Abstract

Background: Anopheles gambiae, the principal vector of malignant malaria in Africa, occupies a wide range of habitats. Environmental flexibility may be conferred by a number of chromosomal inversions non-randomly associated with aridity, including 2La. The purpose of this study was to determine the physiological mechanisms associated with the 2La inversion that may result in the preferential survival of its carriers in hygrically-stressful environments.

Methods: Two homokaryotypic populations of A. gambiae (inverted 2La and standard 2L+) were created from a parental laboratory colony polymorphic for 2La and standard for all other known inversions. Desiccation resistance, water, energy and dry mass of adult females of both populations were compared at several ages and following acclimation to a more arid environment.

Results: Females carrying 2La were significantly more resistant to desiccation than 2L+ females at emergence and four days post-emergence, for different reasons. Teneral 2La females had lower rates of water loss than their 2L+ counterparts, while at four days, 2La females had higher initial water content. No differences in desiccation resistance were found at eight days, with or without acclimation. However, acclimation resulted in both populations significantly reducing their rates of water loss and increasing their desiccation resistance. Acclimation had contrasting effects on the body characteristics of the two populations: 2La females boosted their glycogen stores and decreased lipids, whereas 2L+ females did the contrary.

Conclusion: Variation in rates of water loss and response to acclimation are associated with alternative arrangements of the 2La inversion. Understanding the mechanisms underlying these traits will help explain how inversion polymorphisms permit exploitation of a heterogeneous environment by this disease vector.

Background

In tropical environments, water availability can be a major factor limiting insect distribution [1]. As such, the geographic and seasonal range of the mosquito Anopheles gambiae, the principal African vector of malaria, depends on its ability to survive in arid environments [2]. Its small size results in a large surface area to volume ratio over which evaporative water loss can occur. The activity of
adult mosquitoes exacerbates the risk of water loss, yet the scale of malaria morbidity and mortality attest to the mosquito's success throughout most of the continent [3].

It is thought that A. gambiae owes its broad distribution to a number of polymorphic chromosomal inversions within its genome, which are proposed to confer a diverse array of adaptations to the species as a whole. The patterns of inversion polymorphism shift both geographically and seasonally. Inversion frequencies have been associated with factors such as aridity [4,5], larval habitat preference [6,7], Plasmodium infection rates [8] and insecticide resistance [9]. In particular, some inversions are so strongly associated with climatic factors that climate-based models can be used to predict the presence/absence of these inversions with a high degree of accuracy [10]. However, the physiological mechanisms by which these inversions confer an adaptive advantage in a given environment remain unknown.

One inversion particularly strongly linked to aridity clines in West and Central Africa is 2La. The 2La inversion is absent in the southern parts of Nigeria and Cameroon and progressively increases in frequency, reaching fixation in the arid north of these countries [5,11]. It is hypothesized that this inversion has captured a set of alleles, which together confer an advantage to its carriers in arid conditions [4,12]. Accordingly, the 2La inversion may facilitate A. gambiae survival and consequent malaria transmission in the more xeric parts of its distribution, and may have contributed to the range expansion of this disease vector from the forest fringe into the savanna [13].

Dehydration stress, a particular threat to small arthropods in arid environments, can be limited by one of three mechanisms: increasing body water stores, reducing the rate at which water is lost, or increasing the tolerance to water loss. By modifying any of these traits, a mosquito can increase its resistance to desiccation (DR). In Drosophila melanogaster, various traits may evolve in response to selection for enhanced DR [see [14] and references therein]. However, a common finding is that water loss rates tend to be lower in xeric species than their mesic counterparts [15], an extreme example of which is seen among desert beetles [16]. Water loss rate is also known to vary in Culex pipiens mosquitoes, which drastically reduce their rates of water loss during diapause [17].

Recent studies have revealed variation in DR among Anopheles. Anopheles arabiensis, a sibling species to A. gambiae, inhabits the more arid parts of the A. gambiae species complex range in tropical Africa, and is more resistant to desiccation than A. gambiae s.s. [18]. This difference was shown to be due to higher body water stores in A. arabiensis, but any connection between this trait and the chromosomal inversions that distinguish both species has not been explored. Within A. gambiae s.s., variation in DR has been associated with molecular forms in Mali named M and S [19], but the physiological mechanisms responsible for this variation and their relationship to karyotype differences between forms have not been investigated. Although these studies suggest the presence of genetic variation for DR among members of the A. gambiae species complex, the contribution of individual inversions to this phenotype has yet to be examined.

The current study explores variation in DR that is specifically associated with the 2La inversion of A. gambiae s.s. and may explain its differential distribution in the field. To isolate the effect of this inversion on the mosquito’s physiology, the laboratory strain of A. gambiae was polymorphic solely for the 2La inversion, but fixed and standard for all other inversions. A recent and complementary study by White et al [12] adopted a genotypic approach to identify two regions of high genetic differentiation between alternative arrangements of 2La, encompassing >200 genes within the inversion. The present phenotypic approach may help implicate candidate genes within the inversion that contribute to enhanced aridity tolerance, while shedding light on the mechanisms allowing A. gambiae to exploit environmental heterogeneities and adapt to a changing climate.

**Methods**

**Colonies**

Two homokaryotypic sub-strains of A. gambiae used in this study were created from a parental strain that is polymorphic for 2La but fixed and standard for all other inversions. The parental strain (SUCAM) originated early in 2005 from a cross between CAM (2R+/2La+) and SUA (2R+/2La-) colonies, both representing the M molecular form of A. gambiae derived from regions near Yaoundé, Cameroon and Suakoko, Liberia, respectively. After approximately 36 generations of intermating within SUCAM (assuming one generation per month), homokaryotypic sub-strains, named SUCAM 2La and SUCAM 2La+, were created in April 2008 by identifying the 2L karyotype of live adults using DNA from one leg. On the morning of adult emergence, mosquitoes were cold anesthetized, amputated of one rear leg and isolated in a numbered vial. Each leg was ground in lysis buffer [20] and PCR was performed using 2La and 2La+ specific primers [21]. By early afternoon, the 2L karyotype of each mosquito was known, allowing placement of each mosquito in the appropriate cage. Selection was terminated when each population cage contained at least 70 individuals. Experiments were initiated after three generations. All mosquito populations were maintained at 27°C and 80% RH, except where otherwise noted. Mosquitoes tested at emergence had no access to sugar. Other mosqui-
toes were provided a 10% solution of corn syrup ad lib. All experiments were conducted on female mosquitoes only, given their role in malaria transmission and the importance of longevity to that role. Measurements were performed at various adult ages because water or energy storage strategies as well as stress resistance mechanisms change with age and may take trajectories that are population specific. All experiments were replicated three times on successive generations. Each replicate consisted of at least 20 mosquitoes per population, age group and acclimation regime. Total sample sizes are indicated for each experiment described below.

**Body mass, energy and hydration state**

Body characteristics and energy reserves were determined on the day of emergence and at 2, 4, 6 and 8 days post-emergence (always ~10 am for convenience and repeatability). Mosquitoes of a given age were individually weighed on a microbalance (Mettler Toledo, OH, USA; acc. 0.2 μg), dried overnight at 70°C and reweighed. Lipid content per mosquito was obtained by reweighing the dry carcass after soaking it in hexane overnight on a shaker. Glycogen content was obtained on pools of five mosquitoes, following methods modified from Van Handel [22]. Briefly, dry carcasses were ground in 200 μL of 2% sodium sulfate, and 200 μL of methanol was added, followed by vortexing, centrifugation at 4000 rpm and removal of the supernatant. The pellet containing glycogen was redissolved in 1 mL water and 1/10th was used for quantification (amount per half mosquito). Anthrone solution (750 μg anthrone in 530 mL 72% sulfuric acid) was then added to each tube up to 1.5 mL, tubes were heated at 90°C for 17 min, and glycogen was quantified spectrophotometrically at 625 nm. Overall, a total of 60 mosquitoes per age group and per population were analyzed. For glycogen, 40 additional mosquitoes were used to obtain a total sample size of 20 per age group and population.

**Desiccation resistance**

Desiccation resistance (DR) of adult females at 0, 4 and 8 days post-emergence was determined using a method previously described in Gray and Bradley [18]. On a given day individual females were CO2-anesthetized, weighed, then placed in a glass vial sealed with a foam stopper, Dri-rite® and Parafilm® (RH < 10%). Experiments on emerging mosquitoes were initiated at 8 am and those on 4- and 8-day old mosquitoes were initiated at 12 am, for logistical purposes. The sealed vials were returned to the insectary and survival was assessed hourly until all mosquitoes were dead. A mosquito was declared dead when it could no longer fly or right itself. At this point it was reweighed and placed in the drying oven overnight. Dry mass was obtained the following day. A total of at least 60 females per age group and population was analyzed.

**Acclimation effects on body mass, energy, hydration and desiccation resistance**

On the day of emergence, replicate adult populations were placed in either the humid insectary or an insectary maintained at 60% RH and 30°C (“dry environment”), where they remained for eight days. The characteristics of eight day-old females reared in either the dry or humid environment were compared. The purpose was to investigate each population’s ability to acclimate to a more stressful environment, albeit one almost certainly experienced by *A. gambiae* at the drier extremes of its geographic range [23]. At least 60 mosquitoes were tested for each population and each acclimation regime.

**Statistical analyses**

The effects of desiccation and acclimation on body characteristics were analysed by linear factorial mixed-effects models under a nested design, with population (i.e., karyotype), acclimation, or age as fixed effects, and replicates, population within replicates, and acclimation or age within populations as random effects. For those analyses where the mosquito age was used as a covariable, the statistical significance of differences in water loss rate, initial water content and water content at death between populations (POP factor) in relation to age effects (AGE covariable) was assessed by generalized linear models (GLMs) with POP and AGE and their interaction nested within replicates (factor REPEAT). Given the approximately hyperbolic shape of the relationship in most cases, the best fitting GLM models, judged according to the Akaike Information Criterion (AIC), were those with an inverse link function, gamma errors, and the age covariable square root-transformed. Parametric survival regression was used to test the effect of population (2La+3 vs. 2La), initial water content, water content at death, and water loss rate on survival under desiccation stress. Survivorship was modeled using the Weibull distribution. As all mosquitoes eventually died during the desiccation resistance test, survival times were not censored. All analyses were performed with the software R v.2.9.2 [http://www.r-
**Results**

**Body characteristics of 2La and 2L+a populations**

Figure 1 shows the change in dry mass (A), dry mass specific body water (B), lipid (C) and glycogen (D) in both populations from emergence to 8 days post-emergence. Overall there was no difference between populations in dry mass ($P = 0.396$), water content ($P = 0.219$), lipid ($P = 0.641$), or glycogen ($P = 0.205$). Dry mass strongly increased in the first 2 days while specific body water content dropped, then both remained stable at later ages. Lipid and glycogen both increased during the first week of adult life, although lipid increased progressively while glycogen stores were mostly boosted in the 2 days post-emergence.

**Desiccation resistance**

The survival of 2La females submitted to desiccation stress remained constant across the first two age groups tested, and then decreased (14.7, 14.7, and 13.3 hours for ten-erals, 4-days old, and 8-days old mosquitoes, respectively). Conversely, the survival of 2L+a females increased with age (11.6, 12.6, and 13.9 hours, respectively). This pattern, plotted in Figure 2, resulted in statistically significant differences in survival between the two karyotypes in the first two age groups (ten-erals and 4-days old), with survival leveling off to the same average between the two populations in 8-days old mosquitoes (increase in deviation caused by removal of the POPULATION × AGE interaction term from the full regression model = 14.1; d.f = 2; $P < 0.001$; Figure 2).

To explore the effect of initial water content, water content at death, and water loss rate on survival under desiccation stress, these explanatory variables were fitted as covariates in a regression model containing the population (karyotype) and its interaction with each covariate as terms of the full model. All three explanatory variables had a highly significant effect on survival ($P < 0.0001$ in all cases). Table 1 shows that initial water content increased survival (positive regression coefficients), whereas water content at death and water loss rate decreased it (negative regression coefficients). The probability of death (the baseline hazard; intercepts in Table 1) was significantly different between karyotypes only in the case of the ten-eral and 4-day old age groups, confirming the result of the previous survival analysis (cf Figure 2). Table 1 also shows the statistical significance of the difference in the relative contribution to the hazard of water loss rate, initial water content and water content at death between the two populations (asterisks in Table 1). However, the effect of the explanatory covariates on karyotypes and age groups in Table 1 is confounded by body mass, given the highly significant correlation of dry mass with all three (initial water content: Pearson product-moment correlation coefficient $r = 0.73$, 95% confidence interval 0.68-0.77, d.f. = 436, $P < 0.0001$; water content at death: $r = 0.71$, 95% C.I. 0.66-0.75, d.f. = 436, $P < 0.0001$; water loss rate: $r = 0.68$, 95% C.I. 0.63-0.73, d.f. = 436, $P < 0.0001$). Accordingly, these variables were standardized with respect to dry mass, and their mass-specific values were plotted as a function of time after emergence (Figure 3). The statistical significance of differences between karyotypes in relation to age effects was assessed by generalized linear models (Table 2). Based on these analyses, the mass-specific water loss rate was significantly greater in 2L+a compared to 2La tenerals. However, at 8 days post-emergence the pattern was reversed (Figure 3A), as indicated by the statistically significant interaction term in Table 2. Mass-specific initial water content was similar at emergence, but it diverged between 2L+a and 2La karyotypes later (marginally non-significant interaction, Table 2), with 2La having proportionally more than 2L+a (Figure 3B). Mass-specific water content at death significantly decreased with age in a similar pattern for both karyotypes (Figure 3C, non-sig-nificant interaction in Table 2). Thus, differences in survival between karyotypes were accounted for by lower water loss rates at emergence and increased water contents at 4 days in 2La individuals. By 8 days post-emergence, differences in survival might have disappeared because the larger initial water content in 2La karyotypes was counterbalanced by a higher water loss rate compared to 2L+a karyotypes.

**Effects of prior acclimation to dry conditions**

Prior acclimation significantly increased desiccation resistance of 8-day old A. gambiae (from 15.1 to 18.4 hrs on average, as estimated from survival regression; Figure 4). However, the response was similar for both karyo-types, resulting in no significant difference in survival between 2La and 2L+a (Figure 4), as shown by the non-sig-nificant interaction between karyotype and acclimation factors in Table 3.

Of the three explanatory variables, only standardized water loss rates significantly decreased in both populations following acclimation (Table 4, and Acclimation factor in Table 5). The difference between karyotypes in baseline water loss rate under normal, i.e. non-acclimated, conditions that was found in the previous desiccation resistance experiment for 8-day old mosquitoes was confirmed by this experiment (Karyotype factor in Table 5). The interaction between karyotype and acclimation, how-ever, was not statistically significant, indicating that the response to acclimation was similar in both 2La and 2L+a karyotypes (Table 5). A marginally non-significant difference between karyotypes in initial water content, regardless of acclimation, was found (Table 5); this is in
Figure 1
Change in female *A. gambiae* body characteristics with age, by karyotype. Solid lines represent 2L+a females; broken lines represent 2La females. Bars represent S.E.
agreement with the difference in initial water content between karyotypes at 8 days post-emergence found in the previous desiccation resistance experiment.

In terms of body characteristics, only mass-specific lipid and glycogen content were significantly affected by acclimation (Tables 6, 7). In both cases, the response differed according to karyotype (significant interaction terms in Table 7): 2La karyotypes decreased lipids but increased glycogen content; 2La+ karyotypes increased lipids but decreased glycogen content following acclimation (Table 6). Differences in dry mass and standardized water content were not statistically significant (Table 7), although the larger average water content of 2La vs. 2La+ karyotypes was consistent with results from the previous desiccation resistance experiment.

Discussion
This study sought to establish whether the 2La inversion in A. gambiae confers a physiological advantage under arid

Figure 2
Survivorship by karyotype (2La, continuous lines; 2La+, dashed lines) of female A. gambiae at three age groups when submitted to a desiccation resistance test. The insert shows the estimated value of the scale parameter (± standard error) of the Weibull hazard function for the six populations (black bars: 2La, light grey bars: 2La+). Larger values of the scale parameter correspond to lower hazards and increased survival. Shape parameter of the full regression model = 0.291.

Table 1: Comparison of survival curves for 2La+ and 2La karyotypes of A. gambiae females at different ages when submitted to a desiccation resistance test

| Parameter                  | Tenerals | 4-days old | 8-days old |
|----------------------------|----------|------------|------------|
|                            | 2La+     | 2La        | 2La+       | 2La        | 2La+       | 2La        |
| Intercept                  | 2.424    | 2.660***   | 2.460      | 2.698***   | 2.597      | 2.550      |
| Initial water content      | 0.0025   | 0.0024     | 0.0017     | 0.0014***  | 0.0014     | 0.0013     |
| Water content at death     | -0.0028  | -0.0022*   | -0.0020    | -0.0015**  | -0.0012    | -0.0011    |
| Water loss rate            | -0.0227  | -0.0360*** | -0.0172    | -0.0188*   | -0.0213    | -0.0172*** |
| Shape                      | 0.0471   | 0.0707     | 0.0880     |            |            |            |

Significant differences: * P < 0.05; *** P < 0.01; **** P < 0.001
Shown for the explanatory covariables are fitted regression coefficients of the scale parameter of Weibull hazard functions. Asterisks denote significant differences between pairs of coefficients from the two populations.
Table 2: Analysis of deviance for the effect of karyotype (POP) and days after emergence (AGE) on mass-specific water loss rate, initial water content, and water content at death during desiccation resistance tests of A. gambiae females.

| Response                     | Source                  | d.f. | Deviance | Residual d.f. | Residual Deviance | F     | P     |
|------------------------------|-------------------------|------|----------|---------------|-------------------|-------|-------|
| Water loss rate              | NULL                    | 437  | 32.911   |               |                   |       |       |
|                              | Repeat                  | 3    | 1.147    | 434           | 31.764            | 5.22  | 0.104 |
|                              | Repeat:POP              | 4    | 1.564    | 430           | 30.200            | 5.57  | 0.095 |
|                              | Repeat:AGE              | 3    | 4.110    | 427           | 26.090            | 22.42 | 0.015 |
|                              | Repeat:POP*AGE          | 3    | 2.616    | 424           | 23.474            | 15.75 | 0.024 |
| Initial water content        | NULL                    | 437  | 29.115   |               |                   |       |       |
|                              | Repeat                  | 3    | 1.091    | 434           | 28.024            | 5.63  | 0.095 |
|                              | Repeat:POP              | 4    | 0.299    | 430           | 27.724            | 1.16  | 0.470 |
|                              | Repeat:sqrt(AGE)        | 3    | 11.582   | 427           | 16.143            | 102.12| 0.002 |
|                              | Repeat:POP*sqrt(AGE)    | 3    | 0.731    | 424           | 15.412            | 6.70  | 0.076 |
| Water content at death       | NULL                    | 437  | 39.913   |               |                   |       |       |
|                              | Repeat                  | 3    | 2.718    | 434           | 37.195            | 10.57 | 0.042 |
|                              | Repeat:POP              | 4    | 0.085    | 430           | 37.110            | 0.25  | 0.895 |
|                              | Repeat:sqrt(AGE)        | 3    | 23.155   | 427           | 13.956            | 236.15| 0.000 |
|                              | Repeat:POP*sqrt(AGE)    | 3    | 0.365    | 424           | 13.590            | 3.80  | 0.151 |

Figure 3
Changes with age in mass-specific values of (A) water loss rate, (B) initial water content, and (C) water content at death by karyotype in female A. gambiae submitted to desiccation stress tests. 2La, closed circles and continuous lines; 2L+a, open circles and dashed lines. Symbols and error bars represent means ± 95% confidence intervals. Lines depict the fitted generalized linear models. All values on the ordinate are in μg·μg⁻¹. Water loss rates are expressed in μg·μg⁻¹·hr⁻¹.
conditions. Indeed, it was demonstrated that young adult females carrying the 2La inversion resist desiccation longer than their 2L+a counterparts. Furthermore this difference in DR is age specific: the difference between populations was most pronounced on the day of emergence and still present in four day-old adults, but absent by eight days. The first few days of adult life include flying away from the emergence site, maturation of multiple organs, cuticle hardening, swarming, mating and orienting towards suitable feeding areas [26]. The first day of adult life may be particularly stressful as any fluid losses occurring can only be countered after dusk, when young mosquitoes begin seeking for nectar sources [27]. These activities subject mosquitoes to stressful and potentially lethal environments; the difference in DR may confer a competitive advantage to the 2La karyotype that could help explain its high frequency in arid habitats.

Table 3: Analysis of deviance of the effect of prior acclimation on the survival of 8-day old A. gambiae females in desiccation resistance tests.

| Source                  | d.f. | Deviance | Residual d.f. | -2*LL   | P   |
|-------------------------|------|----------|---------------|---------|-----|
| Null Model              | NA   | NA       | 379           | 2421.00 | NA  |
| Replicate               | 3    | 67.918   | 376           | 2353.08 | <0.001 |
| Replicate/Karyotype     | 4    | 3.631    | 372           | 2349.45 | 0.458 |
| Replicate/Acclimation   | 4    | 64.646   | 368           | 2284.80 | <0.001 |
| Replicate/Karyotype * Acclimation | 4 | 3.265 | 364 | 2281.54 | 0.515 |

LL: log-likelihood

DR is determined by initial and final body water contents as well as the rate of body water loss. The measurement of all three parameters revealed that emerging females of either karyotype differed most strongly in their rate of water loss. Specifically, teneral 2La females had a significantly lower water loss rate than their 2L+a counterparts. In fact, when comparing age groups, 2La females had a similar rate of water loss at emergence and later ages, whereas 2L+a females experienced their highest rate of water loss at emergence.

Variation in water loss rate most likely involves modifications in the physical characteristics of the main barrier to water loss, the cuticle and/or its waxy surface. Although water loss can also occur via the spiracles during respiration, it is thought that respiratory water loss contributes very little to overall water loss in insects [28-31].

**Figure 4**

*Effect of prior acclimation on desiccation resistance of 8-day old A. gambiae.* Survivorship curves of (A) non-acclimated and (B) acclimated females by karyotype (2La, continuous lines; 2L+a, dashed lines).
also contributes to water loss. *A. gambiae* discharge excess fluids during the first 5 min post-eclosion to facilitate flight [32]. In addition, diuresis follows feeding and is thought to be stimulated by abdominal distension [26]. However, it is unlikely that the observed differences in water loss rate were due to diuresis after feeding, as mosquitoes had no access to liquids during the desiccation bout.

**Modulating cuticular water loss**

The cuticle may be the principal source of dehydration and the one that, if modified, can most promote water conservation. Cuticular water loss can be reduced by variation in the quantitative or qualitative production of cuticular hydrocarbons, which are the main components of the waxy layer covering the epicuticle. Beetles, scorpions and other arthropods living in desert environments manage to reduce cuticular permeability by producing more waxes than animals found in mesic environments [33,34]. *Culex pipiens* mosquitoes also increase the amount of cuticular lipids in preparation for winter diapause [17]. The chain length of cuticular hydrocarbons, which correlates positively with melting point temperature, may also influence the waterproofing qualities of the waxy layer by affecting its stability at high ambient temperatures [35]. Cuticular hydrocarbon analyses of *A. arabiensis* and two molecular forms of *A. gambiae* collected from Burkina Faso uncovered no qualitative differences in chain length within or among groups, but quantitative differences in hydrocarbon abundance were noted [36]. It is possible that these differences reflect local climatic conditions, but they are probably not associated with alternative arrangements of the 2La inversion, which is fixed in *A. arabiensis* and similarly fixed or nearly so in *A. gambiae* populations from the dry savanna of Burkina Faso, where most specimens were collected.

Another possible mechanism to modulate cuticular water loss may involve cuticular proteins. Until now these proteins, which are structurally important components of the cuticle, have never been implicated in cuticular waterproofing. Yet recent work suggests that cuticular protein genes can be up-regulated by environmental stress, including desiccation [37]. Interestingly, a chromosomal region within inversion 2La that is implicated in its maintenance [12] contains a cluster of 39 genes encoding cuticular proteins of the RR-2 consensus, a major family of structural components of the hard cuticle [38]. Following an appropriate regimen of stress, differences between populations in the expression profiles of gene(s) encoding these cuticular proteins could be suggestive of their involvement in cuticular permeability.

**The influence of body water content on DR**

Although body water content was not consistently different between populations, it is apparent from the desiccation results for four day-old mosquitoes that this trait can have significant effects on DR (Figure 3). Body water var-

| Table 4: Effect of prior acclimation on mass-specific responses (means ± SEM) by alternative karyotypes (2L+ and 2La) in desiccation resistance tests of *A. gambiae* females. |
|---------------------------------------------------------------|
| **Response** | 2L++ Non-acclimated | 2La | 2L++ Acclimated | 2La |
| Water loss rate | 0.099 ± 0.002 | 0.113 ± 0.003 | 0.071 ± 0.002 | 0.092 ± 0.002 |
| Initial water content | 2.43 ± 0.05 | 2.61 ± 0.06 | 2.20 ± 0.05 | 2.65 ± 0.06 |
| Water content at death | 1.13 ± 0.01 | 1.18 ± 0.02 | 1.08 ± 0.02 | 1.14 ± 0.02 |

| Table 5: ANOVA results describing the effect of prior acclimation on mass-specific responses by *A. gambiae* females to desiccation resistance tests. |
|---------------------------------------------------------------|
| **Response** | d.f. | Sum Sq | Mean Sq | F | P |
| A. Water loss rate | Karyotype | 1 | 0.0098 | 0.0098 | 18.34 | 0.013 * |
| | Acclimation | 1 | 0.0292 | 0.0292 | 54.49 | <0.001 *** |
| | Karyotype * Acclimation | 1 | 0.0004 | 0.0004 | 0.80 | 0.385 |
| B. Initial water content | Karyotype | 1 | 1.2044 | 1.2044 | 5.48 | 0.079 |
| | Acclimation | 1 | 0.0874 | 0.0874 | 0.40 | 0.545 |
| | Karyotype * Acclimation | 1 | 0.2262 | 0.2262 | 1.03 | 0.325 |
| C. Water content at death | Karyotype | 1 | 0.09704 | 0.09704 | 3.79 | 0.123 |
| | Acclimation | 1 | 0.05002 | 0.05002 | 1.95 | 0.200 |
| | Karyotype * Acclimation | 1 | 0.00031 | 0.00031 | 0.01 | 0.922 |
ies throughout the day; it is lost by transpiration, respiration and excretion and is replenished by fluid absorption during feeding. Measurements of body characteristics including body water content (Figure 1) were performed at 10 am, whereas mass measurements prior to the desiccation assays were performed before 8 am for teneral mosquitoes and at 12 am (midnight) for 4 and 8 day-old mosquitoes. As these measurement times were consistent between replicates, they allow for preliminary comparisons of daily variation in body water stores between 2La and 2L+a populations. Whereas both populations had the same body water content at 10 am, water content differed between populations at the other time points, hinting at the possibility that alternative karyotypes may not manage their body water stores in the same way throughout the 24 h period. A. gambiae are nocturnally active and quiescent during the day; it is possible that differences in the timing (or frequency) of feeding (drinking) before entering daily quiescence may lead to differential survival of alternate karyotypes. Testing of this hypothesis will require investigating the diurnal pattern of sugar (or nectar) feeding in both populations.

**Acclimation effects on DR and energy stores**

Adult females of both populations were reared in an environment mimicking more arid conditions (both warmer and drier) to test the hypothesis that 2La females were better equipped than their 2L+a counterparts to respond to hygrically stressful environments. In fact, both groups effectively acclimated to this environment by reducing their rate of water loss, suggesting a phenotypic plasticity in water loss rate that is not associated with alternative arrangements of the 2La inversion. Furthermore, both groups increased their body size, a change leading to decreased ratio of surface area to volume, which is advantageous in dry environments as it reduces the mass specific rate of water loss [39,40]. On the other hand, changes in other body characteristics following acclimation suggest karyotype-specific effects on energy stores that may affect stress resistance. Glycogen is thought to enhance DR by

| Table 6: Effect of prior acclimation on body characteristics (means ± SEM) of alternative karyotypes (2L++ and 2L+) in desiccation resistance tests of A. gambiae females |
|-----------------------------------------------|
| Response | Non-acclimated | Acclimated |
|----------|----------------|------------|
|          | 2L++ | 2L+/a | 2L++ | 2L+/a |
| Dry mass (μg) | 610 ± 18 | 632 ± 19 | 664 ± 15 | 646 ± 15 |
| Water content (μg·μg⁻¹) | 1.621 ± 0.016 | 1.673 ± 0.014 | 1.571 ± 0.015 | 1.669 ± 0.016 |
| Lipid content (μg·μg⁻¹) | 0.295 ± 0.005 | 0.278 ± 0.004 | 0.333 ± 0.005 | 0.268 ± 0.004 |
| Glycogen content (μg·μg⁻¹) | 0.156 ± 0.006 | 0.159 ± 0.006 | 0.146 ± 0.005 | 0.167 ± 0.006 |

1Standardized by dry mass

| Table 7: ANOVA results describing the effect of prior acclimation on body characteristics of alternative karyotypes (2L++ and 2L+) in desiccation resistance tests of A. gambiae females. |
|-----------------------------------------------|
| Response | d.f. | Sum Sq | Mean Sq | F | P |
|----------|------|--------|---------|---|---|
| A. Dry Mass | | | | | |
| Karyotype | 1 | 168 | 168 | 0.01 | 0.925 |
| Acclimation | 1 | 28230 | 28230 | 0.005 | 0.179 |
| Karyotype * Acclimation | 1 | 6467 | 6467 | 0.81 | 0.371 |
| B. Water Content | | | | | |
| Karyotype | 1 | 0.0469 | 0.0469 | 2.40 | 0.196 |
| Acclimation | 1 | 0.0051 | 0.0051 | 0.19 | 0.624 |
| Karyotype * Acclimation | 1 | 0.0105 | 0.0105 | 0.54 | 0.498 |
| C. Lipid Content | | | | | |
| Karyotype | 1 | 0.0316 | 0.0316 | 18.00 | 0.013 * |
| Acclimation | 1 | 0.0043 | 0.0043 | 2.53 | 0.150 |
| Karyotype * Acclimation | 1 | 0.0161 | 0.0161 | 9.43 | 0.007 ** |
| D. Glycogen Content | | | | | |
| Karyotype | 1 | 0.00288 | 0.00288 | 7.72 | 0.050 * |
| Acclimation | 1 | 0.00026 | 0.00026 | 0.17 | 0.694 |
| Karyotype * Acclimation | 1 | 0.00170 | 0.00170 | 4.56 | 0.049 * |

1Standardized by dry mass
increasing water storage, as it binds up to 5 times its weight in water [39,41]. Glycogen stores increase in response to selection for DR in D. melanogaster [42] and both are significantly correlated among several populations [43]. Lipid storage correlates with starvation resistance [44], another possible adaptation to aridity. These traits did not differ among females of alternative karyotypes in the absence of acclimation. However, the populations responded differently to acclimation: 2La+a females boosted their lipid stores and decreased glycogen content while 2La females decreased lipids and increased glycogen. This resulted in significant difference in life history strategy. Lipid is an efficient form of energy storage as for the same mass of stored product, lipid yields 10 times more energy than glycogen (particularly since glycogen is stored with water) [39]. Lipid stores are boosted in some female mosquitoes in preparation for winter diapause [45,46]. A similar strategy has been noted for winter diapause in A. gambiae sensu stricto and A. arabiensis, using climate data. Proc Biol Sci 1995, 265:847-854.

A. gambiae

We would like to thank Marcia Kern for invaluable assistance in establishing the new colonies and Dr. Timothy Bradley for critical reading of the manuscript. Research was supported by NIH grant ROI A1076584 to NB. KR received support from the College of Science and the Glynn Family Honors Program at the University of Notre Dame.

References

1. Sutherst R, Maywald G, Skarratt D: Predicting insect distributions in a changing climate. In Insects in a changing environment. Edited by: Harrington R, Stork N. London: Academic Press; 1995:9-91.
2. Lindsay SW, Parson L, Thomas CJ: Mapping the ranges and relative abundance of the two principal African malaria vectors, Anopheles gambiae sensu stricto and An. arabiensis, using climate data. Proc Biol Sci 1995, 265:847-854.
3. Snow RW, Oumombo JA: Malaria. In Disease and mortality in Sub-Saharan Africa 2nd edition. Edited by: Jamison DT, Feachem R, Makgoba MW, Bos ER, Baingana FK, Hofman KJ, Rigo KO. Washington, D.C.: World Bank; 2006:195-213.
4. Powell JR, Petraca V, della Torre A, Caccone A, Coluzzi M: Population structure, speciation, and introgression in the Anopheles gambiae complex. Parasitologia 1999, 41:101-113.
5. Coluzzi M, Sabatin A, Petraca V, Di Deco MA: Chromosomal differentiation and adaptation to human environments in the Anopheles gambiae complex. Trans R Soc Trop Med Hyg 1979, 73:483-497.
6. Toure YT, Petraca V, Traore SF, Coulibaly A, Maiga HM, Sankare O, Sow M, Di Deco MA, Coluzzi M: The distribution and inversion polymorphism of chromosomally recognized taxa of the Anopheles gambiae complex in Mali, West Africa. Parasitologia 1998, 40:477-511.
7. Manoukis NC, Powell JR, Toure MB, Sacko A, Edillo FE, Coulibaly MB, Traore SF, Taylor CE, Besansky NJ: A test of the chromosomal theory of ecotypic speciation in Anopheles gambiae. Proc Natl Acad Sci USA 2008, 105:2940-2945.
8. Petraca V, Beier JC: Intraspecific chromosomal polymorphism in the Anopheles gambiae complex as a factor affecting malaria transmission in the Kisumu area of Kenya. Am J Trop Med Hyg 1992, 46:229-237.
9. Brooke BD, Hunt RH, Chandre F, Carnevale P, Coetzee M: Stable chromosomal inversion polymorphisms and insecticide resistance in the malaria vector mosquito Anopheles gambiae (Diptera: Culicidae). J Med Entomol 2002, 39:568-573.
10. Bayoh MN, Thomas CJ, Lindsay SW: Mapping distributions of chromosomal forms of Anopheles gambiae in West Africa using climate data. Med Vet Entomol 2001, 15:267-274.
11. Simard F, Ayala D, Kamdem GC, Pombi M, Etouna J, Ose K, Fotsing J-M, Fontenille D, Besansky NJ, Costantini C: Ecological niche partitioning between Anopheles gambiae molecular forms in Cameroon: the ecological side of speciation. BMC Ecology 2009, 9:17.
12. White BJ, Hahn MW, Pombi M, Cassone BJ, Lobo NF, Simard F, Besansky NJ: Localization of candidate regions maintaining a common polymorphic inversion (2La) in Anopheles gambiae. PLoS Genet 2007, 3:e217.
13. Coluzzi M, Sabatin A, della Torre A, Di Deco MA, Petraca V: A polymorphic chromosome analysis of the Anopheles gambiae species complex. Science 2002, 298:1415-1418.
14. Telsonis-Scott M, Guthridge KM, Hoffmann AA: A new set of laboratory-selected Drosophila melanogaster lines for the analysis of
of desiccation resistance: response to selection, physiology and correlated responses. J Exp Biol 2006, 209:1837-1847.
13. Benoît JB, Denlinger DL: Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. J Exp Biol 2007, 210:217-226.
14. Gray EM, Bradley TJ: Physiology of desiccation resistance in *Anopheles gambiae* and *Anopheles arabiensis*. Am J Trop Med Hyg 2005, 73:553-559.
15. Lee Y, Meneses CR, Fofana A, Lanzaro GC: Desiccation resistance among subpopulations of *Anopheles gambiæ* s.s. from Selinkeny, Mali. J Med Entomol 2009, 46:316-320.
16. Le CE, Frost BW: Morphological stasis in the *Eurytemora affine* species complex (Copepoda: Temoridae). Hydrobiologia 1992, 231-233:179-188.
17. White BJ, Santolamazza F, Kamau L, Pombi M, Grushko O, Moulineenyi, Mali. J Med Entomol 2009, 46:316-320.
18. Hadley NF, Schultz TD: Evolution of water conservation mechanisms in *Drosophila*. J Exp Biol 2003, 206:1183-1192.
19. Cloudsley-Thompson JL: Thermal and water relations of desert beetles. Naturwissenschaften 2001, 88:447-460.
20. Benoît JB, Denlinger DL: Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. J Exp Biol 2007, 210:217-226.
21. Grey EM, Bradley TJ: Physiology of desiccation resistance in *Anopheles gambiae* and *Anopheles arabiensis*. Am J Trop Med Hyg 2005, 73:553-559.
22. Lee Y, Meneses CR, Fofana A, Lanzaro GC: Desiccation resistance among subpopulations of *Anopheles gambiæ* s.s. from Selinkeny, Mali. J Med Entomol 2009, 46:316-320.
23. Benoît JB, Denlinger DL: Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. J Exp Biol 2007, 210:217-226.
24. Lee Y, Meneses CR, Fofana A, Lanzaro GC: Desiccation resistance among subpopulations of *Anopheles gambiæ* s.s. from Selinkeny, Mali. J Med Entomol 2009, 46:316-320.
25. Bates D: Fitting linear mixed models in R. R News 2005, 5:27-30.
26. Clements AN: The biology of mosquitoes. In Development, nutrition and reproduction Volume 1. 1st edition. London, New York: Chapman & Hall; 1992.
27. Foster WA, Takken W: Nectar-related vs. human-related volatiles: behavioural response and choice by female and male *Anopheles gambiæ* (Diptera: Culicidae) between emergence and first feeding. Bull Entomol Res 2004, 94:145-157.
28. Gray EM, Chown SL: Bias, precision and accuracy in the estimation of cuticular and respiratory water loss: a case study from a highly variable cockroach, *Perisphaeria sp.* J Insect Physiol 2008, 54:169-179.
29. Bosch M, Chown SL, Scholtz CH: Discontinuous gas exchange and water loss in the keratin beetle *Omorgus radula*: further evidence against the water conservation hypothesis? Physiol Entomol 2000, 25:309-314.
30. Quinlan MC, Hadley NF: Gas exchange, ventilatory patterns, and water loss in two lubber grasshoppers: quantifying cuticular and respiratory transpiration. Physiol Zool 1993, 66:628-642.
31. Williams AE, Rose MR, Bradley TJ: Using laboratory selection for desiccation resistance to examine the relationship between respirational pattern and water loss in insects. J Exp Biol 1998, 201:2945-2952.
32. Goma LK: The exudation of fluid by the newly emerged of the mosquito, *Anopheles gambiae* Giles. Entomologist 1964, 97:233-239.
33. Toolson EC, Hadley NF: Cuticular permeability and epicuticular lipid composition in two Arizona vejovid scorpions. Physiol Zool 1977, 50:323-330.
34. Hadley NF, Schultz TD: Water loss in three species of tiger beetles (Cicindela): correlations with epicuticular hydrocarbons. J Insect Physiol 1987, 33:677-682.
35. Gibbs AG, Chippendale AK, Rose MR: Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster*. J Exp Biol 1997, 200:1821-1832.
36. Caputo B, Dani FR, Horsin GL, NFale S, Diabate A, Turillazzi S, Coluzzi M, Costantini C, Piresman AA, Petrara V, della Torre A: Comparative analysis of epicuticular lipid profiles of sympatric and allopatric field populations of *Anopheles gambiae* s.s. molecular forms and *An. arabiensis* from Burkina Faso (West Africa). Insect Biochem Mol Biol 2007, 37:389-398.
37. Zhang J, Goyer C, Peltier Y: Environmental stresses induce the expression of putative glycine-rich insect cuticular protein genes in adult *Leptinotarsa decemlineata* (Say). Insect Mol Biol 2008, 17:209-216.
38. Rebers JE, Willis JH: A conserved domain in arthropod cuticular proteins binds chitin. Insect Biochem Mol Biol 2001, 31:1083-1093.
39. Schmidt-Nielsen K: Animal physiology: adaptation and environment. 5th edition. Cambridge [England]; New York, NY, USA: Cambridge University Press; 1997.
40. Hadley NF: Water relations of terrestrial arthropods. San Diego: Academic Press; 1994.
41. Archer MA, Bradley TJ, Mueller LD, Rose MR: Using experimental evolution to study the physiological mechanisms of desiccation resistance in *Drosophila melanogaster*. Physiol Biochem Zool 2007, 80:386-398.
42. Gravi JS, Toolson EC, Jeong LN, Vu LN, Rose MR: Desiccation, flight, glycogen, and postponed senescence in *Drosophila melanogaster*. Physiol Zool 1992, 65:268-286.
43. Djawdan M, Chippendale AK, Rose MR, Bradley TJ: Metabolic reserves and evolved stress resistance in *Drosophila melanogaster*. Physiol Zool 1998, 71:584-594.
44. Chippendale AK, Chu TJ, Rose MR: Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. Evolution 1996, 50:753-766.
45. Bowen MF: Patterns of sugar feeding in diapausing and non-diapausing *Culex pipiens* (Diptera: Culicidae) females. J Med Entomol 1992, 29:843-849.
46. Mitchell CJ, Briegel H: Inability of diapausing *Culex pipiens* (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. J Med Entomol 1989, 26:318-326.
47. Storey KB: Life in the slow lane: molecular mechanisms of estivation. Comp Biochem Physiol A Mol Integr Physiol 2002, 133:733-754.
48. Charlwood JD, Vir J, Billingsley PF: Dry season refugia of malaria-transmitting mosquitoes in a dry savannah zone of east Africa. Am J Trop Med Hyg 2000, 62:726-722.