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RESEARCH ARTICLE

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

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ABSTRACT

Aestivation and dispersive migration are the two strategies evoked in the literature to explain the way in which malaria vectors *Anopheles coluzzii* and *A. gambiae* survive the harsh climatic conditions of the dry season in sub-Saharan Africa. However, the physiological mechanisms regulating these two strategies are unknown. In the present study, mosquito species were exposed to controlled environmental conditions mimicking the rainy and dry seasons 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, variations of which are usually considered to be hallmarks of aestivation and dispersion mechanisms in various insects. Our results showed that ecophysiological and morphometric adjustments are made in both species to prevent water losses during the dry season. However, the usual metabolic rate modifications expected as signatures of aestivation and migration were not observed, highlighting specific and original physiological mechanisms sustaining survival in malaria mosquitoes during the dry season. Differences in epicuticular hydrocarbon composition and AKH levels of expression were found between the permanent and temporary *A. 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

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INTRODUCTION

In western Africa, malaria is mainly transmitted by the bite of two anthropophilic female mosquito species, *Anopheles coluzzii* Coetzee 2013 and *A. gambiae* Giles 1902. Both species can occupy a large range of ecological niches, but their densities follow the changes in 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 a reduction in mosquito populations (Dao et al., 2014; Mamai et al., 2015). Only the mosquito *A. coluzzii* can persist in these locations all year long, as it can also exploit permanent larval breeding sites such as 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 *A. coluzzii* for larval breeding (temporary, which dry up at the onset of the dry season, or permanent) may be explained by its aptitude for expressing different adaptive phenotypes related to the local breeding-site characteristics (Gimonneau et al., 2012; Hidalgo et al., 2015a, 2016). Recent work has suggested that *A. coluzzii* mosquitoes collected from temporary larval breeding sites may 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 *A. coluzzii* mosquitoes exploit permanent breeding sites, they are considered to be '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 *A. gambiae* exploits temporary larval breeding sites only. Using population dynamics studies, it has been proposed that these mosquitoes do 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 insects overwintering diapause, metabolic rate depression should be one of the most significant physiological traits characterizing aestivation (Benoit, 2010; Storey and Storey, 1990). Decrease of the metabolic rate has already been observed to prevent water losses and increase fitness in several insect species inhabiting arid environments (Chown, 2002; Hadley, 1994; Rourke, 2000). Yet,

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List of symbols and abbreviations

AKH	adipokinetic hormone
L_w	wing length
ODS	onset of the dry season
RS	rainy season
$\dot{V}_{CO_2}$	rate of carbon dioxide release
$\dot{V}_{H_2O}$	rate of water loss

this pattern has not been observed in field-sampled *A. coluzzii* (Huestis et al., 2011, 2012), even in populations assumed to be 'strong aestivators'. When sampled from the field during the rainy season and at the onset of the dry season, the high inter-individual variability of mosquito phenotypes, resulting from uncontrolled age, trophic and reproductive states, may have prevented the observation 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, has already been linked to desiccation resistance in several arthropod species (Blomquist and Bagnères, 2010; Gibbs and Rajpurohit, 2010; Stinziano et al., 2015). In particular, a large amount of long-chain hydrocarbons correlates with increased cuticle hydrophobicity, and we expect that such adjustments occur in anopheline species at the onset of the dry season. In parallel, the catabolism of specific organic compounds, such as stored glycogen, which generates a significantly higher amount of bounded water than that generated by any other energetic substrates (Schmidt-Nielsen, 1997), can contribute to a reduction in body water losses. The catabolism of glycogen by glycogen phosphorylase is under the control of the adipokinetic hormones (AKH) (Wilps and Gäde, 1990; Gäde, 2004; Ziegler et al., 2011), variations in which may signal changes in metabolic activity at the start of winter for dormant insects (Hahn and Denlinger, 2011). Similarly, variations in AKH levels may depict changes in desiccation resistance over the year in anopheline species, and increased amounts of this peptide can be expected in *A. 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 increase in *AKH* gene expression levels in female *A. 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 *A. coluzzii* and *A. gambiae*: (1) female *A. gambiae* express a dispersive behaviour to escape the harsh conditions of the dry season, whereas *A. coluzzii* females only virtually disappear and aestivate, and (2) the intensity of the aestivation phenotype in *A. 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 season (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 peptide mRNA expression levels (Anoga-AKH-I, Anoga-AKH-II and their putative receptor Anoga-AKH-R) and cuticular hydrocarbon fingerprints were measured. These parameters were compared (1) between RS and ODS conditions for each mosquito population, and (2) between populations. We expected (1) enhanced abilities to prevent body water losses at ODS in *A. coluzzii* females, (2) a decreased metabolic rate (which would also help in preventing body water losses) at ODS in the aestivating females of *A. coluzzii*, but not in those of *A. gambiae*, in which we expected to measure an increase in CO_2 release, (3) differences in *AKH* gene expression levels between populations and experimental conditions that would reveal different metabolic mobilisation and, hence, different survival strategies (aestivating versus dispersive), and (4) seasonal changes in hydrocarbon composition of the cuticle in both species, notably in aestivating *A. coluzzii* specimens. Finally, we expected distinct ecophysiological phenotypes between populations of *A. coluzzii* according to their site of collection (i.e. temporary or permanent breeding-sites areas).

MATERIALS AND METHODS

Mosquito populations and rearing conditions

Mosquito populations

Experiments were conducted using two mosquito populations of *A. coluzzii*, and one population of *A. gambiae*. The two populations of *A. 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), *A. coluzzii* is the prevailing mosquito species (Gimonneau et al., 2012), and it breeds year-round in rice fields (i.e. permanent population). By contrast, at Soumousso (11°01′N, 04°02′W), population dynamics of *A. coluzzii* match the seasonal variations of local climatic conditions (Dabiré et al., 2007). The population of *A. gambiae* used in this study was established at the Institut de Recherche en Sciences de la Santé (IRSS) insectary in 2009 using females of temporary populations collected from the village of Soumousso. This population thrives in sympatry with populations of *A. coluzzii*.

The three populations were maintained under the same insectary conditions (27°C, 70% humidity, 12 h:12 h light:dark cycles) prior to being used for the analysis (approximately six generations). The three populations were maintained in separate rearing rooms to avoid any crossbreeding.

Experimental rearing conditions

The three mosquito populations were reared from eggs to adults in two climatic chambers (Binder KBF720 VWR international S.A.S., Radnor, PA, USA) programmed to reproduce the natural daily fluctuations of rearing conditions experienced by mosquitoes during RS (N=1 climatic chamber) and ODS (N=1 climatic chamber). Programmed daily temperature and relative humidity conditions mimicked those in the field, as earlier used by Hidalgo et al. (2014, 2015b, 2016) and Mamai et al. (2014). Moreover, variation of day length was also accounted for using hourly recorded light intensity data during the RS and ODS conditions with a weather monitoring station (Weatherlink; Davis Instruments, Hayward, CA, USA) (Fig. S1). 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 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 were 4–6 days 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 3–5 days old), females were

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deprived of food and water in order to avoid any bias in carbon dioxide ($\dot{V}_{CO_2}$) and water loss rate ($\dot{V}_{H_2O}$) measurements. The first batch corresponded to mosquitoes used for $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ 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 hydrocarbon analyses.

Gas exchange, flight and allometric measurements

Metabolic ($\dot{V}_{CO_2}$) and water loss ($\dot{V}_{H_2O}$) rates

The $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ of females from the three populations were measured using a flow-through respirometry and a CO_2–H_2O gas analyser (Sable Systems Las Vegas, NV, USA; Li-7000 CO_2/H_2O infrared gas analyzer, Li-Cor-Biosciences, Lincoln, NE, USA). 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 analyser system was reached). Females that were 4–6 days old were placed in a 200×400 mm (length×width) glass chamber. The chamber was connected to the gas analyser, and flushed with a constant flow of air at a rate of 200 ml min^{-1}. 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 humidity controller system, respectively. The programmed conditions corresponded to the hottest and driest periods of the day for each season, during which females are resting (from 12:00 to 16:00 h). Hence, the relative humidity and temperature of the flowing air were 18% and 34°C for the ODS conditions, and 70% and 29°C for the RS conditions, respectively.

Each run lasted 100 min, during which $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ were recorded. Following an acclimation period of 25 min, measurements of $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ 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. 3×10 min). Data were analysed using the Expedata software (Sable Systems International V.1.0.1). $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ of mosquitoes were calculated by subtracting the control values from those measured with mosquitoes in the chamber. After each run, the viability of the females was checked before they were snap frozen and dried for 3 days at 60°C, and weighed (Sartorius, Göttingen, Germany; 0.1 mg accuracy). For each run, $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ were averaged over each 15 min period and expressed in µl min^{-1} mg^{-1} dry mass to assess mass variability between species and conditions (Gray and Bradley, 2006; Huestis et al., 2011, 2012).

Flight activity

The flight activity, which may influence the rates at which CO_2 and H_2O are released by females, was recorded during the $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ analyses. Females were recorded using an HD Camera (Sanyo, Osaka, Japan). A total of 44 batches of four pooled female mosquitoes (i.e. 176 females, from six to nine batches by experimental condition and population) were analysed using the ImageJ 1.41.0 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Allometric measures

The body size of females was estimated using their wing length (L_w). Immediately after the $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ 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 (×20, Leica DFC425). L_w was measured from the alula to the wing tip (Charlwood, 1996) using ImageJ 1.41.0 software to an accuracy of ±0.001 mm. Damaged wings were discarded from the analysis. L_w was divided by the dry mass of mosquitoes to estimate the surface to volume ratio of the specimens. A total of 158 wings were measured (i.e. 24–36 per experimental condition and mosquito population).

AKH-related gene expression variations

RNA extraction and cDNA synthesis

For each population and experimental condition (RS, ODS), three to four samples of 10 randomly pooled 4-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 RNA of each sample was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) coupled with the RNeasy Kit (Qiagen, Hilden, Germany). Samples were then treated with DNase I (Ambion) following the manufacturer's instructions. RNA amounts were quantified by spectrophotometry at 260 nm (Nanodrop2000, Thermo Scientific, Waltham, MA, USA). 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.

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 as its expression was stable in all samples, whatever the experimental condition or the anopheline population tested. Further, mRNA of the two *AKH* genes *Anoga-AKH-I* and *Anoga-AKH-II*, and the *AKH* receptor gene *Anoga-AKH-R* (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 primer (10 µmol l^{-1}) and 3 µl of RNA-free water. The qPCR program and conditions have been described in Bigot et al. (2012). The cycle threshold values (C_t values) for both the reference gene and the target genes were determined using the Light-Cycler® 480 software (Roche, France). The average C_t value of each technical triplicate was used to normalize candidate gene expression levels to the geometric mean of the reference gene level, using the Q-Gene software (Simon, 2003).

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 4-day-old females, so that the minimum sample 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 under nitrogen. Dried extracts were conserved at −20°C until further processing. Upon analysis, samples were re-suspended in 50 µl of

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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 *Anopheles gambiae*.

Primer	Direction	Sequences (5′–3′)
Actin	FOR	CTGGACTTCGAGCAGGAGAT
Actin	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

dichloromethane containing 60 ng of *n*-tetradecane (internal standard). Samples were analysed by gas chromatography (GC) on a HP 6850A chromatograph equipped with a HP-5MS fused silica column (30 m×0.25 mm; film thickness 0.25 mm; Chrompack, Les Ulis, France) and an ionization flame detector (FID). We used the procedure described by Trabalon et al. (1996). Briefly, the oven temperature was increased from 100 to 200°C at a rate of 5°C min^{-1}, and from 200 to 320°C at a rate of 3°C min^{-1}. The composition of the total hydrocarbon extract of each sample was identified by gas chromatography–mass spectrometry (GC–MS) on a HP 1890A equipped with a HP-5MS column. Only clearly defined peaks were used for profile characterization and quantitative analyses (peak 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^{-1} extract injected (ng μl^{-1}). Quantification was done by GC–FID analysis using internal and external fatty acid standards (C16:0, C18:0, C18:1 and C18:2), methyl esters (palmitate, stearate) and *n*-alkanes (C13 to C34) (Sigma®).

Data analysis

All statistical procedures were conducted using the R 3.1.1 statistical software (https://www.r-project.org/). Before analysis, normality of the data distribution and homoscedasticity of variables were verified using Shapiro–Wilk and Bartlett's tests, respectively. Accordingly, further analysis was done using parametric or non-parametric tests. For all analyses, the 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 between the levels of significant factors.

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

Generalized linear models (GLM, quasi-Poisson error and logit function) were used to investigate the variations of metabolic rate ($\dot{V}_{CO_2}$, expressed as $\mu l\ min^{-1}\ mg^{-1}$ dry mass), using rearing conditions, anopheline populations, flight activity level, $\dot{V}_{H_2O}$, wing size and surface to volume ratio as explanatory variables. A second GLM was used to investigate the amount of water loss in females ($\dot{V}_{H_2O}$, expressed as $\mu l\ min^{-1}\ mg^{-1}$ dry mass) with the same above-mentioned variables (except that $\dot{V}_{H_2O}$ was replaced by $\dot{V}_{CO_2}$). Because of a significant effect of the surface to volume ratio on $\dot{V}_{H_2O}$ (d.f.=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 (d.f.=2, χ^2=5.91, P<0.05), but was similar across their population origins (d.f.=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 $\dot{V}_{H_2O}$ 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 analysed using two-way ANOVA procedures, where the experimental condition and the mosquito population were used as explanatory variables.

The cuticular hydrocarbon concentrations (x) were first $\log_{10}$ transformed [$\log_{10}(x+1)$] 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 multivariate ANOVA (MANOVA) was performed to examine qualitative differences in cuticular hydrocarbon profiles between anopheline populations and experimental conditions. Two-way ANOVAs (where the experimental conditions and the 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 between the mosquito populations and rearing conditions. The degrees of freedom between and within groups, together with the F-value, were reported for each LDA axis. The significance of the distribution of the LDA was assessed using a Monte Carlo test with 10,000 permutations (P<0.001).

RESULTS

Gas exchange, flight and allometric measurements

Metabolic ($\dot{V}_{CO_2}$) and water loss ($\dot{V}_{H_2O}$) rates

Overall, the three populations displayed equivalent $\dot{V}_{CO_2}$ (GLM, d.f.=2, χ^2=0.15, P=0.46). However, this parameter was significantly influenced by the rearing conditions (GLM, d.f.=1, χ^2=7.45, P<0.01; Fig. 1). The difference was particularly striking for *A. coluzzii* mosquitoes from the temporary population exposed to ODS conditions: females were characterized by an important increase in their $\dot{V}_{CO_2}$ (Fig. 1). A similar trend, yet non-significant, was observed for the permanent population of *A. coluzzii* (GLM, d.f.=2, χ^2=5.11, P=0.07). $\dot{V}_{H_2O}$ significantly differed between the three populations, and according to the environmental conditions (Table 2). $\dot{V}_{H_2O}$ of all populations significantly decreased when mosquitoes were exposed to ODS conditions (Table 2; Fig. 1). The decrease in $\dot{V}_{H_2O}$ from RS to ODS was highest in females of *A. gambiae* (Fig. 1).

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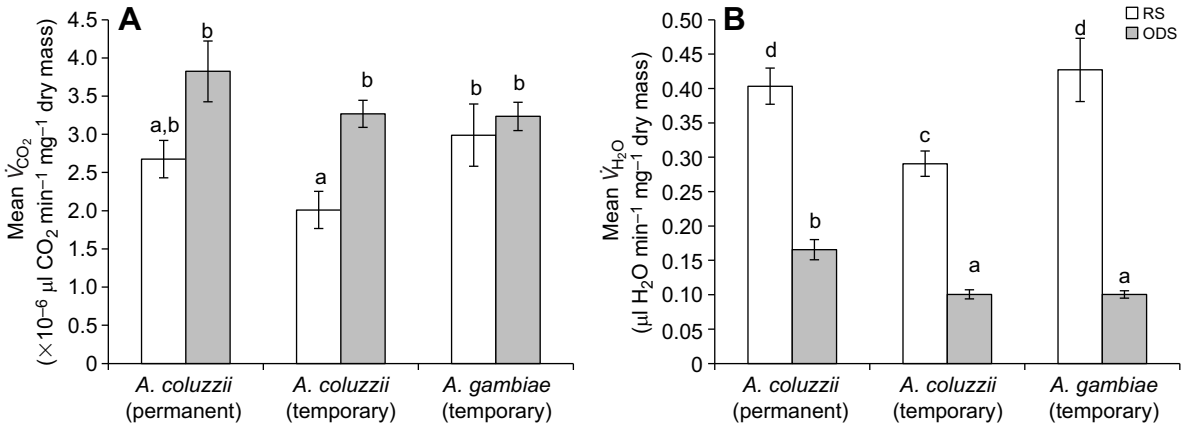

Fig. 1. Metabolic and water loss rates in *Anopheles coluzzii* and *A. gambiae*. Mean±s.e.m. rates of (A) CO_2 release ($\dot{V}_{CO_2}$, µl min^{-1} mg^{-1} dry mass) and (B) H_2O loss ($\dot{V}_{H_2O}$, µl min^{-1} mg^{-1} dry mass) of the three anopheline populations under rainy season (RS, white bars) and onset of the dry season (ODS, grey bars) environmental conditions. Different letters represent significant differences between experimental modalities at P<0.05 (GLM); n=8, 6 and 9 samples of four pooled females (RS), and n=7, 8 and 6 samples of four pooled females (ODS) from the permanent population of *A. coluzzi* and from temporary populations of *A. coluzzii* and *A. gambiae*.

Flight activity

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

Allometric measures

Analyses showed that both wing size and female dry mass significantly differed between the three populations (ANOVA, d.f.=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, d.f.=1, F=5.4, P<0.05; Fig. 3B), and this effect was mainly due to the significant increase in the dry mass of *A. gambiae* females reared in ODS conditions compared with those reared in RS conditions (Fig. 3B). Overall, the surface to volume ratio of females differed between populations (ANOVA, d.f.=1, F=13.0, P<0.001; Fig. 3C) but not between rearing conditions (ANOVA, d.f.=1, F=0.26, P=0.61).

A significant interaction between environmental conditions and populations was observed (ANOVA, d.f.=2, F=4.8, P<0.05), suggesting that the impact of the environmental conditions on the surface to volume ratio differed between the populations. Indeed, only females of *A. 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 was due to the significant increase in dry mass of the corresponding females (see above, Fig. 3B).

Table 2. Results of the GLM computed on $\dot{V}_{H_2O}$ with experimental conditions, anopheline populations, $\dot{V}_{CO_2}$, flight activity and surface to volume ratio as explanatory variables

Effects	d.f.	χ^2	P-value
Environmental conditions	1	76.97	<0.001
Population	2	7.96	<0.05
$\dot{V}_{CO_2}$	1	0.39	0.53
Surface to volume ratio	1	91.04	<0.001
Flight	1	0.05	0.82
Environmental conditions:population	2	5.21	0.07
Environmental conditions:surface to volume ratio	2	5.91	<0.05

Can flight activity and allometry influence female $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$?

$\dot{V}_{CO_2}$ was not influenced by wing size, flight activity or the surface to volume ratio (GLM, d.f.=1, χ^2=1.62, P=0.20; χ^2=0.47, P=0.49; χ^2=0.08, P=0.49, respectively).

$\dot{V}_{H_2O}$ was influenced by the variation in the surface to volume ratio of the mosquitoes, but not by the variation in their wing size and flight activity (Table 2). For all populations and for both rearing conditions, $\dot{V}_{H_2O}$ increased linearly with the surface to volume ratio of females (Fig. 4). This linear relationship in mosquitoes exposed to RS was different from that in mosquitoes exposed to ODS (Fig. 4), as supported by the significant interaction terms between surface to volume ratio and rearing conditions (Table 2).

AKH-related gene expression variations

Expression levels of the *Anoga-AKH-R* and *Anoga-AKH-I* genes differed across the populations (ANOVA, d.f.=2, $F_{AKH\text{-}R}$=5.95, $F_{AKH\text{-}I}$=2.80, P<0.05), but not those of *Anoga-AKH-II* (ANOVA, d.f.=2, F=2.11, P=0.16). Expression levels of the three genes were

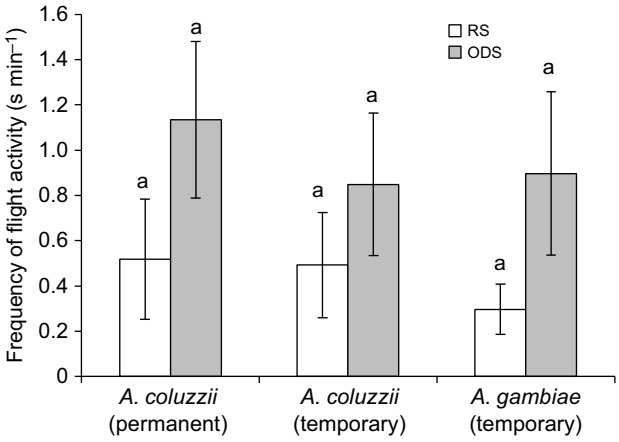

Fig. 2. Mean±s.e.m. rates of flight activity frequency (in s min^{-1}) recorded in the three anopheline populations under RS and ODS environmental conditions. Different letters represent significant differences between experimental modalities at P<0.05 (ANOVA). Samples are the same as those in Fig. 1.

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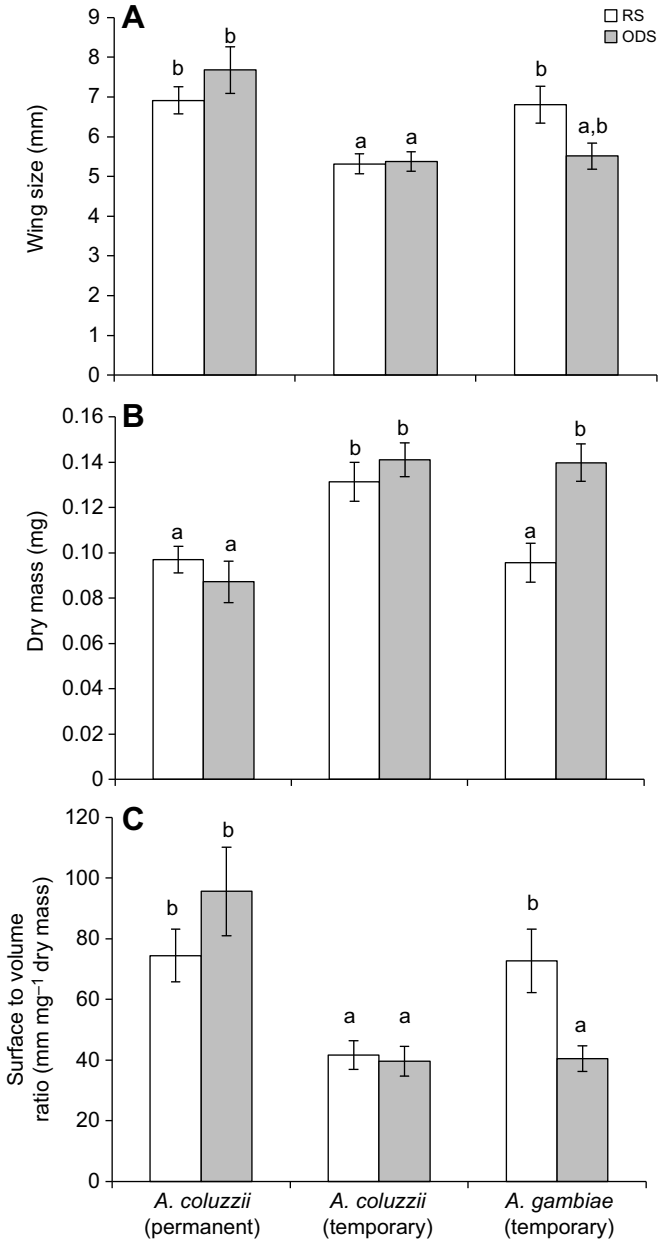

Fig. 3. Allometric measures of *A. coluzzii* and *A. gambiae* under RS and ODS conditions. Means±s.e.m. of (A) wing size (in mm), (B) dry mass (in mg) and (C) surface to volume ratio (in mm mg^{-1} dry mass). Different letters represent significant differences between the experimental modalities at $P<0.05$ (ANOVA); $n=32$, 24 and 36 samples (one female per sample; RS), and $n=28$, 32 and 24 samples (one female; ODS) from the permanent population of *A. coluzzi* and from temporary populations of *A. coluzzii* and *A. gambiae*.

not significantly influenced by the rearing conditions, although a significant interaction term between rearing conditions and population was found (ANOVA, d.f.=2, *Anoga-AKH-R*: $F=15.76$, $P<0.001$; *Anoga-AKH-I*: $F=8.33$, $P<0.01$; *Anoga-AKH-II*: $F=5.53$, $P<0.05$).

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

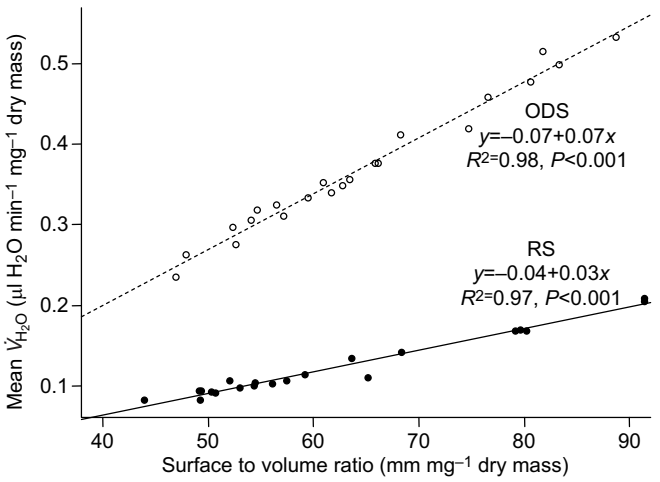

Fig. 4. Linear regression between female surface to volume ratio (in mm mg^{-1} dry mass) and $\dot{V}_{H_2O}$ (in µl min^{-1} mg^{-1} dry mass) under RS (solid line) and ODS (dashed line) environmental conditions. Sample sizes are 22 for ODS and 21 for RS.

which mRNA levels significantly increased for both populations from temporary sites, whereas a decrease (although marginally significant) was observed for *A. coluzzii* from permanent breeding sites (Fig. 5C).

Cuticular hydrocarbon fingerprints

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

Distinct cuticular hydrocarbon profiles were measured across 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 between groups was 19.31 times higher than the variation within groups (Fig. 7). LD1 mainly separated female *A. 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 *A. coluzzii* population, and increased amounts of 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; Fig. S2). The second axis (LD2) accounted for 22.43% of the total inertia, and the variation between groups was 5.06 times higher than the variation within groups. LD2 mainly separated female *A. coluzzii* from temporary breeding sites reared under ODS conditions from all other groups (Fig. 7). Increased amounts of *n*-nonacosane were measured in the ODS-reared temporary population of *A. coluzzii*, and increased amounts of *n*-heptacosane were measured in RS-reared temporary and permanent *A. coluzzii* (Fig. 7; Fig. S2).

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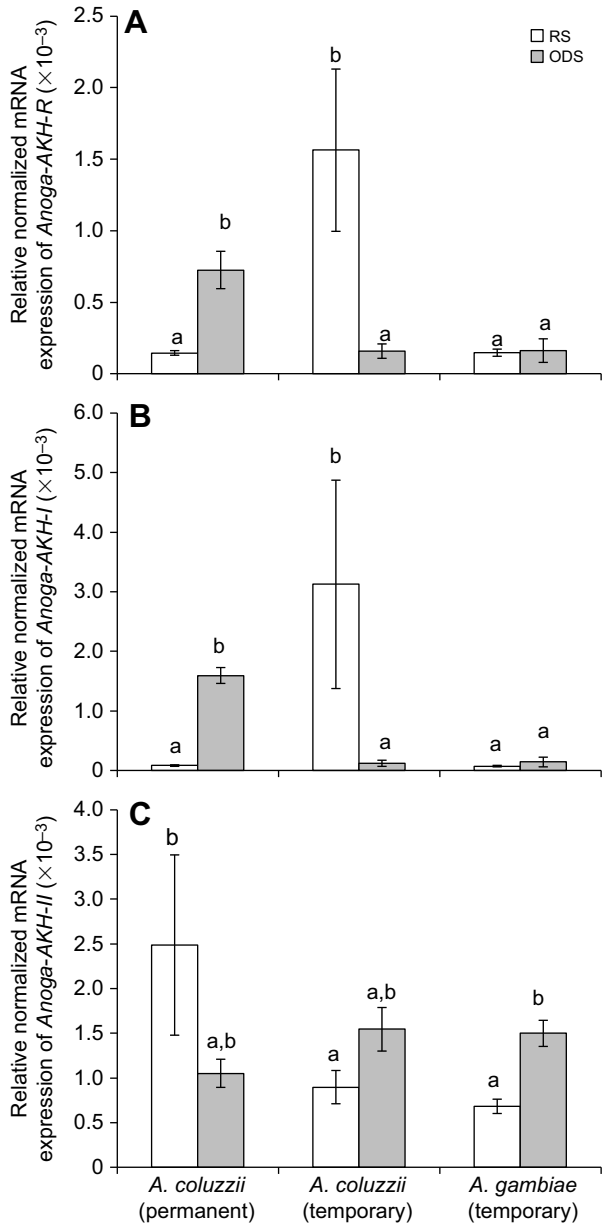

Fig. 5. Relative normalized mRNA expression levels (means±s.e.m.) in *A. coluzzii* and *A. gambiae* under RS and ODS conditions. (A) *Anoga-AKH-R*, (B) *Anoga-AKH-I* and (C) *Anoga-AKH-II* expression levels. Different letters represent significant differences between experimental modalities at $P<0.05$ (ANOVA); $n=3$, 3 and 4 samples of 10 pooled females (RS), and $n=3$, 3 and 3 samples of 10 pooled females (ODS) from the permanent population of *A. coluzzi* and from temporary populations of *A. coluzzii* and *A. gambiae*.

DISCUSSION

The present work was aimed at characterizing and comparing the influence of environmental conditions in sub-Saharan Africa at ODS and RS on phenotypic adjustments displayed by females of *A. gambiae* and *A. 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, relative humidity and photoperiod) experienced by the mosquitoes during their development. Phenotypic adjustments also varied according to the population and the larval breeding-site dynamics (temporary versus permanent).

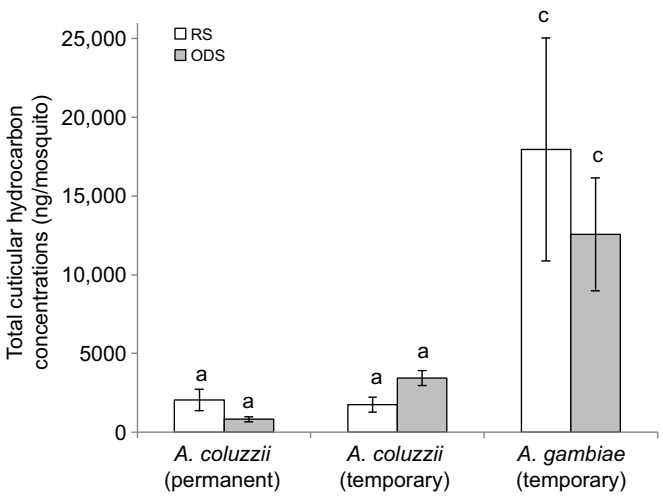

Fig. 6. Means±s.e.m. of the total amount of cuticular hydrocarbons (in ng/mosquito) in the three anopheline populations under RS and ODS environmental conditions. Different letters represent significant differences between experimental modalities at $P<0.05$ (ANOVA); $n=4$, 4 and 3 samples of four pooled females (RS), and $n=5$, 7 and 5 samples of four pooled females (ODS) from the permanent population of *A. coluzzi* and from temporary populations of *A. coluzzii* and *A. gambiae*.

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

According to studies 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 *A. coluzzii* reared under ODS conditions, as these mosquitoes are assumed to enter a dormant state named aestivation. Conversely, metabolic rate should have been increased in female *A. gambiae*, as they are supposed to exhibit a dispersive flight strategy. Surprisingly, $\dot{V}_{CO_2}$ of *A. 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 *A. 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 $\dot{V}_{CO_2}$ fingerprints we found in 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 shown 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 in female mosquitoes, possibly underlining the increased demand for energetic substrates (i.e. ATP) to flee the desiccating conditions (migration) or resist locally (aestivation). The increase in flight activity – only marginally significant – of the *A. coluzzii* females at ODS may have also contributed to an increase in $\dot{V}_{CO_2}$ values.

By contrast, female *A. gambiae* did not show significant variation in their metabolic rate from RS to ODS conditions, indicating that they express lower plasticity levels than those of *A. coluzzii* and put

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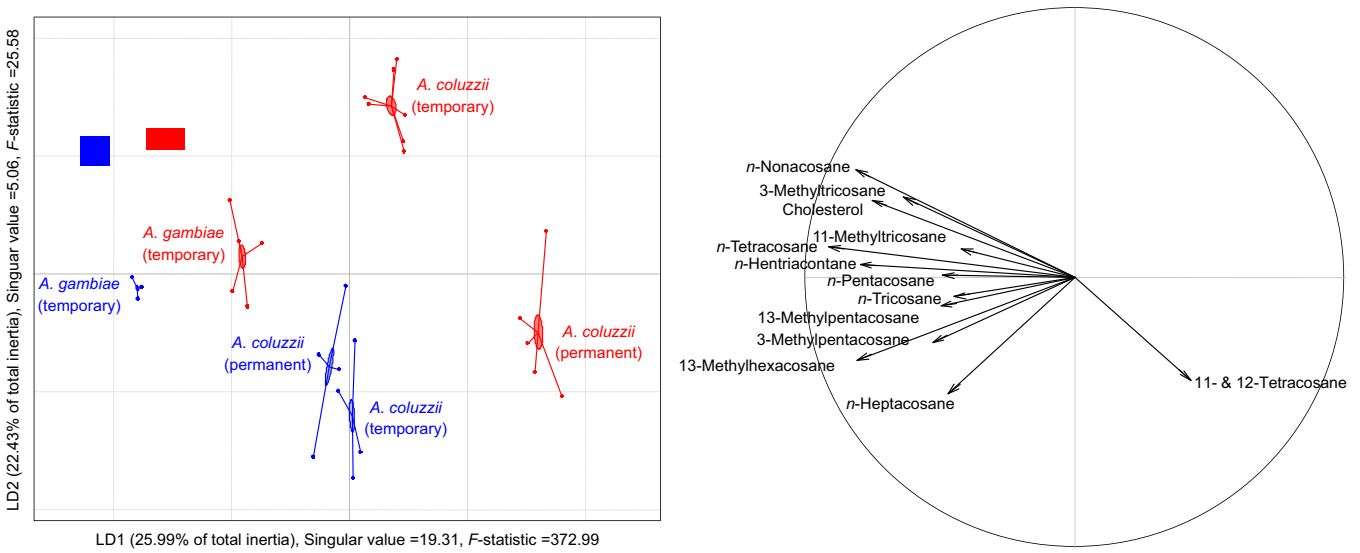

Fig. 7. Sample projection of the three anopheline populations reared under RS and ODS conditions on to the first discriminant plane of the linear discriminant analysis (LDA). Blue and red samples represent specimens reared under RS and ODS conditions, respectively. The singular values correspond to the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

in place distinct physiological mechanisms to cope with hot and dry conditions, as we initially suggested.

Mosquitoes exhibit different ways to prevent body water loss at ODS

The increase in $\dot{V}_{CO_2}$ observed at ODS in *A. coluzzii* females should contribute to an increase in its desiccation level, as gas exchanges are supposed to increase water losses in insects. Yet, our results did not support this assumption, as $\dot{V}_{CO_2}$ variations observed in all mosquitoes at both RS and ODS conditions did not influence their water loss rates ($\dot{V}_{H_2O}$). Previous studies have shown that metabolic rate fluctuations in insects 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 through adjustments of cuticular permeability, as cuticular transpiration is the principal way in which insects experience desiccation (Benoit, 2010). Yet, in the present study, water losses in mosquitoes significantly decreased in all populations at ODS, suggesting that mechanisms other than adjustments of the metabolic rate contribute to a reduction in anopheline transpiration under such climatic conditions.

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 $\dot{V}_{H_2O}$ in female *A. gambiae* and their surface to volume ratio. Such seasonal allometric change in *A. gambiae* is due to an increase in female 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 in the levels of adipokinetic peptides may also help to enhance desiccation resistance in anopheline mosquitoes, as shown in overwintering dormant insects (Hahn and Denlinger, 2011). In the present study, we measured the expression levels of two *AKH* peptide genes (*Anoga-AKH-I* and *Anoga-AKH-II*) and their putative receptor (*Anoga-AKH-R*). Overall, we highlighted that both *Anoga-AKH-I* and *Anoga-AKH-R* 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-R* expression levels did not match those of *Anoga-AKH-II*. A few years ago, *Anoga-AKH-II* was described to code for an intermediary AKH and corazonin protein named AKH/corazonin-related peptide (ACP) (Kaufmann and Brown, 2006). Functional roles of corazonin remain obscure in insects, although it seems 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 *A. gambiae*. In addition, variation of *ACP* mRNA levels correlates with those observed for *glycogen phosphorylase* mRNA in *Anopheles* species (Hidalgo et al., 2016), suggesting a possible physiological interaction between these two factors in *A. gambiae*. In particular, the increase in *ACP* expression in *A. gambiae* at ODS could help in 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).

By contrast, under ODS conditions, the *AKH-I–AKH-R* couple was over-expressed in the permanent population of *A. coluzzii*, whereas it was under-expressed in the temporary one, and did not show any seasonal variation in *A. gambiae*. Expression levels of *AKH-I* mRNA characterized the distinct molecular response of permanent *A. coluzzii* populations, which continue to be active and to reproduce at ODS. Oddly, these results contrast with those we had previously obtained using 7-day-old females, in which no seasonal differences were observed with the same *A. coluzzii* populations (Hidalgo et al., 2016). Such a difference may be explained by the younger age of the mosquitoes in the present study, in addition to our taking into account photoperiod fluctuations in our experimental rearing. AKH peptides are thought to mediate physiological responses to desiccation, as they allow the release and

transport, from the fat body to the haemolymph, 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 *A. 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 in *AKH-I* and *ACP* expressions may represent valuable markers of the survival strategies and/or stress response mechanisms elicited in *A. coluzzii* and *A. gambiae* populations.

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 in which water loss occurs 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; *A. gambiae* females displayed the highest amounts, whatever the environmental conditions, whereas no difference was observed between the two *A. coluzzii* populations. Interestingly, the total amount of cuticular hydrocarbons did not vary in mosquitoes according to rearing conditions. In insects, desiccation resistance is not related to the total amount of cuticular hydrocarbons a species exhibits, but rather to their relative composition (Arcaz et al., 2016; Gibbs et al., 1997; Gibbs and Rajpurohit, 2010; Kwan and Rundle, 2010; Nelson and Lee, 2004).

We highlighted qualitative hydrocarbon differences between the two populations of *A. coluzzii* on the one hand and within the single population of *A. gambiae* on the other, irrespective of season. The profile of *A. gambiae* females was mainly characterized by higher amounts of (1) cholesterol, a precursor of insect steroids, and (2) methyl-branched alkanes, known to be important semiochemical cues for social and sexual insect recognition (Howard and Blomquist, 2005). Such qualitative differences may be part of the biological basis leading to assortative mating between the species and, to a lesser extent, distinct innate biology and ecology (Niang et al., 2015).

Our data underlined distinct cuticular hydrocarbon adjustments in the two species in response to seasonal changes. Although cuticular hydrocarbons did not change with environmental conditions in *A. gambiae* females, seasonal differences were observed in *A. 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 ribosomal protein 2 (RR2), at ODS in *A. coluzzii* females but not in female *A. gambiae* (Hidalgo et al., 2014).

Distinct seasonal adjustments of cuticular hydrocarbons were observed in *A. coluzzii*, depending on whether they inhabited temporary or permanent larval breeding sites. Temporary populations of *A. coluzzii* displayed an increased amount of *n*-nonacosane ($C_{29}H_{60}$) at ODS, a compound that has already been observed to increase under desiccating winter conditions in the scorpion *Centruroides sculpturatus* (Toolson and Hadley, 1979). Conversely, permanent populations of *A. coluzzii* showed increased amounts of 11- and 12-tetracosane ($C_{24}H_{50}$). The three compounds melt at high temperatures, but *n*-nonacosane has a higher melting point (62–66°C) than that of *n*-tetracosane (48–54°C). Insect literature suggests that diapausing species over-express long-chain hydrocarbons (Benoit, 2010; Kankare et al., 2016) because they improve cuticle impermeability and help in avoiding desiccation (Chung and Carroll, 2015; Gibbs, 2011). Thus, the increased amount of longer hydrocarbon chains in *A. 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, which accumulates in temporary populations of *A. 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 tease out the exact roles of these candidate hydrocarbons, but, at least, their distinct profiles across conditions and populations match the expectation of distinct survival strategies in *A. coluzzii* as a function of the ecology of their larval breeding sites.

Conclusions

Understanding the survival strategies used by malaria mosquitoes and other tropical insects to cope with dry season conditions is a major challenge (Denlinger and Armbruster, 2014; Yaro et al., 2012). Our study identified distinct ecophysiological adjustments in *A. coluzzii* and *A. 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 highlighted the high phenotypic diversity in the *A. gambiae s.l.* species complex. These results are in line with the hypothesis of distinct survival strategies in these species (Yaro et al., 2012). Further investigations are required to examine the exact roles of these markers in mosquito metabolism and biology, but we believe that they could constitute relevant markers to highlight specific dry season strategies developed by 'permanent' and 'temporary' anopheline populations. Altogether, our results bring better understanding of mosquito biology during the dry season, paving the way for alternative methods of malaria control in sub-Saharan Africa.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.H., F.S., R.K.D., K.M.; Methodology: K.H., M.T.; Validation: K.H.; Formal analysis: K.H., D.S.; Investigation: K.H., C.M., D.S., V.B.; Writing - original draft: K.H.; Writing - review & editing: K.H., F.S., D.R., K.M.; Supervision: F.S., D.R., K.M.; Project administration: F.S., D.R.; Funding acquisition: F.S., D.R.

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Data availability

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.174433.supplemental

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