.

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Fig.2 Mean ± s.e.m. rate of flight activity frequency (in second/minute) recorded in the three anopheline populations under the environmental conditions of the rainy season (RS, *white bar*) and the onset of the dry season (ODS, *grey bar*). Different letters represent significant differences between experimental modalities at P value < 0.05 (ANOVA). Samples are the same as those in figure 1.

Fig.3 Mean ± s.e.m. of **A:** wing size (in mm), **B:** Dry mass (in mg), and **C:** surface to volume ratio (in mm/mg of dry mass) of the three anopheline populations under the environmental conditions of the rainy season (RS, *white bar*) and the onset of the dry season (ODS, *grey bar*). Different letters represent significant differences between the experimental modalities at P value < 0.05 (ANOVA). n= 32, 24 and 36 samples (one female per sample) for RS conditions, and n= 28, 32 and 24 samples (one female) for ODS conditions in, respectively, the permanent and temporary populations of *An. coluzzii* and *An. gambiae*.

Fig.4 Linear regression between the females' surface to volume ratio (in mm/mg of dry mass) and H_2O losses (in µl/min/mg of mosquito dry mass) when mosquitoes are reared under the environmental conditions of the rainy season (RS; *filled line*), or the onset of the dry season (ODS; *dashed line*). Samples are the same as those described in Fig. 1 and 3.

Fig.5 Relative normalised mRNA expression level ± s.e. of **A:** Anoga-AKH-receptor; **B:** Anoga-AKH-I; and **C:** Anoga-AKH-II in the three anopheline populations reared under the environmental conditions of the rainy season (RS, *white bar*) and the onset of the dry season (ODS, *grey bar*). Different letters represent significant differences between experimental modalities at P value < 0.05 (ANOVA). n= 3, 3 and 4 samples of ten pooled females for RS; n= 3, 3 and 3 samples of ten pooled females for ODS in, respectively, the permanent and temporary populations of *An. coluzz*ii and *An. gambiae*.

Fig.6 Mean ± s.e.m. of the total amount of cuticular hydrocarbon (in ng/mosquito) in the three anopheline populations reared under environmental conditions of the rainy season (RS, *white bar*) and the onset of the dry season (ODS, *grey bar*). Different letters represent significant differences between experimental modalities at P value < 0.05 (ANOVA). n= 4, 4 and 3 samples of four pooled females for RS; n= 5, 7 and 5 samples of four pooled females for ODS

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in, respectively, the permanent and temporary populations of *An. coluzz*ii and *An. gambiae*, respectively.

Fig.7 Sample projection of the three anopheline populations reared under RS and ODS conditions onto the first discriminant plane of the LDA. *Blue* and *red* samples represent specimens reared under the conditions of the rainy season (RS) and the onset of the dry season (ODS), respectively. The singular values correspond to the ratio of between-class/within-class inertias. The correlations circle plotted in the right panel depicts the normed relation between each cuticular hydrocarbons and the first discriminant plane. Samples are the same as those described in Fig. 6.

Table 1. Nucleotide sequences of the primers used in qRT-PCR reactions for the amplification of Actin, Rps13, Rps7, Rpl5, h3a, CytP450, Tubulin, hsp83, EGFR, 18s, *Anoga-AKH-I, Anoga-AKH-II, and Anoga-AKH-R* in *An. gambiae*.

Table 2. Results of the GLM computed on the VH_2O with the experimental conditions, the anopheline populations, VCO_2, flight activity and surface to volume ratio as explicative variables. Signif. code: < 0.001 "***"; <0.05 "*".

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Figure 1

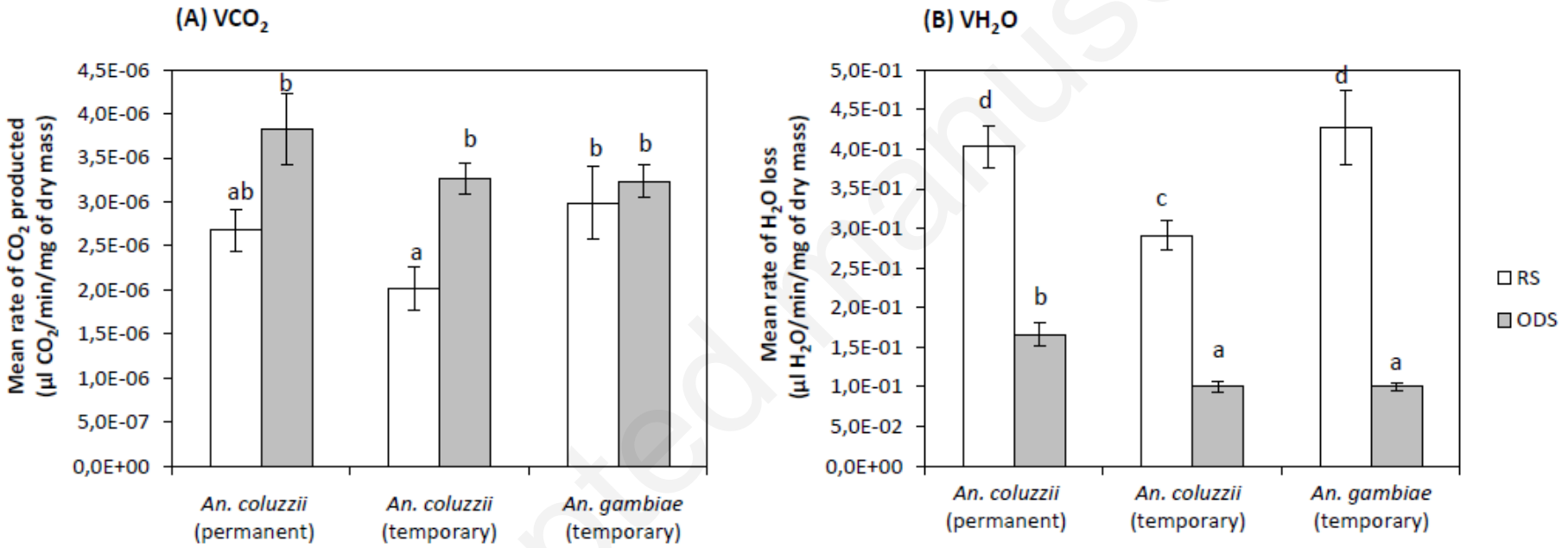

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Figure 2

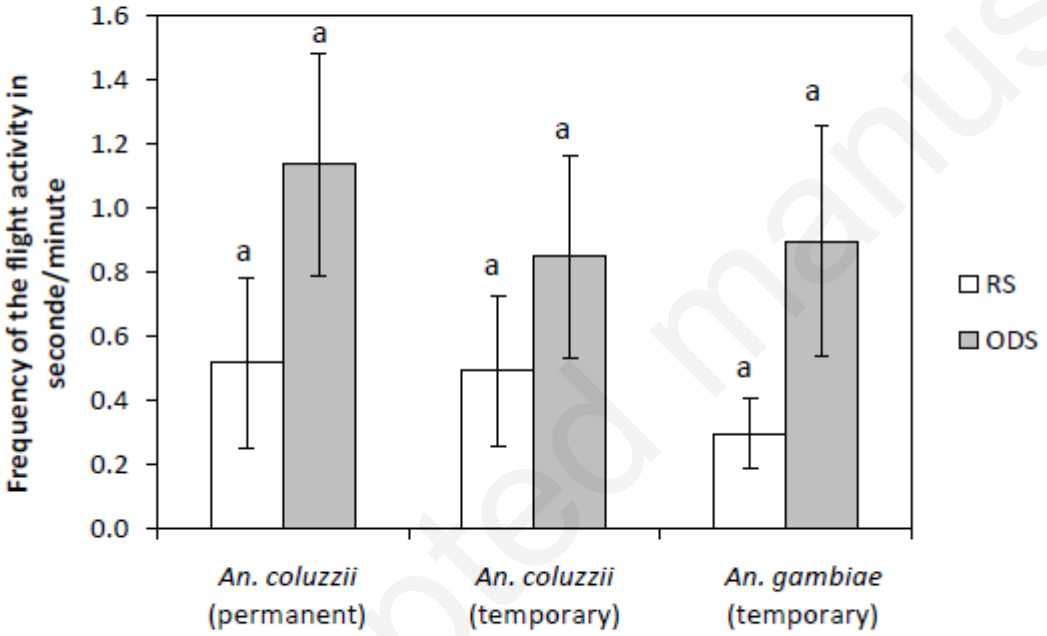

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Figure 3

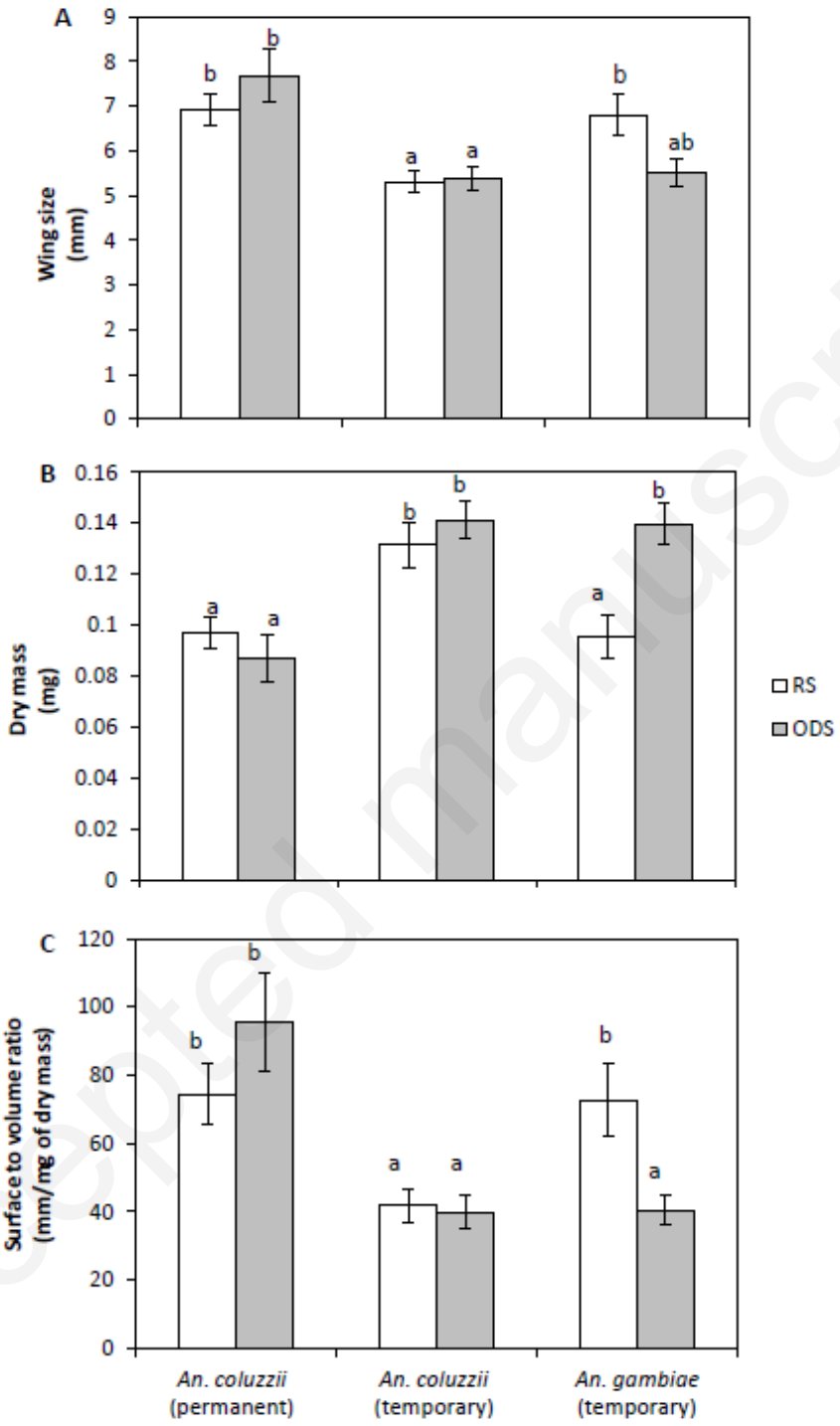

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Figure 4

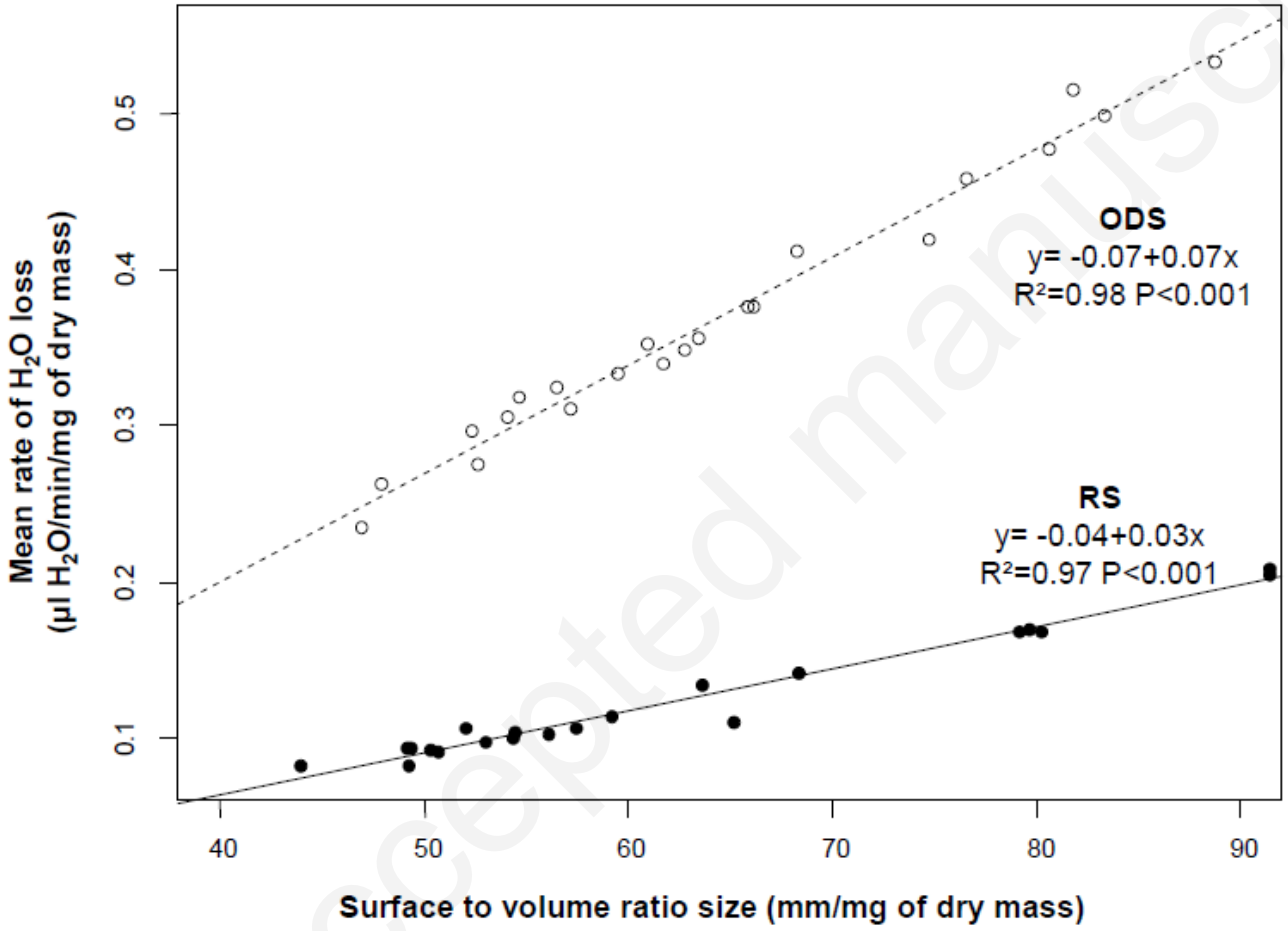

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Figure 5

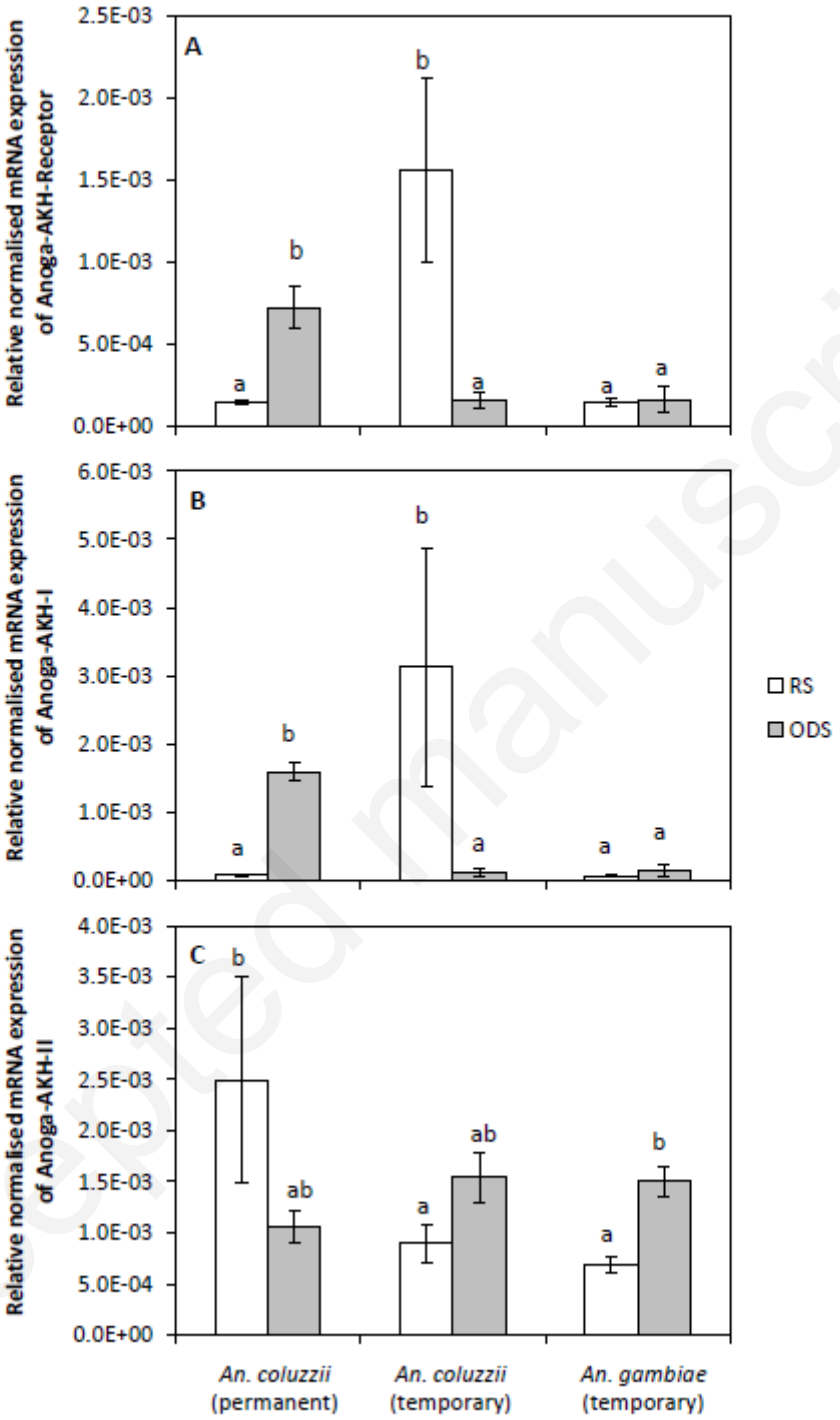

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Figure 6

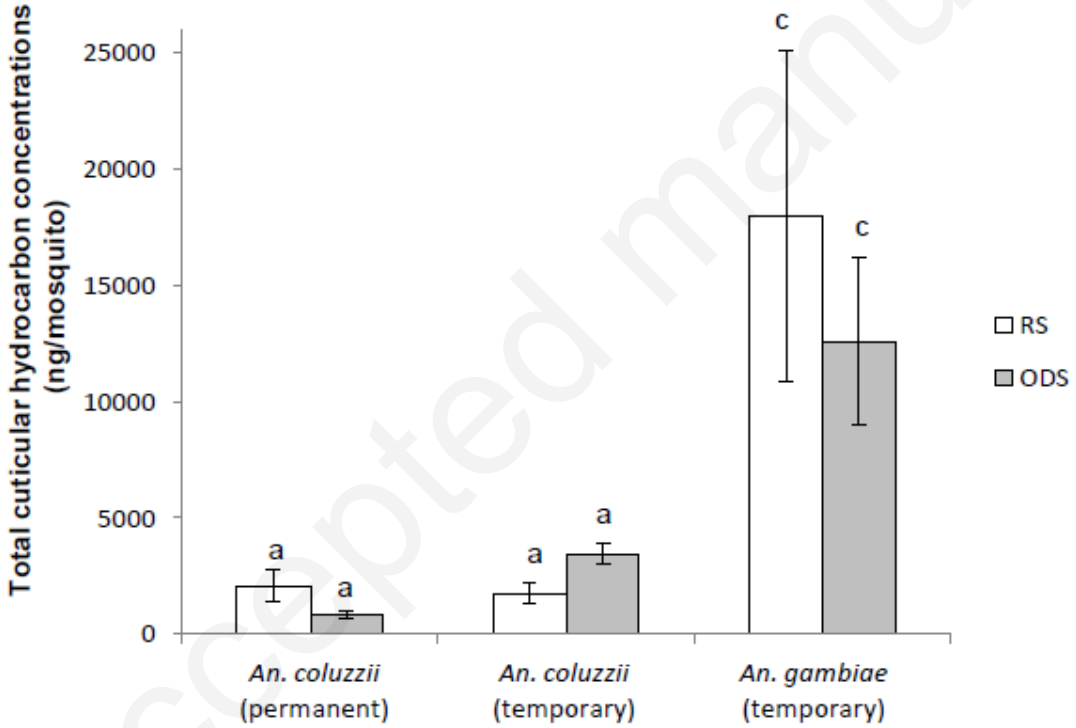

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Figure 7

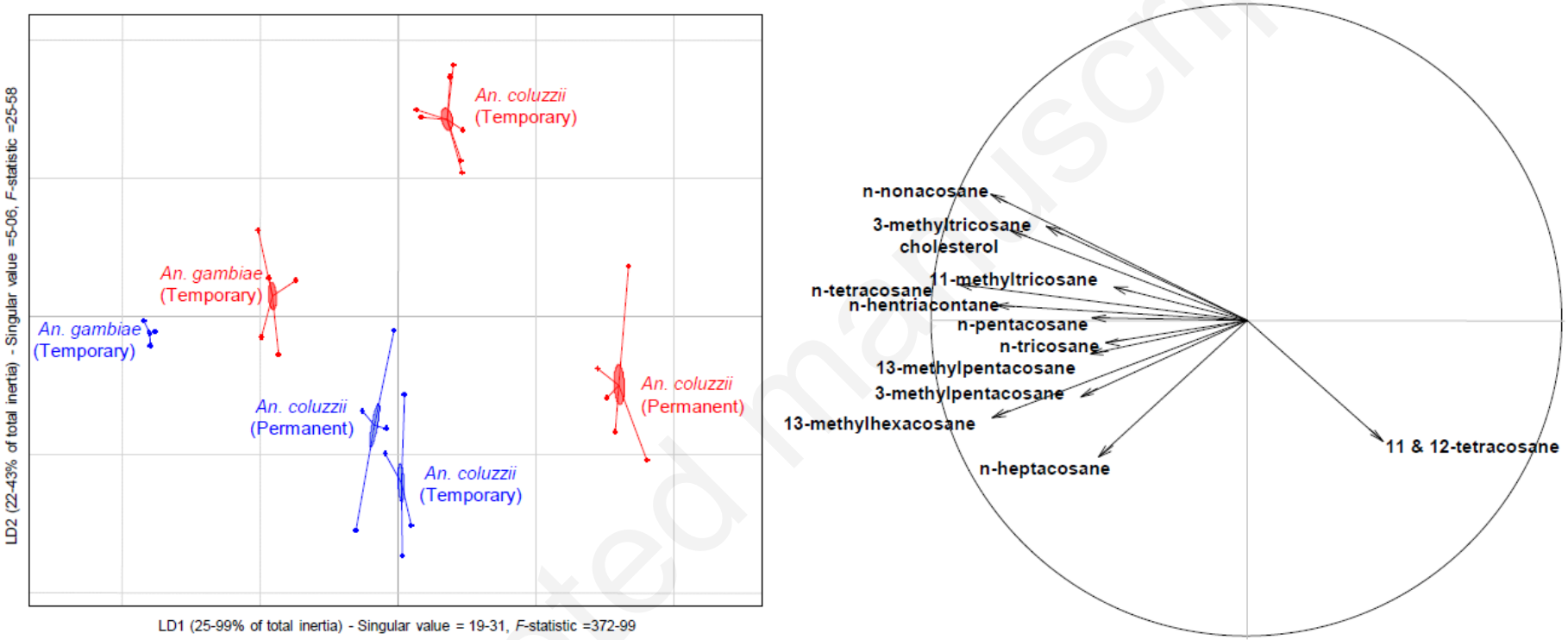

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Table 1. Nucleotide sequences of the primers used in qRT-PCR reactions for the amplification of Actin, Rps13, Rps7, Rpl5, h3a, CytP450, Tubulin, hsp83, EGFR, 18s, *Anoga-AKH-I, Anoga-AKH-II, and Anoga-AKH-R* in *An. gambiae*.

Primer	Direction	Sequences (5'- 3')
Actine	FOR	CTGGACTTCGAGCAGGAGAT
Actine	REV	CGCACTTCATGATCGAGTTG
Rps13	FOR	TATTTCCAAATCCGCGCTAC
Rps13	REV	CATGATACGCAGCACCTTGT
Rps7	FOR	ACCCCAACAAGCAGAAGAGA
Rps7	REV	TACACCGACGCAAAAGTGTC
Rpl5	FOR	GGACTGAACATTCCGCACTC
Rpl5	REV	GATGCCCAGCGAGATGTACT
h3a	FOR	ATCCGTCGGTACCAGAAGTC
h3a	REV	AATGTCCTTCGGCATAATGG
CytP450	FOR	TACCAAATGAAGGGCATGGT
CytP450	REV	AACACCGCGTAATTCAAACC
Tubulin	FOR	AAGCTCGAATTCGCCATCTA
Tubulin	REV	CCAATCAAACGGTTCAGGTT
hsp83	FOR	CTGCGTGAGTTGATCTCGAA
hsp83	REV	ATCGTTCCGAGGTTGTTCAC
EGFR	FOR	GGGAATGTTGCCATCTGTTC
EGFR	REV	GACATTTCCGTACGCAGGTT
18s	FOR	ACCCGCGTCACTACAAAATC
18s	REV	CGGTAGTTTTCGTGTGCTGA
Anoga-AKH-I	FOR	TGCTGATTTGTGCCTCTTTG
Anoga-AKH-I	REV	ATTCCCCAACCCTACCTGAA
Anoga-AKH-II	FOR	CGCTGGACAGGTAACGTTTT
Anoga-AKH-II	REV	GACTCATCCGTTTGCAGTGA
Anoga-AKH-R	FOR	CGTACTATGCGAACGAAACG
Anoga-AKH-R	REV	TGCGCCAACATGATATTGAT

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Table 2. Results of the GLM computed on the VH_2O with the experimental conditions, the anopheline populations, VCO_2, flight activity and surface to volume ratio as explicative variables. Signif. code: < 0.001 "***"; <0.05 "*".

Effects	***ddl***	χ^2	***P*-value**
Environmental conditions	1	76.97	***
Population	2	7.96	*
VCO_2	1	0.39	0.53
Surface to volume ratio	1	91.04	***
Flight	1	0.05	0.82
Environmental conditions : Population	2	5.21	0.07
Environmental conditions : Surface to volume ratio	2	5.91	*

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