Theoretical and Experimental Investigations of the Larval Life History of *Anopheles gambiae*

by

Conan Côté Phelan

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Name: **Conan Côté Phelan**

Degree: **Doctor of Philosophy (Ecology)**

Title of Thesis: ***Theoretical and experimental investigations of larval life history of* Anopheles gambiae**

Examining Committee:

Chair: Jenny Cory, Professor

Bernard Roitberg
Senior Supervisor
Professor

Tony Williams
Supervisor
Professor

Ronald Ydenberg
Supervisor
Professor

Jonathan Moore
Internal Examiner
Assistant Professor
Department of Biological Sciences

Steven Juliano
External Examiner
Distinguished Professor, School of Biological Sciences
Illinois State University

Date Defended/Approved: February 1, 2012

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Abstract

This work addresses how the larval environment of *Anopheles gambiae sensu stricto* affects its growth, development, and survival. This mosquito is a major vector of human malaria in Africa, and how its larval habitat affects its fitness is relevant to epidemiology of this devastating disease. While my experimental work was species-specific, I applied general theory to understanding *An. gambiae's* responses to its environment. I pursued two avenues of enquiry, looking at how larval development and survival are influenced by 1) variable food availability and 2) multiple different variables acting simultaneously.

I began with a theoretical investigation of how variable food affects optimal age and size at emergence under a trade-off between growth and mortality. As a consequence of risk-sensitivity, variable food produced a small delay in emergence and a shallower L-shaped age-size relationship across food levels but did not qualitatively affect predictions for optimal development. I then investigated experimentally how *An. gambiae* responded to increases or decreases in food availability during the larval period and compared the results to predictions from theory. While results were qualitatively consistent with predictions, age at maturity diverged substantially.

To examine how multiple variables interact to affect development, I conducted a factorial experiment manipulating food availability, water depth, and temperature. I detected effects of each of these factors upon age and size at emergence. Furthermore, many of these effects depended on the levels of other factors. In the context of an L-shaped relationship between age and size, some of these interaction-effects could be seen as an outcome of energy budgets of individuals. To directly assess effects on energy, I measured lipid and glycogen of newly-emerged mosquitoes from the experiment. These values suggested that treatments decoupled body size from levels of metabolic reserves. Finally, larval diving activity was recorded during the experiment. Larvae dived more frequently under low food, providing novel evidence that *An. gambiae* dives to forage.

Theory developed in the context of constant environments proved useful for understanding development under changing conditions. Reducing environmental variables to their effects on energy budgets may help elucidate how mosquitoes respond to the variety of environments in nature.

Keywords: mosquito; optimal; age; size; growth; energy

For my children Violet and Avi, wife Laurie,
and Brother Ben.

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Chapter 1.

Introduction

This body of work considers the plastic growth and development of a single mosquito species, *Anopheles gambiae sensu stricto*, in light of a trade-off between growth and mortality that has shaped its life history. Organisms generally evolve within variable environments and consequently adaptations for dealing with uncertainty are ubiquitous. Plasticity in growth and development allows organisms to adjust rates of growth and timing of life history events to maximize fitness in the face of variable levels of resources and risk of death (Kozlowski 1992; West-Eberhard 2003). While *An. gambiae sensu stricto* is a major vector of human malaria in Africa the research presented here has not been conducted for direct application to vector control or epidemiology. Rather, the aim has been to improve basic understanding of larval ecology of this medically important species.

Malaria is caused by blood parasites in the genus *Plasmodium*, which are transmitted among human hosts by mosquitoes from the genus *Anopheles*. Adult female mosquitoes become infected with *Plasmodium* when they take a blood meal from an infected person. They then transmit parasites to new hosts during subsequent blood meals. Somewhat confusingly, *An. gambiae sensu stricto* is one of seven species that make up the *An. gambiae* species complex, more formally referred to as *An. gambiae sensu lato*. This group of species is responsible for transmission of the majority of human malaria in sub-Saharan Africa, and *An. gambiae sensu stricto* is one of the two most competent vector species within the group (Levine et al. 2004). Hereafter, reference to *An. gambiae* indicates the species-proper rather than the multi-species complex. Distribution data for *An. gambiae* are incomplete for but it is thought to occupy a range that runs from Senegal in West Africa southeast through Madagascar (Coetzee et al. 2000; Sinka et al. 2010). See Figure 1.1.

Decision-makers would like to be able to make useful predictions about how the abundance and distribution of mosquitoes that transmit malaria will change over time. Historically, the aquatic larval habitat has been key to managing mosquito populations. Efforts to assess how African malaria vectors will respond to habitat availability and characteristics have largely relied on phenomenological approaches (Chaves & Koenraadt 2010). Researchers identify habitat characteristics correlated with mosquito productivity and make inferences about how populations will respond to changes affecting those characteristics (Ndenga et al. 2011). A complementary approach is to attempt to understand the ecological processes and other mechanisms that underlie population-level responses to the environment. Such a mechanistic approach demands a level of understanding that is often not easily forthcoming but has the potential to improve predictive ability (Buckley et al. 2010). This potential exists because phenomenological models are frequently not applicable to new conditions; biological systems often exhibit non-linear properties that undermine the utility of simply extrapolating from one context to another. This body of work attempts to provide insight into the mechanisms that determine how *An. gambiae* mosquitoes are affected by their larval growing environments.

Larval rearing conditions influence multiple traits expected to influence epidemiology of malaria, including age and size at emergence as well as body condition, or energetic state. Age at emergence (i.e., age at maturity) is important because it affects population dynamics — all else being equal, a shorter time to maturity raises the intrinsic rate of population increase, r. Body size is similarly important for population dynamics because it is positively related to fecundity: bigger mosquitoes have the capacity to bear a greater number of eggs. Additionally, adult body size and physiological state, or condition, are both thought to affect biting rate and size of blood meals, which are influential parameters in models of vectored diseases (Anderson & May 1991; Ma & Roitberg 2008). Body size and condition have both been recently demonstrated to affect attack rate in *An. gambiae* (Roitberg et al. 2010). Size had a positive relationship with attack rate and condition had a nonlinear relationship such that mosquitoes with intermediate levels of reserves attacked the least. Separating the effects that size and condition have upon malaria transmission is often challenging because larger individuals tend to have relatively greater levels of reserves (Briegel

1990; Takken et al. 1998; Blanckenhorn et al. 2007). But it is clear that better condition improves survival, mating success, reproductive output, and other components of fitness (Walker 2008; Aboagye-Antwi & Tripet 2010).

Larvae *of An. gambiae* typically perform best in ephemeral, shallow waters describable as puddles (Gimnig et al. 2002; Ndenga et al. 2011). Such habitats generally have a low risk of predation relative to larger, more permanent water bodies (Minakawa et al. 2004), although evidence of cannibalism has been documented for this species (Koenraadt et al. 2004). As with other mosquito species, larvae go through four instars before spending about a day as non-feeding mobile pupae, after which they emerge from their aquatic habitat. Resource availability during the larval period has a strong effect on the size that an individual *An. gambiae* achieves upon emergence, but its size is fixed once it has undertaken metamorphosis and become a winged adult. Newly emerged, or teneral, *An. gambiae* typically have low levels of metabolic reserves relative to other mosquito species; Hans Briegel, who has conducted decades of physiological research on a variety of mosquito disease vectors has described this species as “skinny” (Briegel 2003).

Across a range of environmental qualities, or growth conditions, many taxa, including *An. gambiae,* show an L-shaped relationship between age and size at life history transitions (Figure 1.2). The basis for this pattern seems to lie in a trade-off between growth and mortality. Growth under poor conditions occurs slowly and the longer an individual remains in its immature state the greater the probability that it will be killed before it has an opportunity to reproduce. This situation creates selective pressure to reduce the time it takes to develop by maturing at a smaller size. The horizontal part of the L-shape reflects a threshold of minimum size (or possibly energy state) required to have some amount of fitness following the life history transition (Day & Rowe 2002). The theory explaining this age-size pattern has been developed in the context of understanding differences among habitats, and it has not been clear whether this theory is useful for understanding growth and development of individuals exposed to change during their development. In my thesis I have used theory surrounding optimal age and size at maturity, briefly described above, to better understand how developing mosquito larvae respond to a changeable, complex environment. In the chapters that follow I pursue two broad avenues of exploration. Chapters 2 and 3 address effects of variability in food resources encountered during development. Chapters 4, 5, and 6 are concerned with whether multiple environmental variables influence age, size, and other responses independently of one another.

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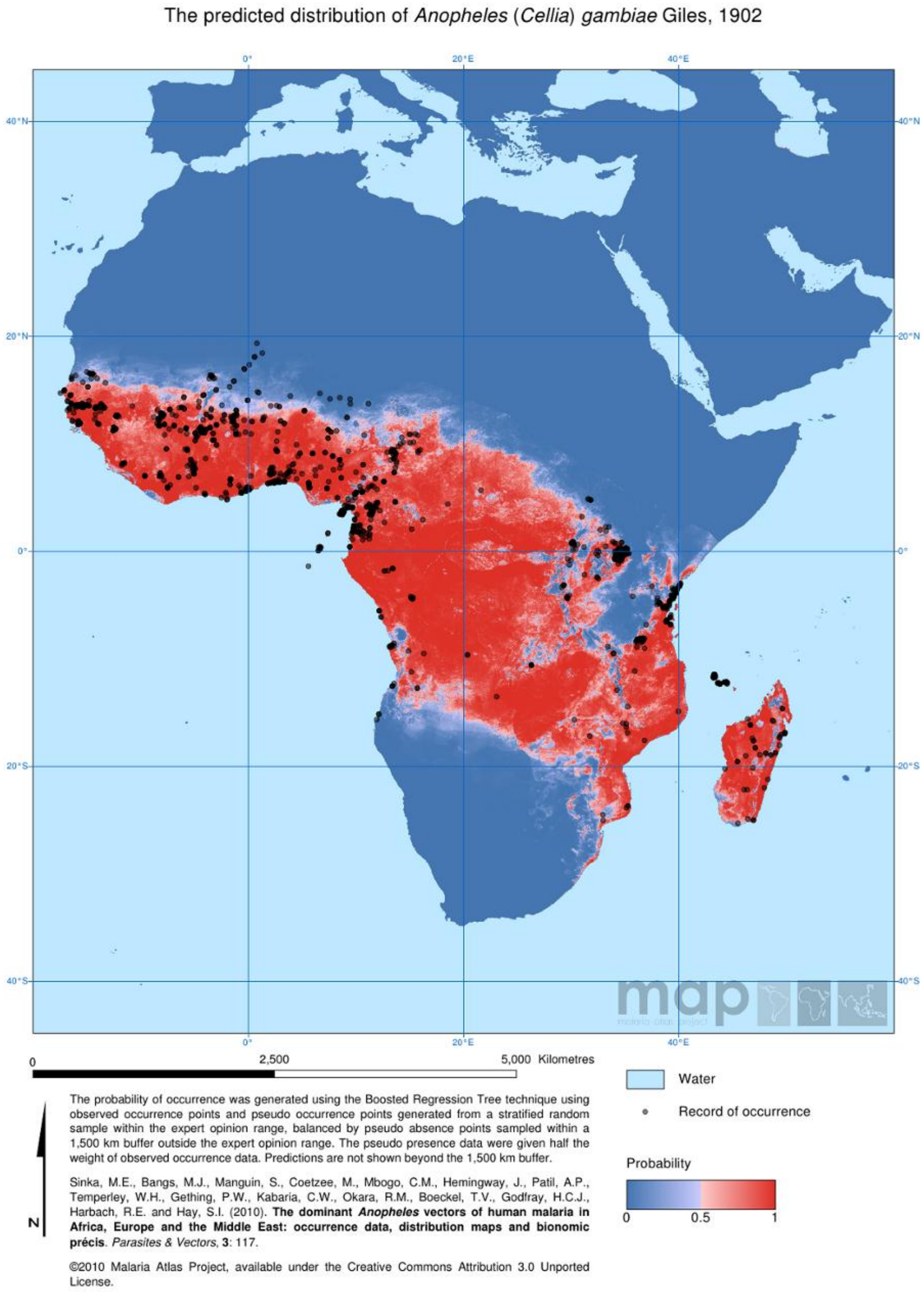

The predicted distribution of *Anopheles* (*Cellia*) *gambiae* Giles, 1902

The probability of occurrence was generated using the Boosted Regression Tree technique using observed occurrence points and pseudo occurrence points generated from a stratified random sample within the expert opinion range, balanced by pseudo absence points sampled within a 1,500 km buffer outside the expert opinion range. The pseudo presence data were given half the weight of observed occurrence data. Predictions are not shown beyond the 1,500 km buffer.

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Boeckel, T.V., Godfray, H.C.J., Harbach, R.E. and Hay, S.I. (2010). **The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis**. *Parasites & Vectors*, **3**: 117.

Figure 1.1. Estimated distribution of Anopheles gambiae sensu stricto (Sinka et al. 2010). Reprinted with permission from http://www.map.ox.ac.uk.

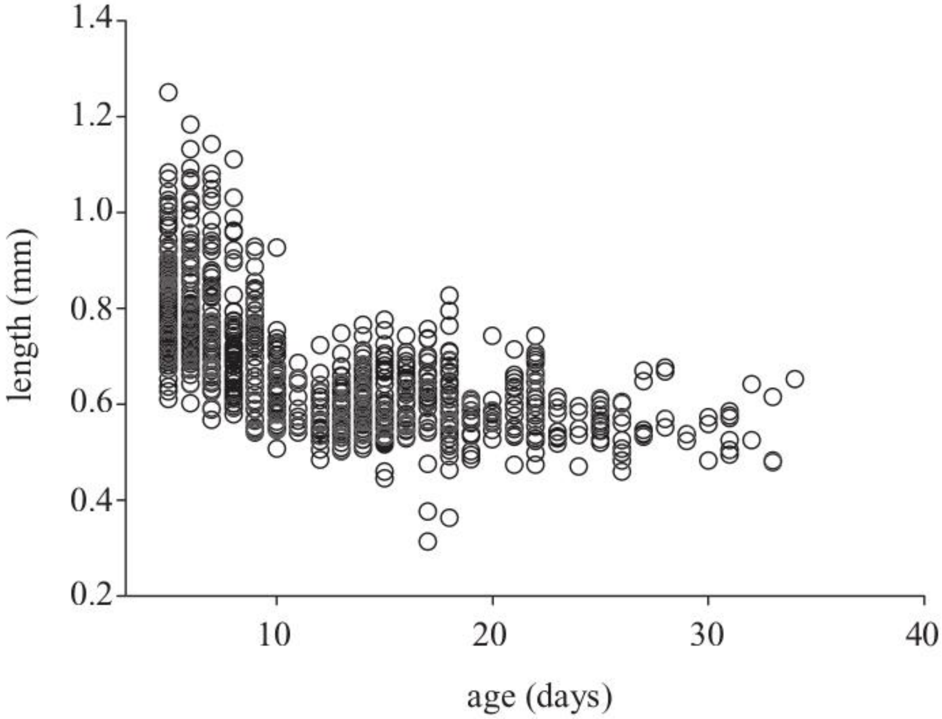

Figure 1.2. Age versus size at maturity in the soil mite Sancassania berlesei (from Plaistow et al. 2004).

These data illustrate a common L-shaped relationship between age and size at maturity resulting from a range of growth conditions. The pattern shown here was generated using three resource levels. Points reflecting early age and large size reflect the best growth conditions while those for late age and small size reflect the poorest.

Connecting statement

The following chapter examines a formal model of optimal age and size at life history transitions developed by Day and Rowe (2002). A computationally intensive technique called dynamic state variable modelling is used to relax the unrealistic assumption that environmental quality is constant and assess the effects of this assumption for model predictions. This effort is concerned with general theory and is not specific to *Anopheles gambiae,* or even mosquitoes per se.

Chapter 2

Dynamic growth and patterns of age and size at life history transitions: the roles of metabolic reserves and submaximal growth

Abstract

A negative, L-shaped relationship between age and size at maturity across a range of growth conditions exists for many species that undergo life history transitions, such as metamorphosis. Day and Rowe (2002) proposed a “developmental thresholds” model to explain this pattern, which assumes constant growth for a given environment. We use a dynamic state variable model to explore the consequences of flexible growth for age and size at the life history transition. To provide ecological context for flexible growth, we consider a metabolic growth trade-off in a fluctuating environment. This exercise yielded a shallower age-size curve and produced a small delay of the life-history transition due to risk sensitivity. These effects were not dramatic, however, and it appears that resource variability and size-dependent metabolic maintenance costs have little qualitative effect on the predicted age-size pattern. Additionally, we found that under our energy-explicit growth the capacity to grow submaximally 1) allowed accumulation of reserves that reduced starvation risk and 2) increased efficiency of resource use under some conditions.

Introduction

Many species with life histories that include important transitions such as metamorphosis or a major habitat shift exhibit a large degree of plasticity in both age and body size at the event. And for many such species, as the quality of the pre-transition growing environment increases, development time decreases and body size at transition increases such that age and size follow an L-shaped pattern (Figure 2.1).

Amphibians represent one of the best-studied examples of this relationship (Wilbur and Collins 1973; Morey and Reznick 2004), although it has been well-documented in other groups including insects (Carpenter 1984; Gebhardt and Stearns 1988; Gimnig et al. 2002), mites (Plaistow et al. 2004), crustaceans (Twombly 1996), fish (Reznick 1990), and birds (Ydenberg 1989).

Growth takes time, thus a trade-off between size and time must exist. So why does the aforementioned prevalent pattern of development appear to not reflect one? Wilbur and Collins (1973) considered this question for amphibians and proposed a minimum size or energy requirement for metamorphosis as an explanation: the better the growing conditions an individual experiences, the sooner it will reach the minimum size or energy level required to leave its nursery habitat. However, good conditions would also cause the individual to remain substantially beyond the threshold in order to take advantage of those conditions and become large. Conversely, individuals that encounter poor conditions would grow slowly and effectively be trapped in their immature habitat and developmental stage until they achieve their transition threshold. Day and Rowe (2002) mathematically formalized and expanded this model to a more general context, providing criteria for optimal age and size at life history transitions that predict the negative age-size pattern. They showed that Wilbur and Collins's explanation works if 1) the optimal point for transition is defined as where incremental fitness gain from staying and growing equals the constant rate of mortality and 2) fitness is considered proportional only to body size above the threshold size (i.e., size accumulated before the threshold doesn't contribute to fitness after it's reached).

However Day and Rowe point out that “one of the primary limitations” of their model is the assumption that growth is a function fixed by the environment. Many organisms flexibly adjust growth rate and timing of developmental events in the face of varying costs and benefits, a classic example being reduced growth in the presence of predators (Anholt and Werner 1995; Fraser and Metcalfe 1997; Peacor 2002; Stoks et al. 2005). We relax the assumption of fixed growth and attempt to evaluate the importance of flexible growth in the context of age and size at the life history transition under Day and Rowe's framework. To make this evaluation, we find it necessary to incorporate size-dependent metabolic maintenance costs into their “developmental thresholds” framework because flexible development is only relevant in light of some state-dependent growth trade-off: without such a trade-off, maximal growth is always optimal. We also allow available energy (i.e., food) to fluctuate because the capacity for flexible growth is only relevant in the presence of environmental variability. Combining this uncertain food supply with a metabolic maintenance cost introduces the possibility of starvation.

We explicitly model usage of variable incoming food energy for growth and maintenance and then use a computational dynamic state variable method (Clark and Mangel 2000) to examine how a capacity for flexible growth influences 1) individual growth and development and 2) emergence patterns across a range of environmental qualities (i.e., food availabilities). For concision we henceforth refer to the life history transition event under discussion simply as emergence, as in the transition of an insect from a larval aquatic habitat and morphology to that of a terrestrial adult. This term is our shorthand for a more general life history event, which may include metamorphosis, or a habitat shift, or both, and where growth either stops or is substantially reduced following the event.

The model

The dynamic state variable (DSV) technique combines stochastic dynamic programming and Monte Carlo simulations, and it is useful for determining optimal behavior where a rate-maximization approach may not yield the optimal solution (Mangel 1989; Clark & Mangel 2000). We use these tools to extend Day and Rowe's (2002) “overhead thresholds” model for optimal age and size at emergence to scenarios in which environmental quality and rate of growth are variable. We begin by describing an energy-explicit growth model that is the basis for our departure from their fixed-growth scenario. We then describe the DSV model that we use to evaluate optimal age and size at emergence across different ecological scenarios.

Growth

We model growth as an allocation of available metabolic energy; some proportion of incoming food energy is converted to size while the rest is either stored or used for metabolic maintenance. For a given time interval t, an individual's reserve of pre-existing energy is ε_{res}, and the amount of food the individual can potentially take in increases with size x. That is, new energy ε_{new} is a decelerating function of x where coefficient g is the proportion of the body used for food collection and absorption (alternately it could be thought of as foraging efficiency), exponent a describes how food acquisition changes with x (where $0 \le a \le 1$), and ν is the density or amount of food in the environment such that

$$\varepsilon_{new} = gx^a \nu \tag{Eq. 2.1}$$

Note however that energy storage capacity is constrained by size x such that $\varepsilon_{max} = x$ and the capacity for energy uptake is $x - \varepsilon_{res}$ Thus the amount of incoming energy that is actually realized ε_{in} is the lesser of ε_{new} and this capacity: $\varepsilon_{in} = \min\{\varepsilon_{new}; x - \varepsilon_{res}\}$. Rather than reflecting the biological reality surrounding uptake and storage capacity of food energy, this constraint limits the rate that energy can be converted to growth: it prevents an individual from unrealistically accumulating enough energy to grow many times its size in a single time interval.

Once food energy is taken in, metabolic maintenance costs ε_{out} are deducted. These costs are a decelerating function of x, with maintenance coefficient m and allometric scaling exponent b ($0 \le b \le 1$) such that

$$\varepsilon_{out} = mx^b \tag{Eq. 2.2}$$

Thus for a given t, an individual's metabolic energy is

$$\varepsilon = \varepsilon_{res} + \varepsilon_{in} \text{-} \varepsilon_{out} .. \tag{Eq. 2.3}$$

If this energy balance falls below the critical level ε_{crit} the individual dies of starvation, a source of mortality absent from the Day-Rowe model. If the individual does not starve then proportion ψ of any remaining energy is allocated to growth and converted into size at efficiency c. (The value of ψ is determined by our dynamic programming algorithm below) Thus, our allocation of energy to growth changes ε such that

$$\varepsilon' = \varepsilon - \psi\varepsilon \tag{Eq. 2.4}$$

and x such that

$$x' = x + c\psi\varepsilon. \quad \text{(Eq. 2.5)}$$

Any energy that remains following allocation to growth serves as a reserve for future growth or maintenance, but notice that because $\varepsilon_{in} \leq x - \varepsilon_{res}$ maintaining such a reserve may be at the expense of energy uptake.

Optimal growth and development

We use a dynamic programming algorithm to assess 1) optimal allocation of energy to growth through time and 2) optimal age and size at emergence. Identically to Day and Rowe, we assume that expected lifetime reproductive output W is proportional to size at emergence. Using this as our ultimate fitness measure, backward induction is used to determine the developmental option that maximizes expected fitness. That is, moving backward in time, how much an individual should grow or whether it should emerge is calculated by maximizing expected fitness for all combinations of size, energy, and time states that an individual can occupy (Clark & Mangel 2000). To implement this process we must assign fitness at terminal time T. In our case $F(x,\varepsilon,T)=0$ and fitness can only be realized by emerging prior to T. If we allow T to be large (i.e., assume an infinite time horizon), after multiple iterations the solutions across t converge and fitness and decisions come to depend only on size and energy state (Mangel 1989), which results in the fitness function

$$F(x,\varepsilon) = \max\left\{\begin{matrix} (1-\beta)^{\theta}F\left(x - x_h, \varepsilon - \theta m x^b\right); \\ (1-\beta)\max\left\{\sum_{k=0}^{K}\Pr\{v\}F(x',\varepsilon')\right\} \end{matrix}\right\} \quad \text{(Eq. 2.6)}$$

The upper term on the right-hand side of this equation is the expected fitness for undertaking emergence, which corresponds to expected lifetime reproductive output W. β is our mortality constant, and θ is the time required for emergence. From Day and Rowe's model, x_h is the developmental threshold, or body size above which fitness can accumulate. Thus $W \propto x - x_h$ when $x \geq x_h$, and $W = 0$ if $x < x_h$ or if metabolic energy upon emergence, $\varepsilon - \theta m x^b$, is less than zero. The bottom right hand term is the expected fitness for staying in the larval environment. $\Pr\{v\}$ is the probability of encountering food amount k given food density v. To get these probabilities we assume v follows a negative binomial distribution, which is unimodal, describes discrete values, and has a mean μ and variance σ where $\mu < \sigma$ (Hilborn & Mangel 1997). We assume environments of different food availability differ from one another by their means but not variances. $F(x', \varepsilon')$ is the expected future fitness from changes in x and ε described above by Eq. 2.4 and Eq. 2.5.

Methods

Our model solves for optimal energy allocation to growth for different combinations of age and size and then describes growth and development of an individual given those best "decisions". Introducing a stochastic environment generates variable predictions of final age and size across individuals. To compare our findings to those of Day and Rowe we solve for optimal allocation and emergence under a stochastic scenario, but for our subsequent growth simulations we fix food at the mean environmental value μ. This produces a "mean" age and size at emergence for each given environment that we can compare qualitatively to their reaction norms. These comparisons are made using a wide range of parameter values and the following results are generally applicable unless specified otherwise.

Day and Rowe considered growth as simple linear or power functions. To compare our results with theirs we initially adjust our parameters to achieve growth identical to their scenarios such that 1) k is constant, 2) $\psi = 1$, and 3) $m = 0$. We create a "perfect" analogue of their model by forcing maximal allocation of energy to growth at each t, thereby bypassing our dynamic programing algorithm. The results are consistent with theirs and thus confirm that our model is analogous: across a range of environmental qualities, age and size at emergence occurs in the L-shaped, negative pattern, and the transition between a vertical orientation for high quality environments to horizontal for low quality environments becomes less abrupt as the developmental threshold increases (see Day & Rowe 2002).

Next we create an "imperfect" Day-Rowe analogue by allowing the dynamic program to solve for optimal growth: growth is now potentially flexible. Under the parameter conditions described above this new version generates the same results as our "perfect" analogue because we have not yet introduced a trade-off for growth. This step ensures that any effects of other changes we make are not simply artifacts of our dynamic programming technique. Finally, we introduce metabolic costs and environmental variability (i.e., let $m > 0$ and k vary) and assess the effects of these changes against the null results of the preceding Day-Rowe analogue.

Results

Maintenance costs

Addition of a size-dependent metabolic maintenance cost introduces a growth trade-off. Potential fitness benefits and increased energy intake from being larger are offset by increased maintenance costs. (Note however that there is still no risk of starvation because variable food is not yet introduced.) If $a \geq b$ growth occurs maximally. If $a = b$ growth is linear. If $a < b$ the maintenance cost results in submaximal growth during later development in low quality environments (Figure 2.2). This last effect results from energetic savings of staying smaller while storing energy for future growth.

A variable world

If we allow food to vary stochastically without maintenance costs we find that emergence shifts to a later time and larger size than under constant food. This happens such that in good environments emergence occurs at a substantially larger size and only marginally later, while for poor environments emergence occurs substantially later and at only a marginally larger size (Figure 2.3A). Further introducing a maintenance cost, and consequentially starvation risk, to this stochastic scenario has little effect on the overall pattern of emergence (Figure 2.3B) but causes growth to occur submaximally (Figure 2.2). The amount that growth differs from maximum increases as resource levels decrease, until growth again approaches maximum at the very lowest levels.

Discussion

When we allow growth to be flexible and food supply to be variable the emergence pattern shifts toward a later age and larger size (Figure 2.2B) as a result of a Jensen's inequality, or risk sensitivity (Caraco 1980; Real 1980). That is, the expected fitness benefit for remaining in a variable growing environment is greater than for a constant environment with the same mean food availability because the fitness returns for food amounts that an individual might encounter are non-linear (i.e., concave upward because growth itself is non-linear). The developing individual is risk-prone because the relatively large payoff for getting lots of food by chance makes it worth postponing the life history transition. This effect becomes larger as variance in food availability increases, but even at high variances it does not appreciably affect the shape of the overall emergence pattern (Figure 2.2B) and is probably not ecologically relevant given typical levels of natural variation in age and size at emergence (Figure 2.1). Optimal age and size depend primarily upon environmental quality, and it seems that allowing the environment to randomly fluctuate around a mean through time ultimately has little effect on the total amount of resources available for growth.

Given that mortality risk drives the emergence pattern of the Day-Rowe model, we were surprised to find that the introduction of starvation risk had little overall effect (see *A variable world*). The reasons for this apparent lack of effect are 1) the risk is only relevant in poor quality environments where starvation would have a substantial probability of occurring, 2) the capacity to grow flexibly allows the individual to maintain an energy reserve that reduces starvation risk, and 3) under some parameter values slower growth actually reduces size-dependent maintenance costs, mitigating both nutritional opportunity costs of being small as well as mortality risk from remaining in the rearing environment (Figure 2.2).

To elaborate this last point, when the allometric energy uptake exponent a is less than cost exponent b in poor environments (see Eq. 2.1 and Eq. 2.2), the presence of a maintenance cost 1) causes growth to occur submaximally and 2) may cause emergence to occur either earlier or later than under a deterministic, fixed-growth scenario (Figure 2.2, Figure 2.3B). The reason for this lies in our energy-explicit growth model (Eq. 2.3). When $a < b$, metabolic costs can increase more than energy uptake as size increases (i.e., $\frac{d\varepsilon}{dx} < 0$). This condition is met beyond size

$$x_{sub} = \left(\frac{agv}{bm}\right)^{\frac{1}{b-a}} \qquad \text{(Eq. 2.7)}$$

after which postponing growth provides benefit by foregoing the relatively high increase in cost of being larger at the expense of a relatively small increase in energy uptake.

(Although stopping growth altogether is not favored because the amount of energy that can be accumulated in storage is constrained by size. See *Growth*.)

The capacity to grow submaximally to lower costs and store energy for future growth largely removes the risk of starvation under our energetic growth model. Starvation is a ubiquitous source of mortality to which all organisms are susceptible and the idea that submaximal growth could manage starvation risk without compromising fitness bears scrutiny. The fact that organisms grow submaximally to manage risk is well established and supported in the context of predation risk associated with foraging activities. However it is somewhat less studied in the context of starvation risk due to variable food availability. (Although Tenhumberg et al. (2000) predicted a relationship between variability in food and mortality due to starvation, they didn't consider how it would influence growth or the overall pattern of age and size at emergence.) Many researchers assume that maximal growth is optimal because organisms should grow as fast as they can in the absence of any growth trade-offs. However, the presence of such trade-offs is likely the norm, thus growth may best be understood in their light. Mangel and Stamps (2001) used a life history model to show how a variety of growth-mortality trade-offs could cause nearly equal fitness across a range of growth rates. And contrary to initial expectations, many experimental studies on compensatory growth show little or no fitness consequence of accelerated growth following a period of retarded growth (Metcalfe and Monaghan 2001; Yearsley et al. 2004; Dmitriew and Rowe 2007) — our model illustrates a possible explanation: early, slow growth has benefits that offset later costs of fast growth.

Our treatment of growth as an allocation of energy to size increase could also be used to explore ecological factors other than size that are relevant to energy budgets. Size and energy tend to be strongly correlated because both are consequences of environmental quality during development, and many studies show a relationship between size and traits that contribute to fitness such as survival and fecundity (Roff 2002). However, size per se is not necessarily the factor that confers the benefit. For example it could be superior immune function or greater energy reserves held over from the nursery habitat. In this work, we use food energy dynamics to explore the consequences of a variable environment because food availability has a conspicuous relationship with metabolic energy and growth and because we expect that most types of organisms experience variation in food supply during development. However, other factors affecting energy uptake and usage (e.g., temperature, parasite load, predation risk) could be similarly used to explore a variety of ecologically relevant consequences of growth and development (e.g., survival, fecundity, immune function).

The assumption that energy uptake exponent a can be substantially less than cost exponent b (see Eq. 1 and Eq. 2) such that submaximal growth would be beneficial may not be realistic. Our growth model incorporates these allometric relationships, which are conventionally used to describe how rates of energy assimilation and metabolism change respectively with size (Reiss 1989). This approach requires more parameters than the equations used by Day and Rowe, potentially decreasing the generality of our model. Values of a and b are highly variable both across and within taxa and even over the course of development (Reiss 1989; Glazier 2005), and how they relate to one and other isn't well documented. If we allow a and b to be equal, size increases linearly, which is not how growth typically occurs. Furthermore, we consider it reasonable that the size an individual can achieve would be limited by energetic constraints in low food environments, which is the outcome of an $a < b$ relationship but not $a \geq b$; indeed, West et al. (2001) propose that, due to the nature of fractal circulatory systems, the size that animals of a given taxa can achieve is limited because metabolic costs always increase with size at a higher rate than does energy assimilation.

DSV modeling has been used extensively to solve for optimal behavior, which has the potential to be extremely labile. But we apply it to the processes of growth and metamorphosis which may not be as flexible as this approach assumes: there must be a limit to how rapidly cellular machinery and integrated developmental processes can start, stop, and change. Flanagin et al. (2000) showed that development in late instar grasshoppers is largely insensitive to increases in food availability, and many organisms that do increase growth rate in response to increases in food availability suffer fitness costs such as lower adult survival or fecundity for doing so (Mangel and Stamps 2001; Metcalfe and Monaghan 2001). If our assumption of dynamic growth is invalid, then 1) starvation risk may be more important than our results suggest here because it may not be possible to adjust growth rapidly enough to mitigate risk, and 2) ages and sizes at emergence that occur in nature may differ substantially from theoretical optima because of developmental constraints (although this point may be somewhat moot because our predictions don't differ substantially from a fixed-growth scenario). Despite our concern that the flexibility of growth is limited, growth rates in nature can be highly discontinuous (most obviously in molting arthropods) and the growth trajectories we predict are relatively smooth, at least up to the point preceding emergence (Figure 2.2).

We assume that growth either stops or is substantially reduced following emergence; but a more general understanding of optimal age and size at emergence also requires consideration of growth-survival trade-offs in subsequent life history stage(s). Growth does not always cease after a life history transition: for example, it is known to increase in alcid birds and salmon (Hansen and Quinn 1998; Ydenberg 1989). In such cases, optimal emergence will depend upon both pre- and post-transition growth and mortality (Werner 1986; Ydenberg et al. 1995). In this larger context, the existence of a life history transition may or may not even be optimal, and if it is, the L-shaped emergence pattern may or may not be favored, depending on how growth and mortality change moving from pre- to post-transition. The “developmental thresholds” model describes age and size at emergence as a plastic response to pre-transition growth conditions; and if pre-transition animals have information on specific post-transition growth conditions and/or mortality risk, conceivably they could also be adapted to adjust the transition event in response to these factors. However, such specific information on post-transition conditions are likely generally unavailable because the transition constitutes a switch to new habitats or resources that the animals have yet to experience.

Finally, an assumption of the larger “developmental thresholds” model is that lifetime reproductive output W is directly proportional to size at emergence, but it is more conventional to assume diminishing fitness returns beyond some size (Mangel and Clark 1988). We don't use this convention to facilitate comparison with the Day-Rowe model. Moreover, we find that the pattern of emergence is far more sensitive to changes in mortality rate than to parameters that directly affect growth (and thus W via x) such that an asymptotic size-fitness relationship has little or no impact on our findings.

Summary

The pattern of emergence for which Day and Rowe offer an explanation is largely unaffected by relaxing the assumption of fixed, environmentally-determined growth and the addition of an energetic growth trade-off. Agreement between their model and our dynamic treatment of it indicates that the criteria they identified as important for optimizing age and size at emergence are useful regardless of whether growth is fixed or flexible. One source of this agreement is an unforseen fitness benefit of slow growth for certain conditions under our energetic growth model. This finding suggests that a trade-off between energy uptake and metabolic costs may be of general importance for understanding animal growth and development. Furthermore, an energy-explicit approach to modeling growth offers a potential tool for better understanding developmental and ecological consequences of environmental factors that affect energy budgets during animal development.

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Figures

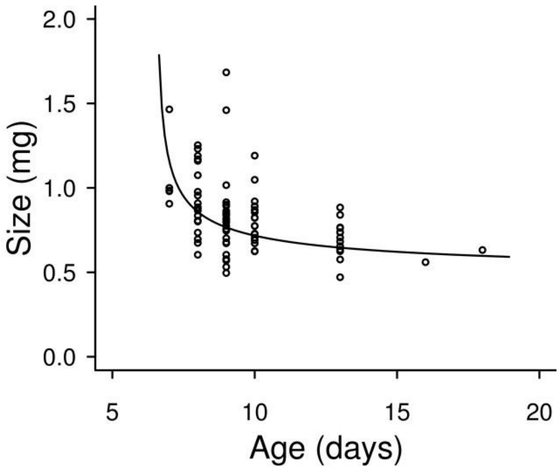

Figure 2.1. Age and size at emergence of lab-reared Anopheles gambiae mosquitoes.

The line illustrates an L-shaped, negative age-size pattern of emergence in which larger individuals achieve life history transition earlier and smaller individuals later. The data themselves illustrate variation in a real system. This common pattern is generated by differences in quality of rearing conditions. Larvae were reared at four different densities. Each container got the same total amount of food per day.

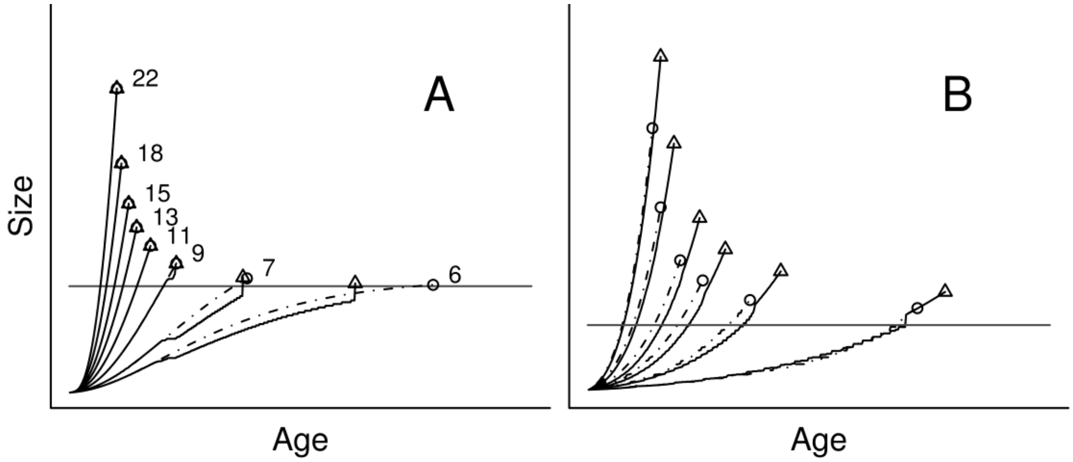

Figure 2.2. Fixed and flexible growth trajectories with emergence events.

Panel A shows fixed and flexible growth trajectories and emergence events under size-dependent metabolic maintenance costs and constant food availability (zero starvation risk). Scenarios with fixed, maximal allocation of food energy to growth are shown as dash-dot lines ending with circles. Scenarios with flexible allocation are overlain in solid black and end in triangles. Numbers show corresponding mean food density ν. For low food environments where $a < b$, submaximal growth during part of development is beneficial because it lowers size-dependent maintenance costs (growth trajectories where ν is 6 and 7). The horizontal grey line is the developmental threshold. $\beta =$ 0:05; θ = 0; $x_h =$ 200; $\varepsilon_{crit} =$ 0; $g =$ 0:1; $a =$ 0:67; $b =$ 1; $m =$ 0:1; $c =$ 0:2. Panel B shows the effect of variable food availability upon growth trajectories and emergence pattern. The dash-dot lines ending in circles show optimal development under the expectation of constant food. The solid lines ending in triangles show optimal development under the expectation of randomly fluctuating food. Growth is potentially flexible for both scenarios. The metabolic benefits of submaximal growth illustrated in panel A (i.e., cost savings) do not apply here because $a = b$ (see *Discussion*). Rather, submaximal growth is optimal because retaining energy that could be used for growth reduces risk of starvation in the face of an uncertain food supply. $\beta =$ 0:05; $\theta =$ 1; $x_h =$ 20; $\varepsilon_{crit} =$ 0; $g =$ 0:05; $a =$ 0:67; b = 0:67; $m =$ 0:1; $c =$ 0:4.

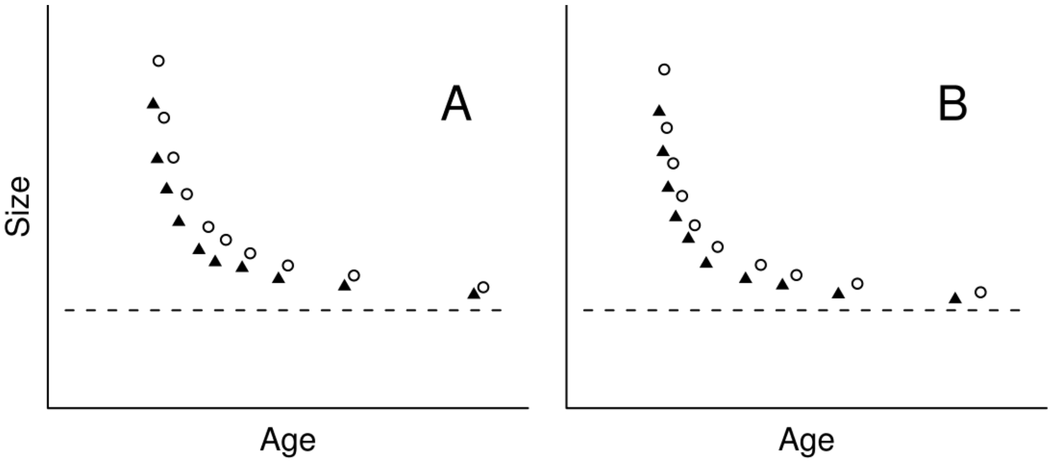

Figure 2.3. ***Optimal age and size at emergence under flexible growth and stochastic food, with and without maintenance costs.***

Panel A, the circles show emergence under a flexible growth and stochastic food scenario where the metabolic maintenance coefficient $m = 0$. The triangles show the corresponding deterministic baseline for comparison. Panel B shows emergence under the same assumptions except that $m = 0.05$. Going from panel A to B, the small increase in distance between the rightmost circles and triangles shows a slight shift in the reaction norm resulting from delayed allocation of resources to mitigate starvation risk. $\beta = 0.05$, $\theta = 1$, $x_h = 20$, $\varepsilon = 0$, $\sigma = 30$, $g = 0.05$, $a = 0.67$, $b = 1$, $c = 0.2$.

Connecting statement

The preceding chapter was concerned with general theoretical implications of variability in resources for optimal age and size at maturity. This next chapter further considers implications of changeable resources by examining how *An. gambiae* adjusts its development in response to changes in food levels over the course of larval development. Results from a rearing experiment in which food availability is manipulated are compared to predictions from theory.

Chapter 3

Environmental variation, plastic development, and adult age and size of *Anopheles gambiae* mosquitoes

Abstract

We reared larvae of the mosquito species *Anopheles gambiae sensu stricto* under upward and downward changes in food availability and observed how these changes affected its age and size at emergence, as well as its survival. We also examined how predictions from a model that had been developed to understand age and size at life history transitions under static resource levels (Day & Rowe 2002) changed in the context of inconstant food, and we further compared these predictions to our experimental results. Our observed ages and sizes at emergence were consistent with a highly flexible developmental program that is adaptive under changing resource levels. Feeding treatments did not affect larval survival. Size at emergence (measured as wing length) corresponded only to the food level that larvae encountered during the latter part of development Age at emergence, however, was influenced by levels encountered earlier in the larval period. Although the model predictions were qualitatively consistent with our experimental results, predicted age at pupation differed substantially from observed. Thus we find that the Day-Rowe model is broadly applicable to within-habitat resource fluctuation, but the model would need to be expanded to address developmental responses to environmental change. We identify and discuss four topics relevant to understanding age and size at life history transitions in response to changing conditions. These are 1) compensatory growth, 2) limits to developmental plasticity, 3) a potential mismatch between supply and demand of metabolic energy, and 4) an evolved strategy for achieving large size in an uncertain environment.

Introduction

For many taxa that undergo metamorphosis, plasticity in age and size at this life history transition follows a distinctive pattern: individuals in good growing conditions undergo transitions earlier and at larger size than those in poor conditions. Across a range of different quality environments the relationship between age and size at metamorphosis often follows an L-shape (Figure 3.1). To explain this relationship for amphibians, Wilbur & Collins (1973) proposed a model of growth and development in which metamorphosis occurs when the fitness benefits from continuing juvenile growth are surpassed by the cost of further delaying metamorphosis and subsequent adult growth and reproduction. Under their framework, good growing conditions will generally favour delaying reproduction to achieve a larger body size, up to a maximum viable size. And poorer conditions will favour initiation of emergence at a smaller size as long as it is greater than some evolved minimum. This model has since been mathematically formalized by Day & Rowe (2002) and offers an explanation for variation in age and size at life history transitions that is applicable to a wide range of taxa (Reznick 1990; Gimnig et al. 2002; Morey & Reznick 2004; Plaistow et al. 2004; for exceptions see Juliano et al. 2004 and Rudolf & Rödel 2006). As with many such models for optimal age and size at life history transitions, Day and Rowe's model is based on a trade-off between growth and mortality (Kozlowski 1992).

Day and Rowe’s growth-mortality model is attractive because it offers a relatively simple, biologically relevant explanation for response to variation among environments. However, it is unclear how well the growth-mortality framework applies to within-habitat variation encountered during development. Growing animals often respond to an increase or decrease in food availability with a corresponding increase or decrease in rate of growth, and they may also adjust their rate of development (i.e. age at the life history transition) in a way that is consistent with the Wilbur-Collins model described above (Hentschel 1999). Such plastic responses to environmental change is ubiquitous. Because the growth-mortality trade-off grounds predictions in the fundamental process of natural selection, it seems reasonable that Day and Rowe’s model would apply to understanding development under environmental change. However, within-habitat variation introduces biological considerations not addressed by their model (e.g. limits to how quickly complex, integrated developmental programs can be adjusted, seasonal time constraints on the developmental period, etc.). Thus there is also large potential for dynamics it does not account for.

Anopheles gambiae sensu stricto provides a good system for studying developmental plasticity. Because this species is the major vector for malaria, a significant amount is known about its larval growth, development, and ecology (Briegel 2003; Fillinger et al. 2009). Like many other mosquitoes, this species exhibits plastic age and size at emergence. Increasing larval food availability among separate larval populations can produce more than a 250% increase in adult body mass and more than a 50% reduction in development time (Gimnig et al. 2002; unpublished data). *An. gambiae's* plastic development is appears to be adaptive for among-habitat variation in resource availability (Gimnig et al. 2002; DeWitt & Scheiner 2004). Differences in the mean quality of separate nursery habitats have a strong effect upon age and size at emergence that are consistent with predictions under the Wilbur-Collins framework described above (Gimnig et al. 2002): constant high food availability results in early emergence at a large size, emergence under constant low food conditions result in late emergence at small size. However, resource levels and larval density of natural habitats vary substantially, not only from one habitat to the next but within habitats through time (unpublished data), and the degree to which *An. gambiae's* developmental plasticity is an adaptation for dealing with within-habitat variation is unclear.

We conducted a rearing experiment to assess changes in age and size at pupation and survival of *An. gambiae* to within-habitat variability in food level during larval development. We then compared these developmental responses to optima predicted using the Day-Rowe model (2002). We had two goals: 1) to characterize *An. gambiae's* responses to environmental changes encountered during development, and 2) to evaluate the Day-Rowe model's usefulness for predicting such responses.

Methods

Rearing experiment

We reared *An. gambiae* larvae under different feeding regimes to test the effect of changes in food availability during development on age and size at pupation. The experiment was conducted in large greenhouse-style buildings with glass roofs and screened (mesh) walls at the International Centre for Insect Physiology and Ecology (ICIPE) in Mbita, Kenya (0°30' S; 34°15' E).

For two days prior to start of treatments, a small amount of liquid fish food was added to the water of a common culture of newly hatched *An. gambiae* larvae. Larvae were reared in small plastic tubs approximately 10 cm in diameter, 6 cm in height. Approximately 80 ml of filtered water (1.5 cm deep) was maintained in them during the experiment. Our unit of replication was tub, each of which were allocated 10 larvae. On April 15, 2006, 10 larvae were transferred from a common culture to each tub and feeding treatments began. From this point onward larvae were fed ground dry fish food flakes (Nutrafin basix staple for tropical fish, Hagen Inc).

Food treatments were either constant daily food throughout larval development or an amount that switched from one level to another. We use L, M, or H to indicate low, medium, or high food levels, which consisted respectively of 1, 2, and 3 mg of food per tub per day. We use a hyphenated notation for our food treatments such that L-H indicates a switch from low to high daily food during the experiment. Furthermore, each switching treatment was conducted either early (April 18 — day four of treatments) or late (April 20 — day six), to give four switching treatments (L-He, L-Hl, H-Le, H-Ll). For consistency we also use a hyphen to indicate constant food levels (controls) as L-L, M-M, or H-H (Table 3.1.). Altogether there were seven treatments and each treatment was replicated 10 times, resulting in 70 tubs. The experiment ran until all larvae had emerged or died, some 16 days later.

After five days of treatment the experimental tubs were checked for pupae every six hours. Pupae were removed by pipette and allowed to emerge in separate containers. Time of pupation was recorded as the start time of the six hour period in which the pupae were discovered. All mosquitoes that survived pupation eclosed before the end of the next day. The resulting adult mosquitoes were killed by freezing, sexed, and wing lengths were measured under a microscope. Mosquito wing length corresponds to adult body size and is commonly used as a size index (Nasci, 1990). Time to pupation is reported as days since April 13 (hatching day), 00:00 hrs. Note that since we did not directly observe time of eclosion we used time to pupation in our analyses even though wing length properly corresponds to age at eclosion (about 24 hours later). This discrepancy does not affect our findings because the duration of the non-feeding pupal stage is constant and would not change the statistical relationships between treatments and responses.

A StowAway TidBit temperature data logger (Onset Computer Corporation) recorded the temperature of a single tub every five minutes during the experiment. The mean water temperature was 23.51± 0.12°C (95% CI), the maximum was 35.53°C and the minimum was 19.39°C.

Statistical analysis

Statistical tests were conducted using R language and environment for statistical computing (R-Development-Core-Team 2012). Tub was our unit of replication and measurements of adult mosquitoes from within tubs were combined to give values for each tub. Females and males were analysed separately because the sexes differ in importance for disease transmission and demographic processes (Steven Juliano, pers. comm.). Thus days to pupation and wing length were analysed separately for each sex. We used linear models to determine the effects of feeding treatments on the two response variables age at pupation and wing length. Counts of larvae that survived through metamorphosis were included as covariates in these analyses. The data for days to pupation did not meet the assumption of equal variances among treatment groups and the generalized least squares method was to address this problem (Zuur et al. 2009). Each tub of ten individuals yielded some percentage of survivors, and survival data were analyzed using a generalized linear model with a binomial error distribution (Crawley 2007). Outcomes of statistical tests were evaluated using a critical α value of 0.05.

Determining model predictions

We used Day & Rowe's (2002) model of optimal age and size at life history transitions to predict emergence of *An. gambiae* across resource levels and then visually compared these theoretical optima to our experimental results. We began this process by reproducing a set of predictions that Day and Rowe used for purposes of model illustration (Figure 3.1.). We then generated predictions of within-habitat variation for our experimental system using parameter values that were either sensible estimates or derived from a subset of our results. We chose values for the constants initial body size w_i (i.e. size following hatching), minimum size to undergo the transition w_c, and shape parameter b for the power growth function by constraining these values to biologically reasonable ranges and systematically exploring the consequences of changing each value across its range. Changing these values did not qualitatively affect our results, so we used values from the middle of each range for the analysis we present here. We estimated three values for k (the parameter for environmental quality, here represented as food level) from our experimental data by forcing three separate growth curves through an observed mean age and size at emergence from the invariant L-L, M-M, and H-H treatments (see Figure 3.2 for parameter values). We then used the Day-Rowe optimality criteria to solve for the prereproductive mortality constant μ from each resulting control growth curve and its observed age at pupation. These estimates of μ were subsequently used to calculate optimal age and size at pupation under switches in resource abundance. Note that even though the Day-Rowe model assumes constant mortality across environments we used mortality estimates specific to a treatment's rate of growth (or k) to solve for optimal age and size; these disparate estimates yielded predictions more consistent with our results (see *Model predictions* below). Mathematical solutions were found using Mathematica (version 6, Wolfram Research).

Results

The rearing experiment

The generalized least squares linear models found that feeding treatment had a significant effect upon days to pupation for both females and males (females $F_{6,61} = 7.34$, $p < 0.001$; males $F_{6,62} = 8.59$, $p < 0.001$). Total number of tub survivors was non-significant for females ($F_{1,61} = 2.92$, $p < 0.093$) and significant for males (F1,62 = 28.14, $p < 0.001$) such that a greater number of survivors corresponded with later pupation.

Feeding treatment had a significant effect upon wing length for both sexes (females $F_{6,61} = 34.72$, $p < 0.00$; males $F_{6,62} = 30.75$, $p < 0.001$). The term number of survivors per tub was significant for the linear models of both sexes (females $F_{6,61} = 13.31$, $p < 0.00$; males $F_{6,62} = 12.46$, $p < 0.001$); for both sexes more survivors in a tub corresponded with shorter wing length.

Mean ages at pupation and mean wing lengths with 95% confidence intervals are listed in Table 3.1. Bivariate plots of age and size with univariate 95% confidence intervals for each sex and treatment are shown in Figure 3.3. Bivariate means with the sexes pooled are plotted in Figure 3.2.

We did not detect an effect of feeding treatment upon the number of larvae that survived to pupate in each tub. The model of survival as a function of treatment was not significantly different from a null model with no coefficients ($p = 0.21$).

Model predictions

Observed mean ages and sizes at emergence for the three constant feeding treatments were broadly consistent with pattern that was predicted using the Day-Rowe model; age and size at emergence follow an L-shape across the different food levels (Figure 3.1, Figure 3.2, Figure 3.1). Using data from these three constant feeding treatment groups we calculated three separate mortality estimates for μ. Estimates for L-L, M-M, and H-H were 0.99, 0.60, 0.48 respectively (note that these estimates do not reflect survival observed from our experiment, rather they represent what larvae have evolved to “expect” in nature for a given food abundance). Using a mean of these three values for a single estimate of μ produced predictions that were inconsistent with our results — predicted sizes at pupation were all intermediate to the observed sizes of our L-L and H-H groups. So when food was at the low level in the food-switching treatments we used the estimate from L-L, and when it was high we used the estimate from H-H (see *Discussion*). This approach produced size predictions that were much more consistent with results. However, mean experimental ages for treatments in which food levels changed departed substantially from model predictions (Figure 3.2). The model predicted later emergence than was observed for both the treatments where food availability switched from low to high (L-He and L-Hl). Conversely, it predicted that emergence would occur much earlier than was observed for high-to-low treatments (H-Le and H-Ll). These discrepancies were consistent across the range of parameter values reasonable for this system.

Discussion

An. gambiae showed plastic development in response to our experimental manipulations of food availability during the larval phase, and this plasticity appears to be an adaptation for dealing with changes in food level during growth (Figure 3.2 and Figure 3.3). In our food-switching treatments, size at pupation appears to have been determined by the level of food that larvae encountered during the latter portion of development. That is, size at pupation always matched that of either the high or low constant food treatments; if the treatment ended with high food the mosquitoes emerged large and if it ended with low food they were small. The effect of the feeding treatments upon age at pupation was less straight forward. Pupation was delayed for L-H treatments relative to H-H, and hastened for H-L treatments relative to L-L (explanations of treatment abbreviations in Table 3.1). Relative to the H-H group the magnitude of the delay in pupation for both the early and late L-H food switches was relatively small. In contrast, both of the H-L switches caused age at pupation to occur substantially earlier than that of the L-L treatment. We detected no differences in physiological survival among the treatments and these results are consistent with adaptive developmental plasticity for handling within-habitat changes in food availability. Fitness costs from delayed pupation in the L-H treatments were likely minimized because the delay in pupation was so small, and earlier pupation of both H-L treatments relative to L-L suggested a benefit from high food early in development.

Bradshaw and Johnson (1995) used an experimental design similar to ours to examine the effects of variable resources for growth and development of the pitcher-plant mosquito *Wyeomyia smithii*. Their results differed from ours in a potentially important way: their treatments didn’t simply produce values for body size at metamorphosis (which they measured as pupal mass) that reflected whichever food level the larvae experienced during the latter portion of development (see previous paragraph). Rather the effect of their switches in food levels produced outcomes that differed from their invariate food treatments with respect to *both* age and size at pupation. This discrepancy between the two sets of results may reflect an important difference between the two species and their ecological contexts; there may be differential adaptation for dealing with environmental variability. *An. gambiae* typically inhabits shallow, often temporary habitats perhaps best described as puddles (Ndenga et al 2011). In contrast, the duration of the larval habitat and food supply encountered by *W. smithii* are relatively reliable characteristics of its pitcher-plant host (Bradshaw & Johnson 1995). Since there were several major methodological differences between our study and Bradshaw and Johnson's it is important to note that the difference in development we see between *W. smithii* and *An. gambiae* may only be apparent; our replicates were tubs containing 10 larvae while theirs were individually-reared larvae, our treatment levels were fixed daily food amounts while their food levels were adjusted by instar, we measured adult wing length while they used pupal mass, the larval duration for *W. smithii* was on the order of 20 to 45 days while for *An gambiae* it was 9 to 12 days, etc.).

Bradshaw and Johnson discussed their results in the context of developmental models originally generated for understanding amphibian systems. Two broad developmental strategies have been proposed to explain observed patterns of age and size at metamorphosis. These are 1) flexible development that generally produces body sizes that are optimal under current growth conditions (Wilbur & Collins 1973) and 2) an inflexible rate of development that has been established by growth conditions early in the larval period (Travis 1984). These can be thought of respectively as “flexible-size” and “fixed-rate” strategies, and they are not mutually exclusive since it is possible for developmental outcomes to be informed by both past and current conditions. Such a hybrid strategy appears to have been the case for Bradshaw and Johnson's experiment; *W. smithii* exhibited some degree of “developmental intertia” such that size at emergence was partially influenced by early larval conditions via developmental rate. In our experiment, early growth conditions did not influence final size *An. gambie*, suggesting that this species' development is highly flexible. The age-size relationships produced by our L-H treatments indicate that developmental rate was not fixed by growth conditions encountered early in the larval period: emergence for both the early and late L-H treatment groups occurred substantially earlier than under the L-L group (Figure 3.2 and Figure 3.3). Similarly, both the H-L groups appear to have emerged later than the H-H group. However, it is important to consider that these apparent discrepancies with the fixed-rate strategy may actually reflect a minimum and maximum viable body size. Fixed maximum and a minimum sizes for metamorphosis appear to be universal in such systems (Bradshaw & Johnson 1995), and our results do not suggest an exception. It is possible that our observed ages of pupation only appear to be inconsistent with the fixed-rate strategy because they reflect instances of special initiation of metamorphosis at upper and lower size boundaries. In retrospect switching treatments that included the medium food level would have provided useful insight into primacy of the flexible-size and fixed-rate strategies in this system.

The pattern of age and size we observed in *An. gambiae* were broadly consistent with predictions from Day and Rowe's (2002) model across a range of continuous food levels. Our three constant food levels (L-L, M-M, and H-H) produced an L-shaped pattern across these levels. Data from these constant food treatments were used to parameterize the model and predict responses to food-switching treatments. These predictions qualitatively matched our experimental results if we assumed that food level determined mortality risk: both early and late L-H treatments yielded the same size as the H-H control but emerged later, and both of the H-L treatments yielded the same size as the L-L control but emerged earlier.

However, several differences between the model's predictions and our results suggest that the model does not fully explain *An. gambiae's* responses to changes in food level (Figure 3.2). The model predicted substantially greater differences than were observed between ages at pupation for the constant feeding treatments and all four switching-treatments. Moreover the two pairs of observed L-H and H-L treatment responses did not differ from predictions in the same way: age at pupation for both of the L-H treatment groups was nearly the same as the H-H group, but the H-L pupations were spread apart in time, both from the L-L group and each other. Such discrepancies are unsurprising because the model's original purpose was qualitative explanation rather than quantitative prediction (Locke Rowe, pers. comm.). Below we discuss several possible, biologically relevant explanations for the departures from prediction we have observed.

The small observed differences in age between both of our L-H food treatments and our H-H control may be caused by growth compensation. Compensatory growth is a game of catch-up in which many animals exhibit a higher-than-usual rate of growth following a period of low growth. This phenomenon is common and has been documented for a wide range of taxa (Mangel & Munch 2005). Theory suggests that such relatively high rates of growth must incur delayed fitness costs but evidence for such costs has been equivocal (Metcalfe & Monaghan 2001; Mangel & Munch 2005; Dmitriew & Rowe 2007). In the context of *An. gambiae*, selection might favour increased larval growth following a delay because 1) earlier emergence may improve access to mates for males (Holzapfel & Bradshaw 2002) and reduce risk of habitat dessication prior to emergence (Juliano & Stoffregen 1994), and 2) emergence at a larger size may improve fecundity for females (Lyimo & Takken 1993) and access to mates for males (Yuval et al. 1993).

In contrast, the large discrepancy between predicted and observed time of pupation for our H-L treatments may represent a mechanistic limit to *An. gambiae's* ability to adjust to its changed environment. Although the fitness costs of maintaining plasticity are thought to be generally low (DeWitt & Scheiner 2004), there are limits to how fast and at what point an organism can adjust its developmental program (Marczak & Richardson 2008). In mosquitoes and other insects, metamorphosis is controlled by a hormonal cascade, which once initiated must run its course and during which growth may or may not occur (Bradshaw & Johnson 1995). Under a flexible-size strategy, this cascade would likely be initiated just prior to metamorphosis. Under a fixed-age strategy, it would likely be initiated earlier in larval development. We included early and late switches in our treatments in an attempt to detect such a transition and better assess limitations on developmental flexibility in *An. gambiae*. Within each of our two experimental directions in food switches, L-H and H-L, our results from the early and late switches did not differ from one another qualitatively. This suggests that any transition between flexible and inflexible development occurred either before our early switches in food level or after our late switches. The outcomes of our food switching treatments were consistent with a flexible-size scenario of development, in which case we expect such a transition to be after our switches. However, as discussed above, it is also possible that our results reflect a fixed-age strategy in which developmental rate is specially adjusted to accommodate minimum and maximum limits for body size. Hentschel (1999) developed a graphical model to predict the point in development where age at life history transitions becomes fixed but our experiment does not provide an explicit test for his model (however, see Howard & Hentschel 2005), Another possible explanation for differences between predicted and observed ages for pupation of H-L treatments could lie in energy budgets. As size increases, so does the metabolic cost of maintaining somatic tissue. Thus, when food switched from high to low, the developing larvae may already have grown bigger than the new environment could easily support. A resulting energetic shortfall may have produced the apparent delay in pupation. However, such a delay would represent a relatively low cost compared to death by starvation (Marczak & Richardson 2008).

Yet another related, possible reason for the later-than-predicted pupation of the H-L treatment groups is that the Day-Rowe model does not account for uncertainty in food availability. The optimality criteria "expects" the larvae to know that once our experimental treatments have switched to the lower food availability that they will not switch back. Clearly this is an invalid assumption if the developmental plasticity we have observed represents an adaptation for fluctuating food. Thus we might expect small individuals under low food (but that had encountered high food in the past) to hold out against the possibility of conditions improving — that is, the fitness benefits of becoming large could favour risk-prone development. This line of reasoning might also be applied to understanding compensatory growth; information about previous low food availability might favour raising growth rates to levels that causes cellular damage (Mangel & Munch 2005) in order to take advantage of ephemeral high food.

A more subtle but potentially informative discrepancy between the model and our findings involves mortality rates. Day and Rowe assumed constant mortality μ across environments. However, we found three substantially different estimates when we solved for μ using their optimality criteria and our observed ages and sizes at emergence for each of the L-L, M-M, and H-H treatments ($\mu = 0.95, 0.60, 0.48$ respectively). These disparate estimates could result from different "perceived" starvation risks among the three food levels. We did not detect differences in survival among our experimental food treatments (nor did Gimnig et al. 2002) but we would not necessarily expect to see one if plastic development is a strategy for managing starvation risk — plastic development compensates for the greater risk. Thus μ might be thought of as the risk of death, including that from starvation, that *would* be experienced without an adjustment in growth rate. Such a definition seems tenuous, however, and understanding age and size at life history transitions in response to variation encountered over the course of an individual's development may require an improved accounting of risk.

Conclusions

Our findings reveal that development of *An. gambiae* is highly flexible in the face of changing resource availability. Under our experimental treatments, final body size was determined by the food level to which larvae were exposed during the latter part of development. In contrast, age at pupation reflected the food level encountered in the early part of development. The resulting pattern of age and size at emergence along with a lack of effect of treatment upon larval survival was consistent with highly flexible, adaptive plastic development. Existing theory for how different environments affect age and size at life history transitions, which is based on a trade-off between growth and mortality, may be useful for understanding how fluctuating conditions within habitats affect age and size at emergence. Day and Rowe's (2002) formalization of the Wilbur-Collins model (1973) provides a framework applicable to understanding qualitatively how development of *An. gambiae* larvae responds to fluctuating resources encountered by individuals within habitats. However, full understanding of this species' plastic development will require a system-specific model. Discrepancies between predictions from the Day-Rowe model and our data suggest four topics that may shape *An. gambiae's* response to changing food availability: 1) compensatory growth, 2) limits to developmental plasticity, 3) a mismatch between energetic supply and demand, and 4) a risk-prone strategy for acquiring fitness from growth in an uncertain environment.

Acknowlegements

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Tables

Table 3.1. ***Mean values with confidence intervals for the experimental treatment groups with corresponding mean days to pupation and adult wing lengths (mm) with 95% confidence intervals.***

n=10. Means and CIs shown for pooled sexes, females, and males. Values for pooled treatment groups are also provided. See *Methods* for details of treatments.

		Sexes pooled		Females		Males	
Feeding treatment	Abbrev	Mean age	Mean w.l.	Mean age	Mean w.l.	Mean age	Mean w.l.
Low food, no switch	L-L	11.00 ± 0.40	2.80 ±0.04	10.93±0.50	2.86±0.07	11.00±0.56	2.75±0.05
Low to high, early switch	L-He	10.03 ± 0.17	3.07 ±0.03	10.05±0.25	3.12±0.04	9.93±0.13	3.00±0.03
Low to high, late switch	L-Hl	10.26 ± 0.30	3.04 ±0.05	10.36±0.48	3.11±0.05	10.21±0.31	2.99±0.08
Medium food, no switch	M-M	9.90 ± 0.14	2.95 ±0.05	9.87±0.16	2.99±0.06	9.86±0.24	2.90±0.05
High food, no switch	H-H	9.82 ± 0.12	3.06 ±0.04	9.83±0.12	3.12±0.03	9.76±0.17	2.99±0.04
High to low, early switch	H-Le	10.65 ± 0.45	2.81 ±0.05	10.60±0.53	2.86±0.06	10.66±0.56	2.78±0.04
High to low, late switch	H-Ll	10.06 ± 0.17	2.82 ±0.06	10.01±0.19	2.87±0.07	10.10±0.31	2.77±0.06
Pooled	na	10.24 ± 0.13	2.94 ±0.03	10.23±0.14	2.99±0.03	10.21±0.15	2.88±0.03

Figures

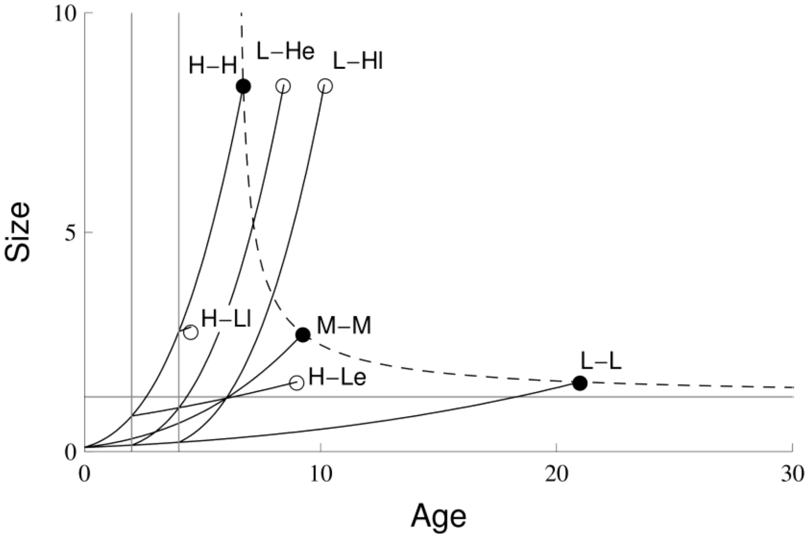

Figure 3.1. Growth trajectories and predicted age and size at life history transition (e.g. pupation) for both constant environments and scenarios where environmental quality changes during development.

Terminal points indicate optimal age and size at transition from the model of Day and Rowe (2002): filled circles show optimal transitions under constant resource levels; open circles show transitions under a switch in resource level that occurred either early or late during development. The vertical lines show time of early and late switches in resource abundance. The horizontal line shows minimum size threshold for life history transition. The curved dashed line shows the reaction norm of transition age and size across a range of different quality environments that are constant. See Day and Rowe (2002) for model details; b = 0.66, wi = 0.1, μ = 0.4, klow = 0.1, kmed = 0.3, khigh = 0.7, and wc = 1.25.

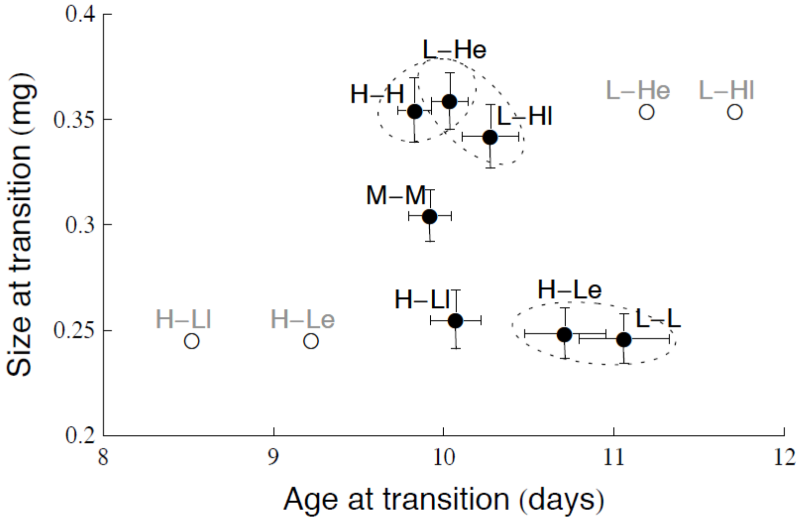

Figure 3.2. Observed and predicted age and size at pupation.

Observed mean days to pupation by mean body size for each experimental feeding treatment group is shown in black. Grey text with open symbols shows predictions for our food-switching treatments from a growth-mortality model with a minimum size threshold (Day & Rowe 2002). $b =$ 0.66, $w_i = 0.01$, $w_c = 0.2$, $k_{low} = 0.109$, $k_{med} = 0.136$, $k_{high} = 0.147$, $\mu_{low} = 0.95$, $\mu_{med} = 0.60$, $\mu_{high} =$ 0.48. Dotted ovals indicate where no difference in both observed days to pupation and wing length was detected. Whiskers indicate univariate 95% confidence intervals. Wing lengths were converted to estimates of body size (mg) according to the relationship $y = 0.0037x^{4.08}$ (Koella & Lyimo 1996).

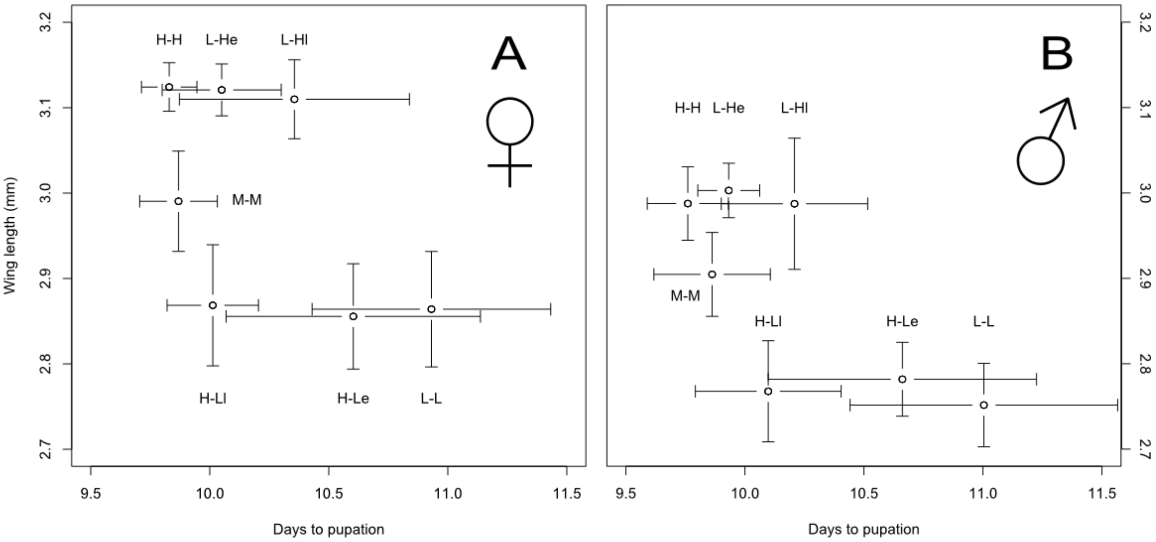

Figure 3.3. Bivariate plot of age at pupation and wing length for seven experimental feeding treatments, with univariate 95% confidence intervals.

Panel A shows values for females, panel B for males. For the treatment codes, upper case letters indicate whether high, low, or medium food levels were administered during the early or latter portions of the developmental period. Lower case letters show whether the timing of switches in food level was early or late. See text for details. Numerical values for these results are shown in Table 3.1.

Connecting statement

This next chapter contains further experimental investigation of how larval rearing conditions influence age and size at emergence of *An. gambiae*. However, this thesis now moves away from questions of variation in resource availability through time and considers the complexity that arises from the presence of multiple distinct environmental variables.

Chapter 4

Multiple environmental factors and larval growth and development of *Anopheles gambiae* mosquitoes

Abstract

Larvae of *Anopheles gambiae sensu stricto* were reared individually to investigate the combined effects of food availability, water temperature, and water depth on growth and development. We applied two levels of each factor for a 2×2×2 design and measured survival, time to emergence, and adult wing length (a proxy for size). Food was the only factor found to affect larval survival, with more food improving survival. All three of our experimental factors as well as sex influenced both time to emergence and wing length, and there were significant statistical interactions among factors. We discuss potential causes for the effects of individual factors as well as their interactions in the context of energy budgets and an evolved life-history. Across a range of environmental qualities the relationship between age at maturity and body size appears to follow an L-shape, which is common for animals with plastic development. This norm of reaction is useful for understanding some of the interactions we found. Furthermore, we observed that temperature appears to cause the L-shaped curve to shift, perhaps adjusting the effective minimum body size and age at emergence.

Introduction

Understanding how populations of the malaria vector *Anopheles gambiae sensu stricto* (hereafter, *An. gambiae*) will respond to large-scale shifts in habitat characteristics resulting from land use and climate change is key to developing appropriate management responses (Afrane et al. 2005; Aboagye-Antwi & Tripet 2010; Chaves & Koenraadt 2010). A basic requirement of this process is to develop a detailed understanding of how *An. gambiae* life history outcomes are affected by environmental factors. The nursery environment of a developing mosquito is made up of any number of variables, many of them potentially interacting. Because of its central role in malaria transmission, there have been multiple efforts to identify productive larval habitats for this African mosquito using associations between habitat characteristics and species abundance (Gimnig et al. 2001; Minakawa et al. 2004; Impoinvil et al. 2008; Fillinger et al. 2009). However, these studies are phenomenological and do not identify mechanisms for responses in larval *An. gambiae* abundance to rearing conditions. Mechanistic models have the potential to yield more accurate predictions about responses to climate change because conditions will likely change beyond the range under which statistical models were derived (Williams & Jackson 2007; Buckley et al. 2010). Understanding how and why different environmental variables influence larval growth and development may improve our ability to predict population responses under novel sets of conditions.

In a recent review of predicted climate change effects on African highland malaria, Chaves and Koenraadt (2010) point out that while there is strong evidence supporting a linkage between climate change and malaria the mechanisms driving this association are unclear. Moreover, they identify an urgent need to determine if specific and causal relationships between climatic variables and malaria exist amongst the diverse results from descriptive surveys. Our ability to identify these relationships has been hampered in part by a single factor approach that ignores the probable interaction of multiple environmental variables operating in concert to influence growth and development. The importance of multiple environmental factors and their interactions on life history outcomes can be difficult to assess and is often overlooked, even in well-studied systems where managers are concerned with population characteristics (Hoxmeier et al. 2009).

Both age and size at metamorphosis of *An. gambiae* are plastic life history traits influenced by multiple factors. These traits provide information important for predicting how populations will change over time because each can strongly affect population growth rates; age at maturity determines generation time while adult size determines fecundity by way of egg capacity. Consequently, how various factors affect age and size at metamorphosis of *An. gambiae* has received some attention. Three such factors that are relevant to climate change include food availability, water temperature of the larval habitat, and water depth. In general, increasing larval food availability decreases time to emergence and increases adult size as well as larval survival (Timmermann & Briegel 1996; Gimnig et al. 2002). For water temperature, Bayoh and Lindsay (2004) found that *An. gambiae* larvae only developed within a range of 18-32ºC and that mortality was lowest around 24ºC. In general, as water temperature of a larval habitat increases both time to and size at emergence decrease (Lyimo et al. 1992). Timmermann and Briegel (1993) considered the effects of water depth and found that as depth in larval rearing containers increased, above a shallow optimum of less than 2 cm, time to emergence increased while adult size and larval survival decreased. Note that food availability and water depth both affect age and size at emergence such that age increases as size decreases, or vice versa. In contrast, a change in temperature typically causes age and size to both change in the same direction. Each of these three factors affects growth and development of *An. gambiae* within the context of this species' complex evolved life history, and it is not clear how, or even whether, any one factor mediates effects of the others.

Age and size at maturity are non-independent traits because, during growth, size is function of time. When plotted across a range of environmental qualities animals that exhibit plastic age and size at metamorphosis often show an L-shaped relationship between age and size (Wilbur & Collins 1973; Plaistow et al. 2004). This pattern has been demonstrated for many animal systems, including *An. gambiae* (Day & Rowe 2002; Gimnig et al. 2002). While it is clear that multiple factors separately influence growth and development, variability in this age-size reaction norm under differences across multiple factors has not been assessed. Do different rearing environments that result from changes in multiple factors merely represent a change in a universal ‘environmental quality’ (perhaps governed by energy available for growth) and thus cause the age-size response to shift along a constant species-specific reaction norm? Or is the reaction norm itself context-dependent such that different factors generate their own distinct curve?

We conducted a factorial rearing experiment in the lab to determine how food availability, water temperature, and water depth, influenced age at emergence, size at emergence, and survival of larval *An. gambiae*. Our goals were to 1) to identify dependencies, or statistical interactions, among factors and 2) evaluate whether *An. gambiae's* responses to our experimental treatments fall out along a single L-shaped age-size reaction norm. Plastic development can be adaptive for dealing with environmental changes that are encountered during the larval period. It is also relevant to differences among discreet larval habitats that remain relatively constant through time. Our factorial rearing experiment examined plastic responses of *An. gambiae* is this latter context of spatial, rather than temporal, variation in rearing conditions.

Methods

We manipulated food level, water depth, and water temperature of individual *An. gambiae* larvae. Each of these three factors was held at two levels (low, high) to give a 2×2×2 design (Table 4.1). We tracked larval survival during development, and upon emergence we recorded days to emergence and measured adult wing length as an index of size (Koella & Lyimo 1996). Larvae were reared individually in cylindrical drosophila vials (diameter 25 mm, height 95 mm). Food levels were 0.2 or 1.4 mg/larva/day (Nutrafin basix staple for tropical fish, Hagen Inc.). Water depths in the rearing vials were maintained at either 2 or 5 cm. Water temperatures were maintained at either 26 or 30°C in a series of eight water baths. Prior to addition of food each day, we used a pipette to remove accumulated food residue and waste material from the bottom of the vial to prevent fouling (M.J. Voordouw, pers. comm).

Larvae were hatched from eggs that originated from a November 25th bloodfeed, 2009. Hatching took place on Nov 28th and constituted the start of the experiment. While held in group culture, larvae were fed ground fish food ad libitum. Larvae were transferred from a group culture into individual vials on November 29. Once the experimental treatments began, a 1 ml suspension of ground food was added to each vial each day (either 0.2 or 1.4 mg/ml). Food was administered by pipette and a magnetic stirrer was used to keep a consistent mixture of food and water prior to transfer. A system of temperature-controlled water baths maintained temperatures in the vials. We had eight baths in total, four at 26°C and four at 30°C. These baths were arranged such that the two temperatures alternated spatially. Vials were held in racks of eight and we were limited by a total of 20 racks. Thus there were a total of 160 vials, with either 16 or 24 vials in each bath. Each rack of eight contained two full sets of food-by-depth treatments, arranged randomly. The number of vials at each temperature was equal. Larvae were fed and checked daily and dates of larval deaths were recorded. Individuals that survived to emerge as adults were freeze-killed, sexed, and their wing lengths measured using a stage micrometer under a dissecting microscope.

Statistical analysis

Statistical analyses were conducted with R version 2.10.1 for Linux (R-Development-Core-Team 2010). Survival was analyzed using a generalized liner model with a binomial error distribution (Quinn & Keough 2002; Crawley 2007). We used two separate linear models to analyze age at emergence and wing length. Including water bath, or tub, as a random factor (see above) did not improve our statistical models and so it was excluded (Pinheiro & Bates 2000). We used the generalized least squares method and R's varIdent function to address homoscedasticity in our data when necessary (Zuur et al. 2009). For all the above models, we conducted backward selection using a critical α of 0.05 to achieve reduced models (Quinn & Keough 2002), while forcing the retention of main experimental factors (thus any non-significant interactions were dropped). Adjusted means were calculated with the effects package in R (Fox 2003). To explore the data in the context of the age-size reaction norm, we conducted a polynomial regression of days to emergence against wing length.

Results

From our generalized linear model, food was the only experimental factor to significantly affect survival ($\alpha = 0.05$, $p < 0.0001$) (Figure 4.1).

From our linear model of age at emergence, all three of our experimental factors as well as sex and the interactions food-by-depth and food-by-sex were statistically significant ($\alpha = 0.05$, Table 4.2). In general, higher food level decreased age at emergence ($F_{1,73} = 247.20$, $p < 0.0001$), higher temperature decreased age at emergence ($F_{1,73} = 61.25$, $p < 0.0001$), and deeper water increased age at emergence ($F_{1,73} = 23.89$, $p < 0.0001$; Figure 4.2 and Figure 4.3). Also, females generally took longer than males to develop ($F_{1,73} = 6.57$, $p < 0.0118$; Figure 4.2 and Figure 4.3). The food-by-depth interaction was such that deeper water depth caused a greater delay in emergence under low food than high ($F_{1,73} = 19.75$, $p < 0.0001$; Figure 4.4). For the food-by-sex interaction, the difference between female and male development times was more pronounced under low food ($F_{1,73} = 3.94$, $p = 0.0498$; Figure 4.4).

For wing length, we found significant relationships for all of our three experimental factors as well as sex and the interactions food-by-temperature, food-by-depth, temperature-by-depth, and depth-by-sex ($\alpha = 0.05$, Table 4.3). For the main effects, more food increased wing length ($F_{1,71} = 129.57$, $p < 0.0001$; Figure 4.5 and Figure 4.6), higher temperature decreased wing length ($F_{1,71} = 48.70$, $p < 0.0001$; Figure 4.5 and Figure 4.6), and deeper water also decreased wing length ($F_{1,71} = 4.24$, $p = 0.0421$; Figure 4.5 and Figure 4.6). And females generally had longer wings than males ($F_{1,71} = 23.42$, $p < 0.0001$, Figure 4.5 and Figure 4.6). For the food-by-temperature interaction, increased temperature produced a much greater decrease in wing length under low food than high ($F_{1,71} = 13.40$, $p = 0.0004$; Figure 4.7). For food-by-depth, an increase in water depth produced a much greater decrease in wing length under high food than low ($F_{1,71} = 10.40$, $p = 0.0017$; Figure 4.7). For temperature-by-depth, increased water depth caused greater reduction in wing length at lower temperature ($F_{1,71} = 6.18$, $p = 0.0147$; Figure 4.7). And for depth-by-sex, females had longer wings than males in shallow water while both sexes had equal wing lengths in deeper water ($F_{1,71} = 18.75$; $p < 0.001$; Figure 4.7).

The response variables age to emergence and wing length are non-independent, and their bivariate means for each of the experimental treatments fell out along a roughly L-shaped pattern (Figure 4.8). This general pattern is common across gradients of environmental qualities for this and other species (see *Introduction*). We conducted a polynomial regression of age at emergence and wing length to approximate the shape of the age-size reaction norm and the model was statistically significant (adjusted $R^2 = 0.1907$, $p < 0.000$). From the observation that this L-shape appears to be repeated within each of our two temperature levels, we added temperature as a covariate and this substantially improved the model (adjusted $R^2 = 0.3408$, $p < 0.000$, Figure 4.8).

Discussion

Our results are broadly consistent with predictions of how each of the experimental variables would separately influence larval age and size at emergence (see *Introduction*): more food decreased age at emergence and increased size; higher temperature decreased both age and size; and deeper water increased age and decreased size (Figure 4.3 and Figure 4.6). However, the combined effects of these variables proved to be non-additive across our treatments (Figure 4.4, Figure 4.7); there were multiple significant interactions and results varied by sex of emerged mosquitoes.

Our finding that *An. gambiae* does not respond to different combinations of environmental factors in an additive way supports the assertion by Chaves and Koenraadt (2010) that for predictive models to be useful, temperature's role in shaping mosquito populations must be assessed across a full range of ecological conditions. However, evaluating the developmental responses of mosquitoes for all relevant combinations of factors and levels is impractical. A more fruitful approach would be to identify the mechanistic causes of interactions among dominant factors. It is unlikely that mosquitoes maintain different rules for every possible set of conditions governing how they develop; rather they probably respond to internal states that are the outcome of environmental conditions, physiology, and their evolved developmental programs. Thus we discuss our results in the context of energy budgets and the observed relationship between age and size.

Throughout our discussion it is important to bear in mind that some of the effects we have detected may have resulted from differential survival among individuals. There is good evidence for plastic development in *An. gambiae* and other mosquito species (Chapter 3), and we generally interpret our results in the context of how larvae may adaptively adjust their developmental program in response to rearing conditions. However some of the effects we have observed may result from differential survival among phenotypes. We detected a significant effect of food level upon survival and there is substantial heritability in *An. gambiae* for both of our response variables, larval development time and body size (Lehmann et al. 2006).

Separate environmental variables

Increasing larval food produced faster development and larger size because there is more matter available to incorporate during growth and more energy to use for metabolic processes. These effects were evident in the overall pattern of age and size at emergence (Figure 4.8). *An. gambiae* generally emerges earlier and larger under high food and later and smaller under low food such that the age-size relationship across multiple food levels appear to follow an L-shape (Timmermann & Briegel 1998; Gimnig et al. 2002). Increasing food availability can produce a 250% increase in body size and 50% reduction in development time (unpublished data). Of the three variables assessed here, food level produced the largest effect upon age at emergence, wing length, and survival (Figure 4.1, Figure 4.3) and was present in four of the six statistically significant interactions, all of which involved two factors (Table 4.2, Table 4.3).

The general effect of our experimental increase in water depth was consistent with an increase in energy expense — essentially the inverse effect of adding food (Figure 4.3 and Figure 4.6). The mechanism for this is not clear. Previous authors have maintained that *An. gambiae* feed from the water surface, and that diving behaviour is for predator avoidance (Kaufman et al. 2006). However, we observed that larvae spend a substantial amount of time at the bottoms of their rearing containers where food accumulates. A possible explanation for this pattern is that larvae dive to forage, and energy or oxygen demands increase with water depth in a manner similar to that of other diving animals (Leeuw 1996; Tuno et al. 2007). Timmermann and Briegel (1993) found that rearing *An. gambiae* in water more than 2 cm deep substantially increased mortality, and in a study of larval diving behaviour Tuno et al. (2004) found increased mortality associated with increased water depth.

The experimental increase in rearing temperature decreased both age at emergence and wing length (Figure 4.3 and Figure 4.6) — this effect differed from the effects of food and water depth in that age and size at emergence both changed in the same direction. This property of temperature on development is prevalent across taxa and is called the temperature-size rule (Angilletta et al. 2004). Increasing rearing temperature for poikilothermic organisms has the effect of speeding up development as well as increasing metabolic requirements (Kooijman 2009). The effect of temperature on developmental rate distinguishes it from our other factors. While an increase in temperature is similar to a food (i.e., energy) reduction with respect to wing length, it also speeds up development and reduces age at emergence, which is inconsistent with a simple reduction in available energy.

Interactions among variables

Considered separately, effects of each of these three experimental factors on larval development seem straight forward. However, statistical interactions we observed show that development of larval *An. gambiae* in a given environment is highly context-dependent. In our experiment, food had the largest effect among our three variables but this may simply be an artifact of the levels we used. While some variables have a strong influence upon age and size at emergence, our results suggest that the relative importance among different factors is contextual; changes in a given environmental variable may have a large effect at one level of a second variable but little, or an opposite, effect at another. And which of the mosquito's life-history traits are affected, age or size at emergence, is similarly context-dependent. The key to understanding interactions among our experimental factors lies in how each factor affects the energy state and developmental rate of larvae, as well as the non-independent relationship between age and size inherent in the age-size reaction norm (see above).

As we discussed earlier, greater food availability increases the amount of energy for growth and development of larvae while being in deeper water reduces the energy. Under the age-size reaction norm (see *Introduction*), more energy for growth generally means earlier emergence at a larger size. An important consequence of the L-shaped age-size relationship is that if growth conditions are generally in the upper part of their range (i.e., energy is generally abundant, so larvae will be in the vertical part of the L), small differences in conditions will yield responses in adult size but not time to emergence, which is minimized. However, if growth conditions are generally poor (the horizontal part of the L), small differences in conditions yield a response in time to emergence but not body size, which is now minimized. That is, under good conditions time to emergence becomes fixed at a minimum value, while under poor conditions size becomes fixed, again at a minimum. The experimental variable temperature is distinct from both food availability and water depth in how it affects both the energy available for growth and basal rate of development. Increased temperature generally lowers energy availability (because it raises metabolic costs) while at the same time increasing rate of development. This property means that the relationship between age and size is different under changes in temperature than it is under changes in food availability or water depth. These effects provide explanations for some of the statistical interactions we've observed among our factors.

Under low food conditions it took larvae longer to emerge when they were in deeper water (Figure 4.4). This result can be understood in the context of an L-shaped age-size reaction norm. Recall that under high environmental quality, larvae emerge within a narrow range of time but across a wide range of sizes (vertical part of the L), while under lower quality growth conditions size of emerging larvae is highly constrained while age at emergence is able to vary (horizontal part of the L). In our experiment, food level determined whether conditions were broadly good or bad. So the reason we saw an effect of depth on days to emergence only under low food was because age at emergence is constrained under the age-size reaction norm at high food (good growth conditions). Another possible explanation for our observed food-by-depth interaction lies in bottom foraging (see above). Perhaps there is an energetic cost to deep water that the mosquito can adjust for by increasing food intake when food is abundant. Deeper water requires more energy to dive for food such that when less food is available, more dives (or longer) are needed to acquire enough food for growth and maintenance: individuals must do more to get more (Boyd 1997). Possibly under high food this extra activity yields enough food to compensate for the extra cost of foraging while under low food it does not.

A food-depth interaction was also evident for wing length, but it followed an inverse pattern to that of age at emergence. A difference in wing lengths occurred between depths only under high food (Figure 4.7). Again, this is likely because the age-size reaction norm allows flexibility in size (i.e., wing length) only under good conditions. Our experimental food level determined the coarse level of environmental quality, and so the effect of water depth was expressed by wing length only under high food because this treatment fell along the vertical component of the age-size curve. Thus age and size at emergence must both be considered for a full picture of the effect of water depth; depth affects age to emergence under high food and wing length under low food.

We also observed an interaction between food and sex for age at emergence (Figure 4.4). Females took substantially longer to develop than males, but only under low food. A simple explanation for this is that females are generally larger than males (Figure 4.6) and under low food it takes a longer time to achieve this size difference.

There was an interaction between food and temperature such that wing length was much smaller at high temperature than low temperature, but only under low food (Figure 4.7). Temperature speeds up developmental rate and increases food requirements such that it can be considered equivalent to a transformation of time (Kooijman 2009). Under high food, larvae may have had enough energy to become as large as possible despite of greater energetic costs experienced at higher temperature. Under low food, however, larvae at the higher temperature may have been forced to adjust their size to the limited energy available. This energetic explanation may be overly simplistic however. Across different organisms the relationship between temperature and development has been called a “puzzle for life-historians” (Berrigan & Charnov 1994), and several competing and complementary theories have been proposed to explain temperature effects upon age and size (Angilletta et al. 2004).

There was also an interaction between temperature and depth. Under low temperature wing length was greater at low depth, while there was no significant difference at high temperature (Figure 4.7). This pattern lacks an obvious explanation. One possibility is that the combination of shallow depth and low temperature was energetically favourable enough to shift this group into the vertical portion of the age-size curve where size becomes less constrained. However, the differences between our experimental levels for temperature and water depth had very similar overall effects on wing length (Figure 4.6), and it is unclear why low temperature would cause a larger difference in wing lengths between the two water depths (or vice versa). One possibility is that the increase in developmental rate brought on by higher temperatures limited time available for growth, and because growth is a non-linear process an increase in developmental time under low temperature allowed for relatively large gains in size under the more favorable depth.

In shallow water only, there was a large difference in wing length between sexes (Figure 4.7). Females are generally larger than males (Figure 4.6) except, it seems, when both are reared in deep water. We also find this pattern difficult to explain. If this were a result of simple differences in energy costs between living in deep and shallow water we would expect to see a similar food-by-sex interaction, but we do not. This suggests that something more complex than higher energy costs in deep water is occurring. We speculate that effects of body size on oxygen budgets could be at work: a higher cost of being underwater for larger mosquitoes might produce this result.

Variable interactions create different age-size reaction norms

We now come to the nature of the age-size reaction norm under a multi-factorial framework. We have seen above that this life-history pattern provides insight into understanding responses in age and size to different combinations of our factor levels. It also appears that temperature may interact with the age-size reaction norm differently from our other two experimental variables. From Figure 4.8, the treatments within each temperature level appear to follow an L-shaped age-size curve, and it to be shifted downward under higher temperature. We conducted a polynomial regression to approximate an L-shaped curve and test whether the data supported a model with a curve for each temperature (ANCOVA) better than a single-curve model. The two-curve model was significantly better. This further illustrates the fundamental difference between how temperature and our other two variables affect development of *An. gambiae*. To explain L-shaped age-size reaction norms Day and Rowe (2002) proposed a mathematical model that used a minimum size or energy threshold to explain the pattern. Under their framework, our result suggests that temperature may adjust the level of such a ‘minimum’ size threshold for *An. gambiae*.

Conclusion

In our factorial experiment of individually-reared *An. gambiae* larvae we found significant interactions among our three experimental variables, food, temperature, and water depth, as well as sex. These interactions reveal dependencies between variables that could improve understanding of how populations of this species respond to environmental change. Furthermore, considering how individual variables affect energy budgets and developmental rate of larvae in conjunction with the norm of reaction for age and size at emergence can provide insight into what otherwise may appear to be idiosyncratic responses of mosquito populations to environmental variability. Changes in food level and water depth were consistent with a generic change in environmental quality, causing age and size to fall out in a roughly L-shaped pattern. However, a change in temperature appeared to shift the reaction norm such that minimum body size, and possibly minimum age to emergence, were temperature-specific.

Acknowledgements

Many thanks to Ekaterina Pokorova for her competent assistance running this experiment.

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Tables

Table 4.1. Treatment levels in the factorial rearing experiment.

Treatment ID	Food (mg/vial/day)	Temperature (°C)	Water depth (cm)
1	0.2	26	2
2	1.4	26	2
3	0.2	30	2
4	1.4	30	2
5	0.2	26	5
6	1.4	26	5
7	0.2	30	5
8	1.4	30	5

Table 4.2. Output from linear model of days to emergence for developing Anopheles gambiae s.s. as a response to food availability, rearing temperature, water depth, and sex.

Generalised least squares was used to address heteroscedasticity between the two food treatment levels (Zuur et al. 2009). The factors presented here were selected using backward elimination until all p-values ≤ 0.05 or only main experimental factors remained (Quinn & Keough 2002).

Source of variation	**DF**	**F-value**	**p-value**
Food	1	247.20	<.0001
Temperature	1	61.25	<.0001
Depth	1	23.89	<.0001
Sex	1	6.57	0.0118
Food x Depth	1	19.75	<.0001
Food x Sex	1	3.94	0.0498
Residuals	73		

Table 4.3. Analysis of wing length for developing Anopheles gambiae s.s. as a response to food availability, water depth, rearing temperature, and sex.

A generalised least squares model was used to address heteroscedasticity between the two food treatment levels (Zuur et al. 2009). The factors presented here were selected using backward elimination until all p-values ≤ 0.05 or only main experimental facors remained (Quinn & Keough, 2002).

Source of variation	**DF**	**F-value**	**p-value**
Food	1	129.57	<.0001
Temperature	1	48.70	<.0001
Depth	1	4.24	0.0421
Sex	1	23.42	<.0001
Food x Temperature	1	13.40	0.0004
Food x Depth	1	10.40	0.0017
Temperature x Depth	1	6.18	0.0147
Depth x Sex	1	18.75	<.0001
Residuals	71		

Figures

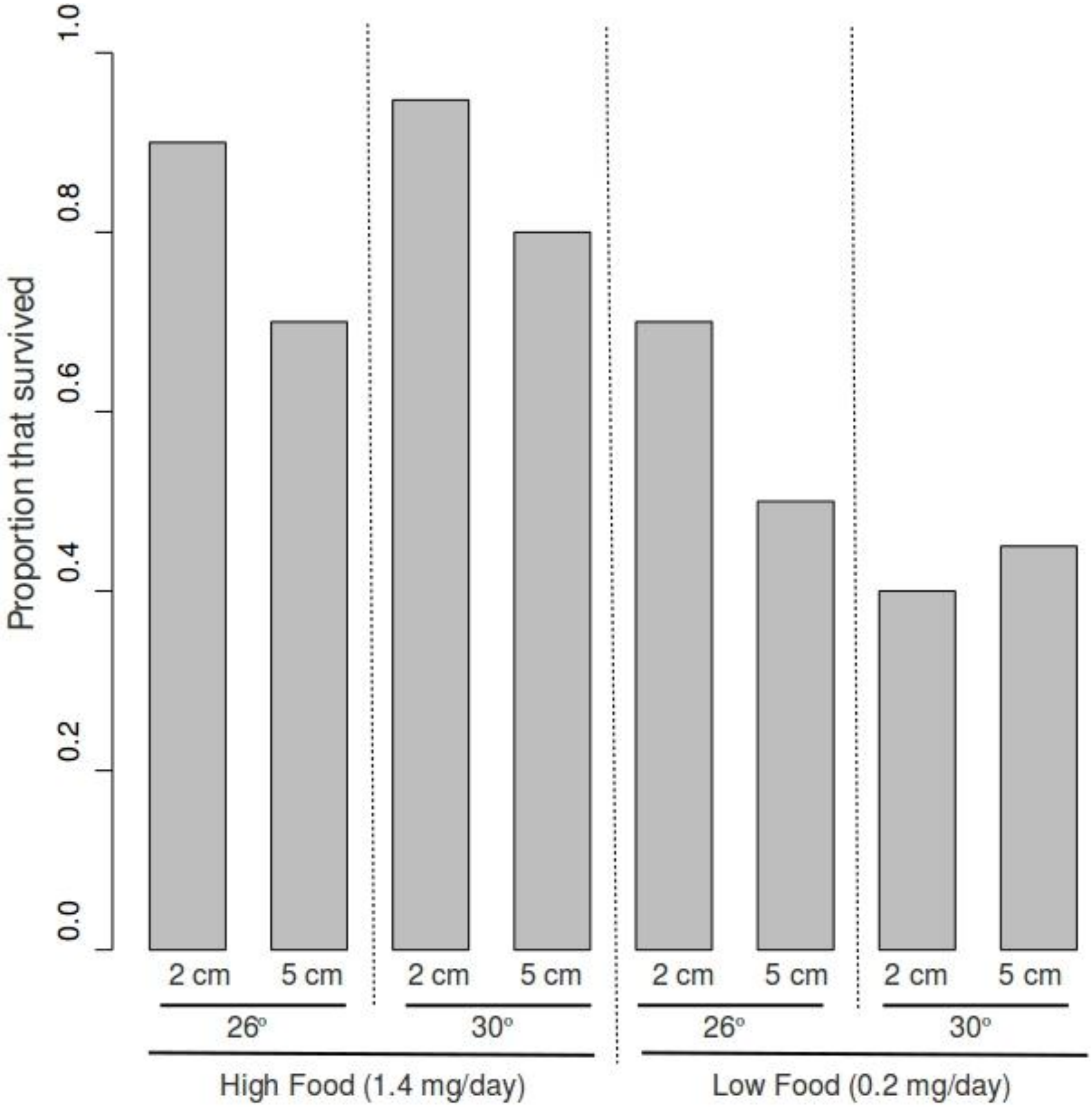


Figure 4.1. Proportion of Anopheles gambiae s.s. larvae from each treatment that survived to emerge as adults.

Levels of each experimental factor are indicated. Food was the only experimental factor to significantly affect survival ($p < 0.001$).

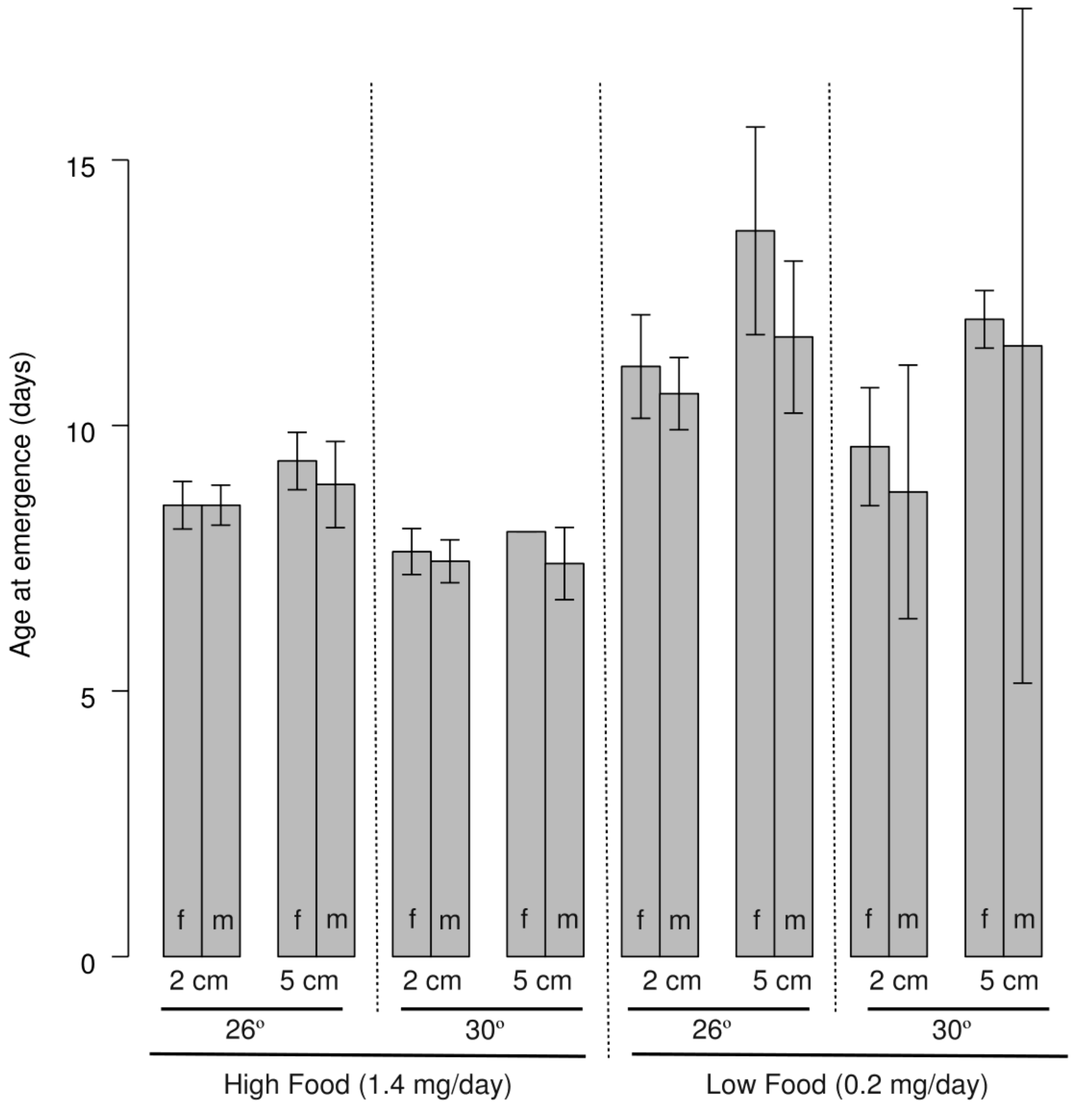


Figure 4.2.* *Mean age at emergence of Anopheles gambiae s.s. larvae for each treatment and sex.

Level of each experimental factor is indicated. Confidence intervals are 95%.

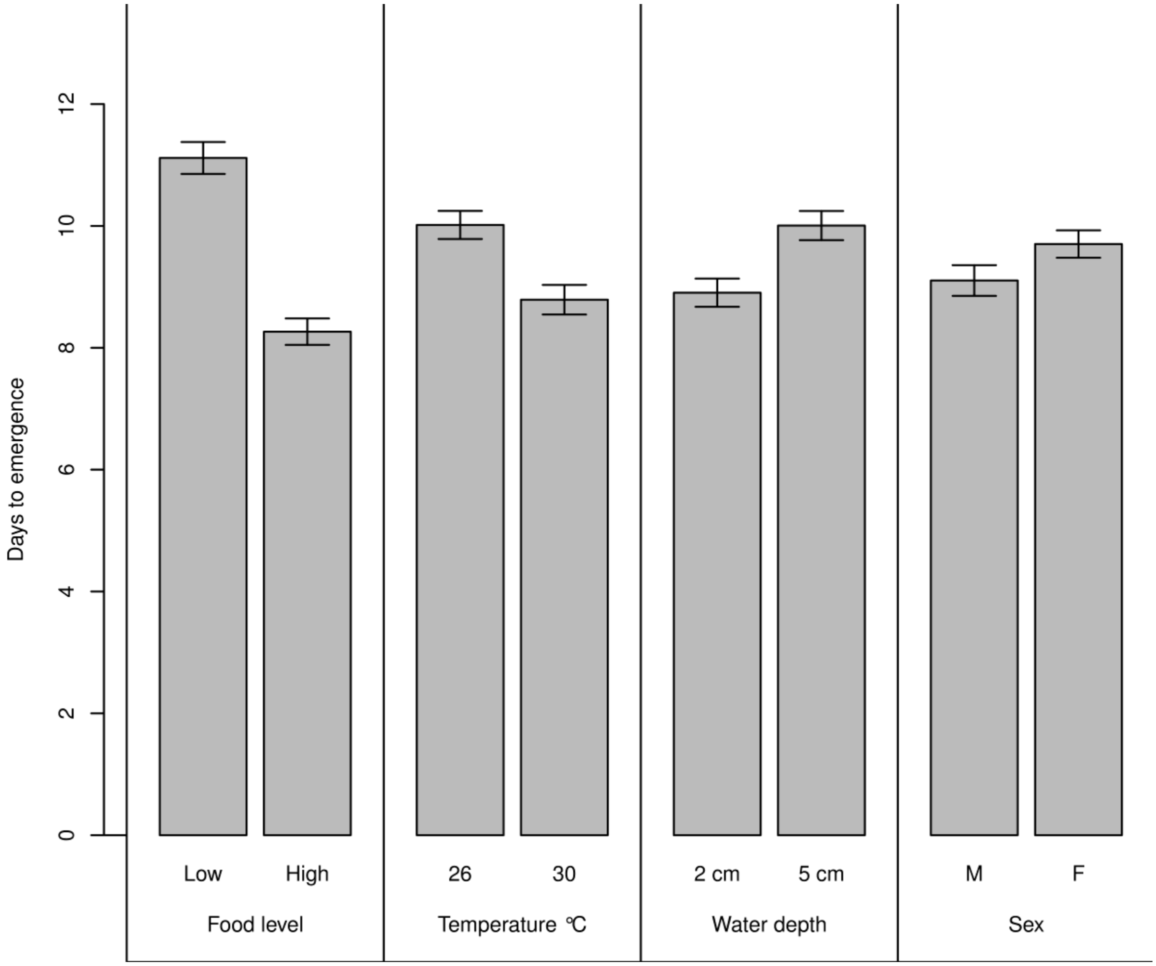


Figure 4.3. ***Adjusted means for age at emergence with 95% confidence intervals, by factor and level, adjusted for effects of the other terms in our statistical model.***

Panels A, B, C, and D show respectively the effects of food level, temperature, water depth, and sex, calculated in R with the effects package (Fox 2003).

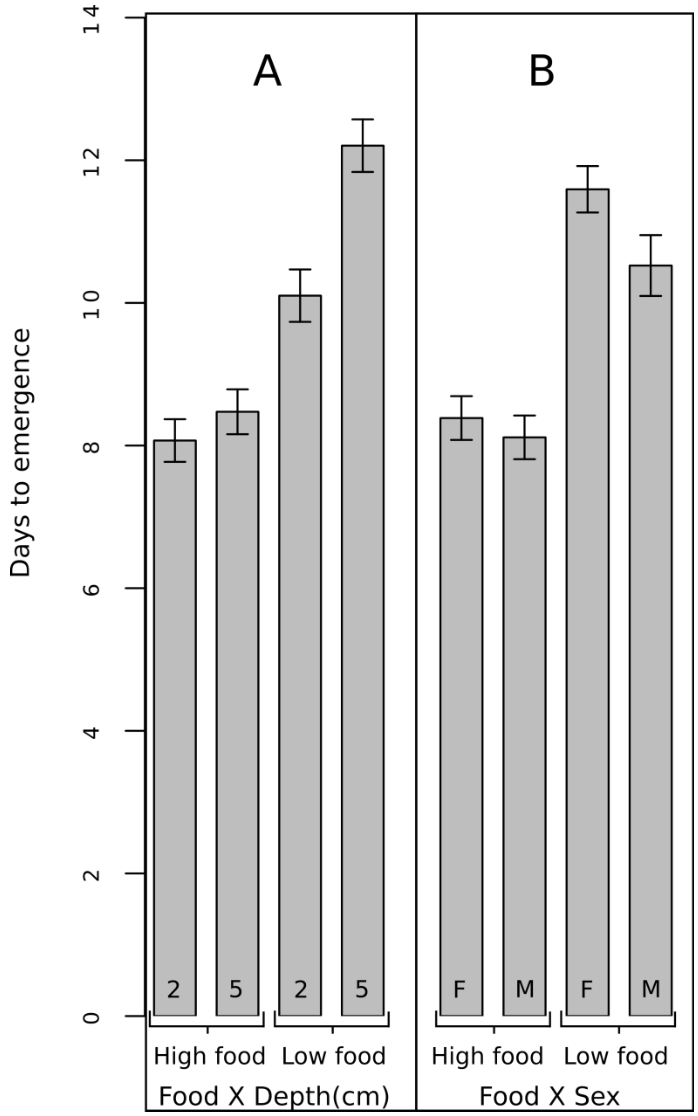


Figure 4.4. Adjusted mean age at emergence by groups showing interactions among factors. Adjusted means and 95% CIs were calculated using the effects package in R (Fox 2003).

Panel A shows a food-by-depth interaction. Panel B shows a food-by-sex interaction.

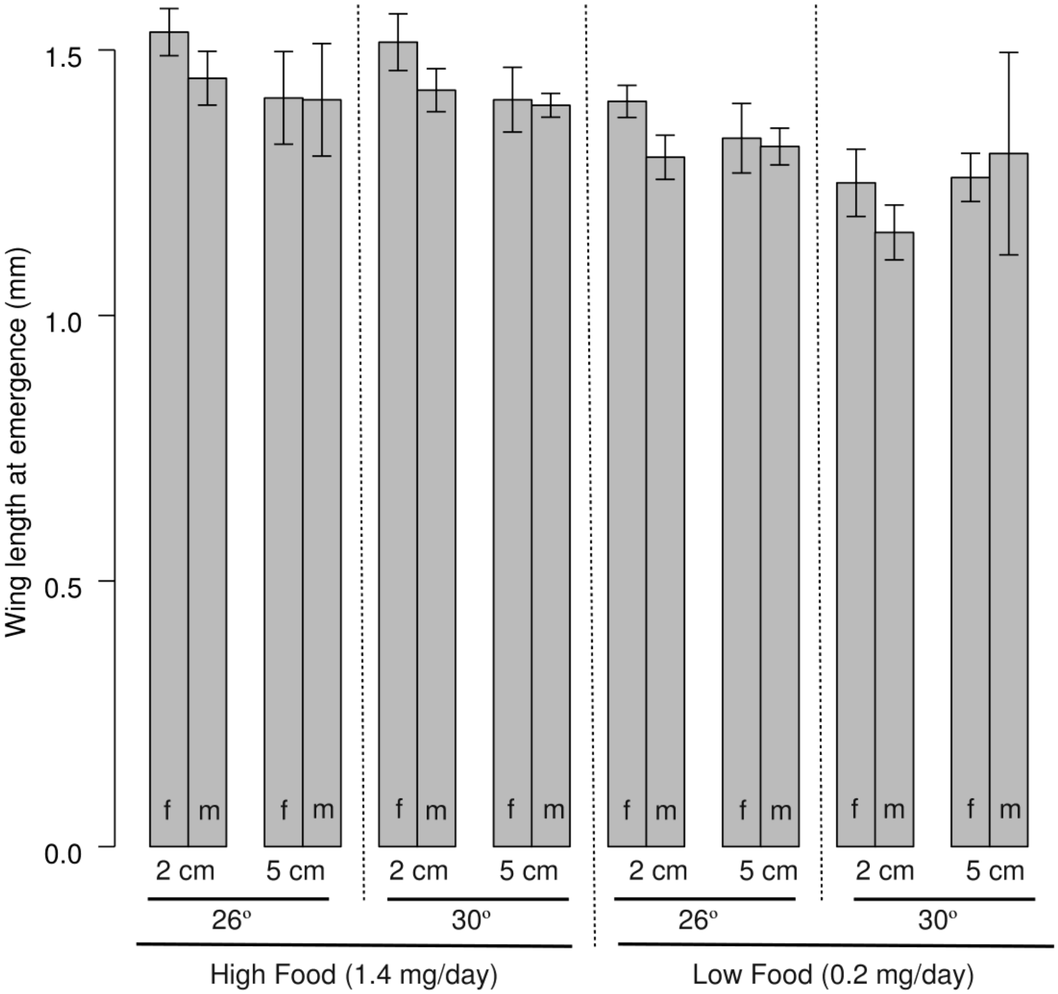


Figure 4.5. Mean adult wing length of Anopheles gambiae s.s. for each treatment and sex.

Level of each experimental factor is indicated. Confidence intervals are 95%.

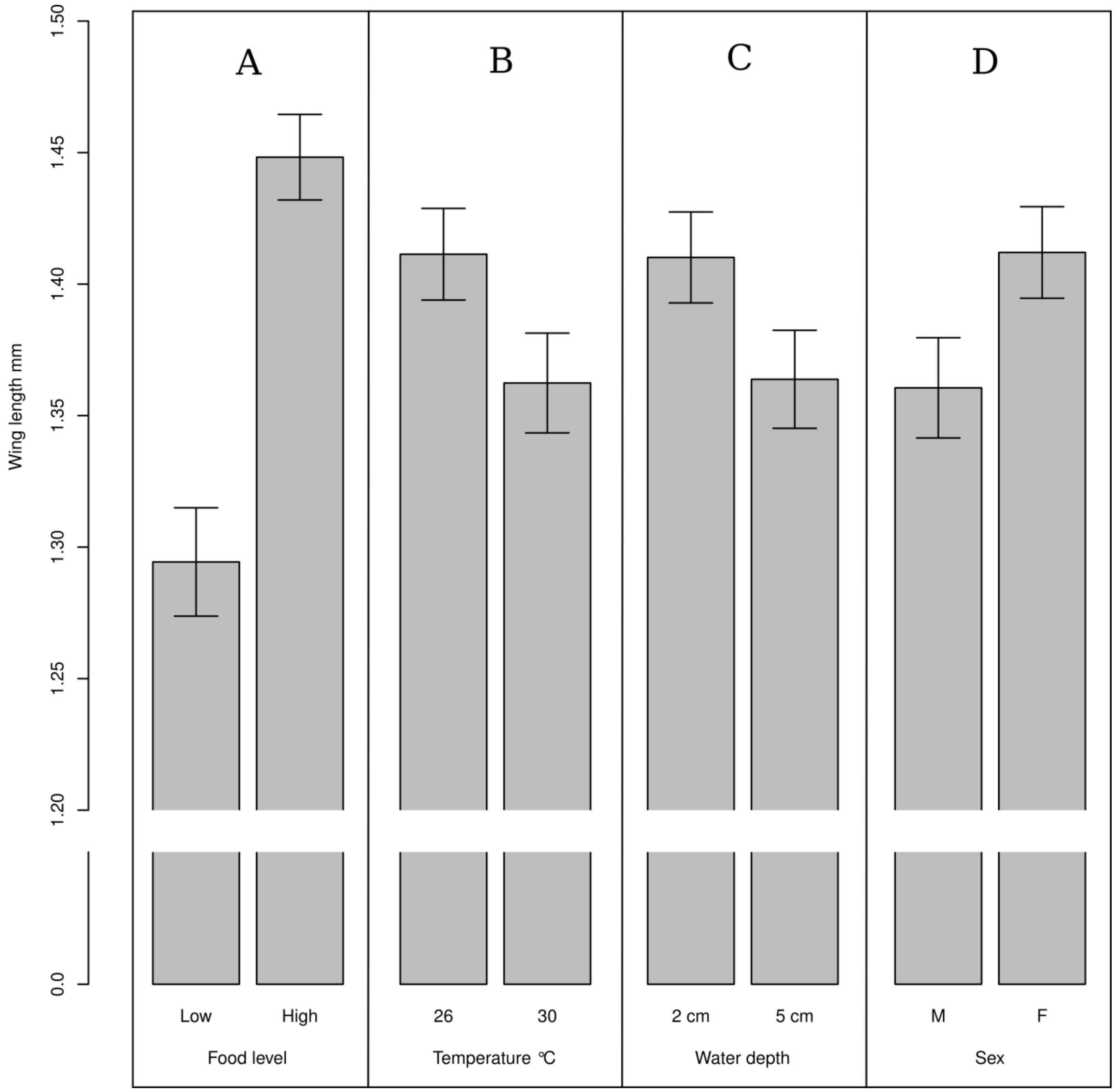


Figure 4.6. ***Adjusted means for wing length with 95% confidence intervals, by factor and level, adjusted for effects of the other terms in our statistical model.***

Panels A, B, C, and D show respectively the effects of food level, temperature, water depth, and sex, calculated in R using the effects package (Fox 2003).

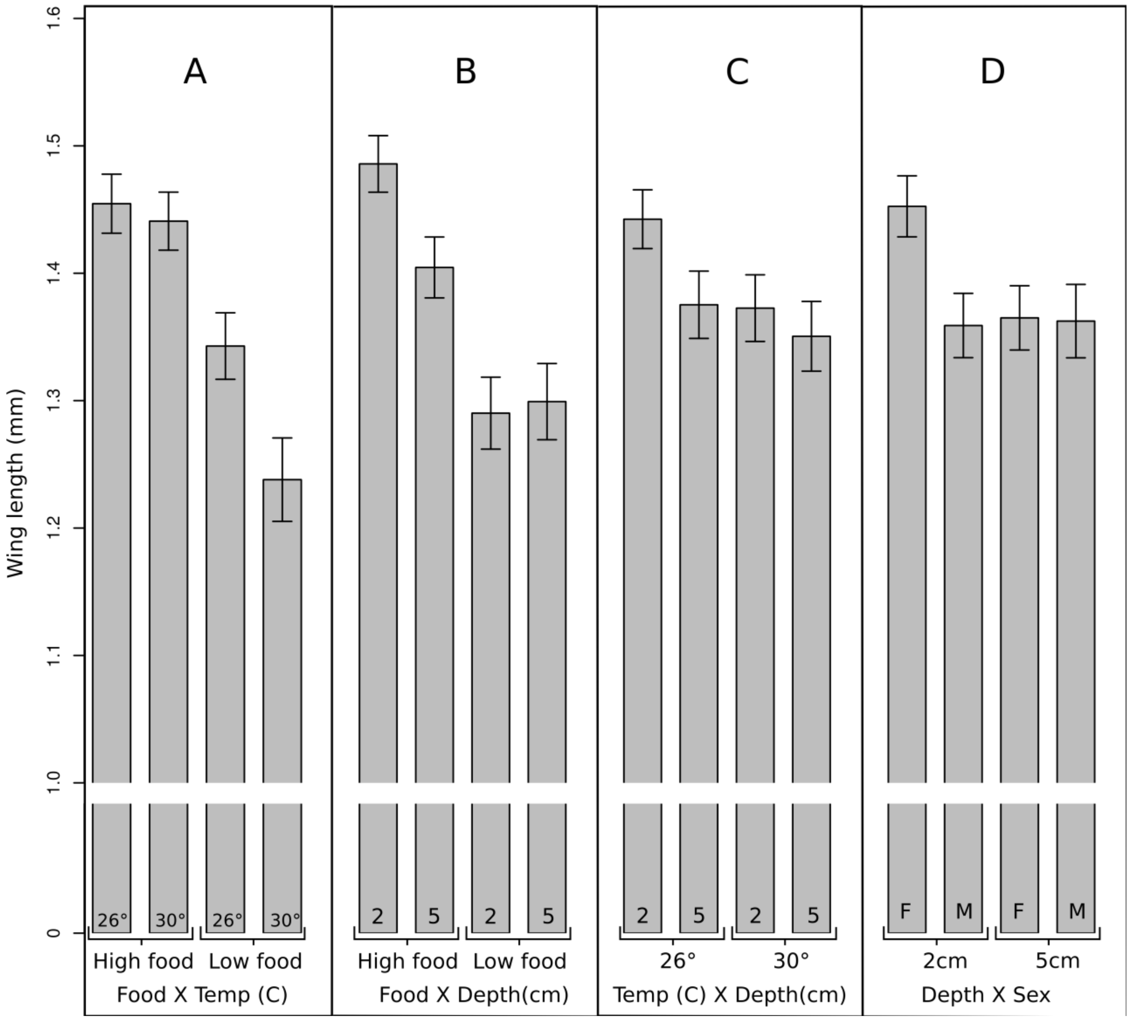
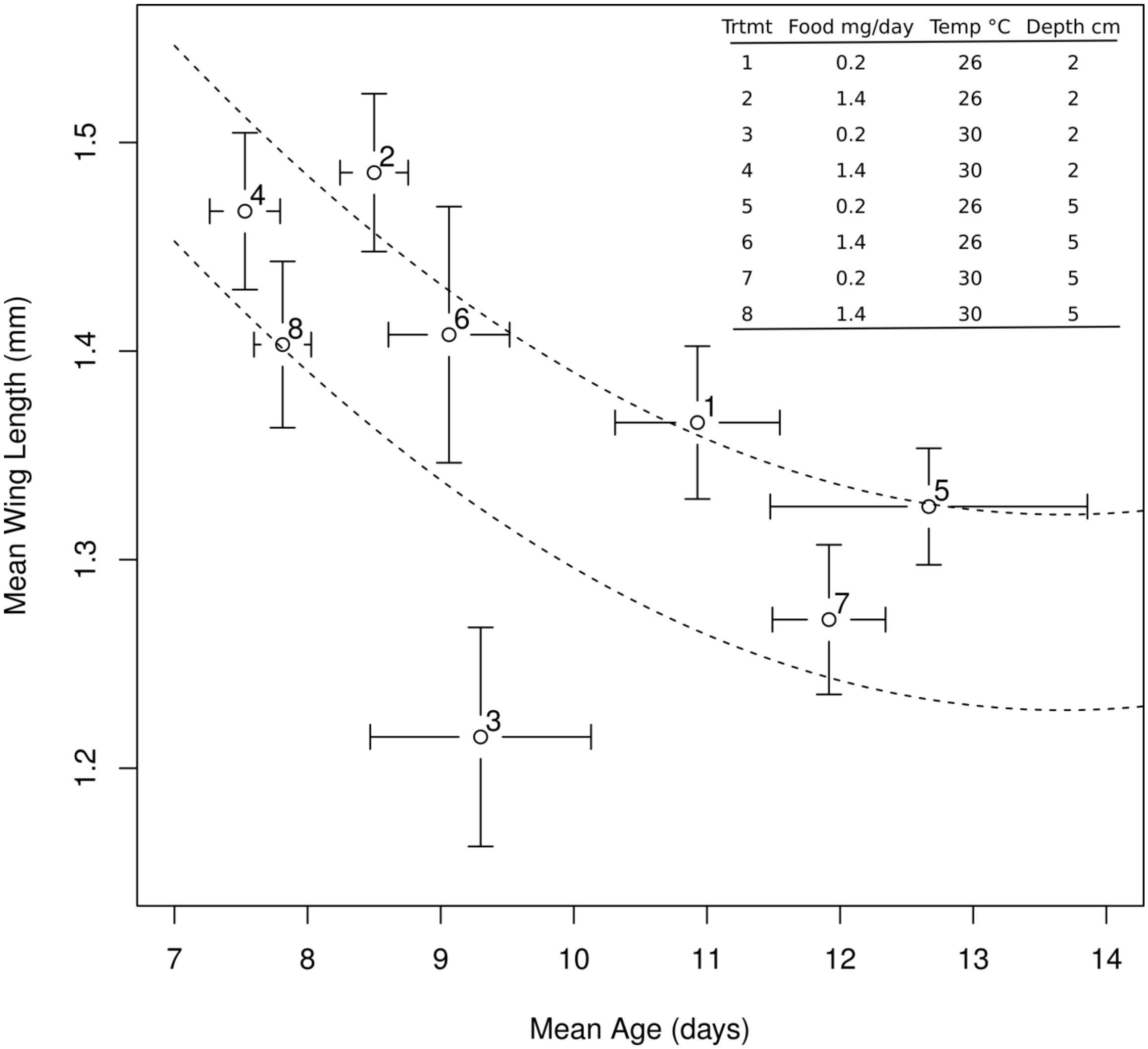


Figure 4.7. Adjusted mean wing lengths by groups showing interactions among factors.

Adjusted means and 95% CIs calculated using the effects package in R (Fox 2003). Panel A shows food by temperature, B shows food by depth, C shows temperature by depth, and D shows depth by sex. High and low food labels correspond to 0.2 and 1.4 mg per larva per day.



Trtmt	Food mg/day	Temp °C	Depth cm
1	0.2	26	2
2	1.4	26	2
3	0.2	30	2
4	1.4	30	2
5	0.2	26	5
6	1.4	26	5
7	0.2	30	5
8	1.4	30	5

Figure 4.8. Bivariate means for age at emergence and wing length of Anopheles gambaie s.s. in response to different food, water depth, and temperature levels.

Confidence intervals are 95%. Treatment ID numbers are shown with corresponding factor levels. Dashed lines show our polynomial linear model with temperature as a covariate. Upper line shows 26°C, lower show 30°C. Data are suggestive of two age-size reaction norms.

Connecting statement

The following chapter reports further results from the factorial rearing experiment described in the preceding chapter. Glycogen and lipid levels of newly-emerged individuals were measured in an attempt to assess effects of larval rearing conditions for initial adult body condition, an important component of fitness and factor influencing malaria transmission.

Chapter 5

Effects of variable larval food, water depth, and temperature for lipid and glycogen levels of adult *Anopheles gambiae sensu stricto*

Abstract

The larval growing environment of mosquitoes determines metabolic constituents of newly emerged individuals. Such constituents, including lipids and glycogen, play a critical role for maturation as well as both short- and long-term survival. Different factors in the larval environment during growth and development potentially interact with one another to affect tissue composition, with consequences for adult performance. We examined the combined effects of larval food availability, water depth, and temperature on lipid and glycogen levels of newly emerged *Anopheles gambiae* mosquitoes, an important African vector of human malaria. We detected no effects on lipids or glycogen. Unexpectedly, we also found no relationship between body size and these two major metabolic storage materials, which is inconsistent with results from other studies. These results are suggestive of a decoupling of body size from reserve levels in response to our experimental treatments. Further work is needed to assess the role of different environmental factors on body composition. We briefly discuss possible mechanisms for different effects from different variables in the larval habitat.

Introduction

The larval growing environment of mosquitoes is comprised of multiple factors, many of which act upon larvae to provide energetic benefits and costs. Consequently, conditions in the aquatic nursery habitat strongly influence levels of metabolic constituents of newly emerged mosquitoes. *Anopheles gambiae sensu stricto* (hereafter *An. gambiae*) is a major vector of malaria, and understanding how larval conditions affect adult body composition is relevant to disease epidemiology and management (Briegel 2003; Russell et al. 2011).

Energy carried over from the larval to adult stages plays an important role in survival and reproduction of mosquitoes. Briegel (1990a) measured reserve levels of both newly emerged and starved adult *An. gambiae* and found a 41% reduction in calories from lipids and a 32% reduction in calories from carbohydrates (glycogen plus oligosaccharides) between these two groups. Immediately following emergence from the aquatic larval environment mosquitoes undergo a maturation process during which constituents carried over from the larval phase are used to prepare for flight, mating, feeding, and reproduction (Briegel 2003). This critical role of teneral reserves for adult development illustrates how environmental conditions of larvae can affect mosquitoes for their entire lifespan (Aboagye-Antwi & Tripet 2010).

The two principal energy storage materials in insects are lipids and glycogen (Klowden 2007). And abundance of these substances has been shown to have a relationship with survival, reproduction, and flight capacity in *An. gambiae* (Walker 2008; Briegel 2003). Lipids represent a range of hydrophobic molecules and are used as the main energy reservoir of animals. The primary lipids found in insects are energy-dense triacylglycerols, and most lipid synthesis and storage occurs in the conspicuous tissues of the fat body (Klowden 2007; Wigglesworth 1984). Oxidation of lipids yields about twice the energy of carbohydrates by weight (9 vs 4 kcal/g). Lipids are calorie-dense but cannot be immediately converted to energy for use by cells. Triacylglycerol must first be converted to glycerol and then fatty acids, which can then join the glycolytic pathway that finally results in energy usage by cells. In contrast, glycogen is a branched-chain polysaccharide used to store carbohydrate in animals. It is less energy-dense than lipids but more immediately available for metabolic processes. Similar to lipids, glycogen is abundant in the fat body. From there, it can be easily converted to glucose and trelahose for release into the hemolymph. It can also be stored in cells where it provides immediate energy to rapidly respiring muscles (Klowden 2007). Because of its ready availability, glycogen is generally thought to be preferentially used to fuel flight in mosquitoes (Clements 1955). However, Briegel (2003) found consistent substantial reductions in lipid content for *An. gambiae* following flight challenges, indicating that such a preference may not hold for this species. Briegel also found that for the well-studied yellow-fever mosquito, *Aedes aegypti*, the amount of lipids accumulated prior to a blood meal determined reproductive potential, while egg production in *An. gambiae* and other *Anopheles* species depended primarily on volume of blood meal rather than accumulated reserves (Briegel 1990).

The amount of lipids and carbohydrates found in *An. gambiae* have been found to be linearly related to their body size (Briegel 1990). And, relative to *Aedes aegypti*, *An. gambiae* has low levels of teneral reserves and seems to lack the ability to accumulate large amounts of storage materials relative to its size (i.e., females do not become “fat”; Briegel 1990a). Environmental conditions of the larval habitat strongly influence adult size of this species, and the isometric relationship between size and reserves suggests that the larval environment will generally affect absolute teneral reserves in proportion to its effect on size (Timmermann & Briegel 1999). That is, better growth conditions produce larger mosquitoes which should increase the absolute, but perhaps not relative, amounts of reserves. Being larger generally confers a number of fitness advantages to animals (Blanckenhorn 2000), and this pattern is evident in *An. gambiae*; larger size has been shown to confer greater fecundity (Briegel 1990; Lyimo & Takken 1993), mating success (in *An. freeborni*; Yuval et al. 1993), immune response (Suwanchaichinda & Paskewitz 1998), adult drought resistance (Aboagye-Antwi & Tripet 2010), and adult survival (Takken et al. 1998). The extent to which these effects are directly caused by physical size (e.g., greater fecundity because of greater egg capacity) and how much they are the product of correlated factors, including abundance of lipids and glycogen, is unclear and often difficult or impossible to determine (Blanckenhorn et al. 2007). Because size and reserves affect fitness of this important disease vector they are relevant to its vectorial capacity and epidemiology of malaria (Russell et al. 2011).

The ability to predict how mosquito populations will respond to environmental conditions would be useful for epidemiologists and managers. The key to developing such models lies in the aquatic larval habitat because it largely determines relevant population-level processes (Fillinger et al. 2004). However, the larval growing environment is comprised of myriad factors, many of which may not affect larval growth and development independently of one another. Efforts to identify key factors controlling abundance and distribution of A*n. gambiae* are ongoing, but a lack of mechanistic understanding of how and why mosquitoes respond to different factors has compromised reliability of predictive models (Chaves & Koenraadt 2010; Li et al. 2011).

Three factors, food availability, water depth, and temperature have each been found to affect growth and development of *An. gambiae* larvae. Greater larval food availability generally produces larger mosquitoes, reduces time to emergence, and improves larval survival (Lyimo et al. 1992; see Chapter 4). Greater water depth has the inverse effects; deeper water produces smaller adults, lengthens development time, and reduces larval survival (Timmermann & Briegel 1993). Within a range of viable temperatures, increased temperature generally reduces adult size, shortens development time, and reduces survival (Lyimo et al. 1992; Bayoh & Lindsay 2004). It is likely that these different effects reflect how each factor influences the energy budgets of larvae. Greater food availability probably causes mosquitoes to mature larger and earlier because food provides the energy needed to grow faster. Conversely, deeper water may cause mosquitoes to emerge smaller and later, probably because it either increases the energetic cost of acquiring food, or reduces its availability, or both. Finally, increased water temperature causes mosquitoes to emerge smaller and earlier because it increases rates of biochemical processes (Kooijman 2009), consequently both speeding up development and raising metabolic costs. However, even if reducing these environmental factors to simple additions and subtractions to the larval energy budget is valid, the mosquito's evolved responses to its energetic circumstances are not necessarily straightforward.

An. gambiae responds to quality of its rearing environments by adjusting two important non-independent life history traits, age and size at maturity (Roff 2002). Across a range of different quality larval environments age and size follow an L-shaped relationship. Individuals reared under poor conditions emerge at a fixed, minimal size but can still vary in development time in response to small differences in environmental quality (the horizontal part of the L, see Chapter 4). In contrast, individuals from high-quality larval environments have a fixed, minimal development time but can still vary in size to some degree from minor differences in quality (the vertical part of the L). This general pattern is prevalent in animals that undergo life history transitions such as metamorphosis (Wilbur & Collins 1973; Day & Rowe 2002). This age-size pattern has consequences for teneral reserves of *An. gambiae*. Because reserves are coupled to body size (Briegel 1990), and body size responds differently to environmental factors depending on the general quality of the environment (i.e., horizontal vs vertical parts of the L), we expect multiple environmental factors to influence absolute amounts of metabolic constituents in a non-independent fashion.

We measured lipid and glycogen levels of newly emerged *An. gambiae* that were reared under varying food availability, water depth, and water temperature during the larval phase. Our experiment included these three factors each at two levels, for a factorial design with eight treatments (2×2×2). The purpose of considering these three factors together was to look for dependencies among their effects on larvae. A second goal was to evaluate how these factors, all of which are relevant to climate change and land use, affected reserve levels of this important malaria vector.

Methods

Larvae were reared individually in a factorial experiment. Food, water depth, and water temperature were each held at one of two levels each for the duration of larval development, for a total of eight treatments. Food was either at 0.2 or 1.4 mg/larva/day, depth was 2 or 5 cm, and temperature was 26 or 30°C. Full details of the experimental design and treatments are described in Chapter 4. Larvae were freeze-killed following emergence. Wings lengths were measured using an ocular micrometer. Lipids and glycogen were measured using the methods described in Walker (2008), which included the preparation of standard curves. These curves provided linear relationships between absorbance and lipid and glycogen concentration that were used to calculate our sample concentrations. To better consider the relative energetic contribution of the two reserve materials we measured, µg of lipids and glycogen were converted to estimates of calories by conversion factors 0.009 and 0.004 calories/µg respectively (Takken et al. 1998). Absolute amounts of metabolic reserves are largely a function of body size (Briegel 1990a; Blanckenhorn et al. 2007). The volumetric measurement winglength^3 is correlated with body size of *An. gambiae* (Koella & Lyimo 1996), and calorie estimates were divided by this proxy to get size-specific caloric content (SSCC), an index of condition (Timmermann & Briegel 1999). (Measured weights were unavailable because dried adults proved too fragile to handle for weighing and our wet weights were compromised by moisture from condensation following freezing.)

Statistical analyses were conducted with R version 2.13.1 (R-Development-Core-Team 2011). Preliminary work revealed that the order in which terms were removed strongly affected results of stepwise backward model selection for our univariate models. To reduce risk of spurious model selection, R's MuMIn package was used to rank linear models by AICc values (small-sample-size corrected Akiake information criterion), from which we inferred the importance of different factors (Dalgaard 2002; Kamil Bartoń 2011). Along with experimental factors, sex was included in the candidate models for effects on lipids and glycogen. For models concerning lipids, processing date was also included as a main effect because it had a strong influence on mean lipid value. Samples were randomized across the two processing dates and this date effect appears to have been a procedural artifact. For other analyses of lipids, values from the date with the higher mean were adjusted downward to remove the date effect. Lipid levels after this transformation were consistent with levels in other studies (Walker 2008).

Because we do not expect lipid and glycogen levels to be independent of one another we conducted a multivariate analysis of variance (MANOVA) to consider lipid and glycogen measures as a combined response to our predictor variables, food, depth, and sex and their higher order terms. Date and winglength^3 were also included in the model as main effects to account for a strong effect of processing date on lipid levels and an expected effect of size on absolute measures of metabolic storage substances. Model selection tools for MANOVA in R are limited so we conducted a simple stepwise model reduction (Dalgaard 2002; Quinn & Keough 2002). All main terms were retained in the final model regardless of significance levels.

Four ordinary least-squares linear models were used to assess how 1) unadjusted lipids, 2) SSCC from lipids, 3) unadjusted glycogen, and 4) SSCC from glycogen were affected by our experimental factors and sex. Further analyses were conducted to assess relationships between body size and reserve levels, as well as relationships between levels of glycogen and lipids. However, we did not explore allometric relationships between body size and lipids and glycogen because these constituents have been shown to have a linear relationship with winglength^3 for *An. gambiae* (Briegel 2003).

Results

A MANOVA considering the combined response of lipid and glycogen detected significant effects from food and processing date (Table 5.1). However, this model was not significantly better than a model that included only processing date ($p = 0.28$). Greater food produced higher levels of lipids and glycogen, which is consistent with expectation, but a clear effect of food on the bivariate lipid-glycogen response was not detected (Figure 5.1). For the analysis of unadjusted lipids, no effects of our three experimental factors or sex were found. For SSCC from lipids the top-ranked model included all three experimental factors, food, depth, and temperature, as well as sex, processing date, and the interaction terms food-by-depth, food-by-temperature, depth-by-temperature, depth-by-sex, and food-by-depth-by-temperature (Table 5.2, Figure 5.2, Figure 5.3, Figure 5.4). No effects of treatments were detected for either unadjusted glycogen or the SSCC from glycogen. We did not detect a relationship between absolute measures of lipids and glycogen ($r = 0.01$, p-value from regression = 0.921). Similarly, there was no relationship between the SSCCs from lipids and glycogen ($r = 0.169$, p-value from regression = 0.150). No relationship was detected between winglength^3, our proxy for body size, and lipids ($r = -0.13$, p-value from regression = 0.254). And no relationship was found between winglength^3 and glycogen ($r = 0.14$, p-value from regression = 0.197).

Discussion

The results of our MANOVA were suggestive of a food effect upon lipid and glycogen levels but were not conclusive, and we found no effects of our experimental factors on three of the four univariate responses we considered (unadjusted lipids and glycogen and size-specific caloric content (SSCC) from glycogen). And, while we did find a statistically significant result for SSCC from lipids (Table 5.2), it seems unlikely that this result actually reflects lipid levels of *An. gambiae*. Rather, it is probably an artifact of the transformation used to calculate SSCC. We detected no effects of treatments or winglength^3 (size) on unadjusted lipid levels, and transforming data that have no relationship with size to account for size is expected to produce values that are inversely related to size. Our results for SSCC from lipids were consistent with such an effect: they were inverse to effects of our treatments upon size (Figure 5.4, see Chapter

4). Moreover, the effects we saw were opposite to our intuitive expectation for treatment effects on reserves. SSCC from lipids were greater under low food than high (Figure 5.2, Figure 5.4), and they were generally greater in deeper water (Figure 5.2, Figure 5.3), which we expect from other studies to be more energetically costly (Timmermann & Briegel, 1993). We also found greater SSCC from lipids for males than females (Figure 5.3), which is again inverse of the size response (although this result is consistent with findings from other studies of teneral reserves for this species (Timmermann & Briegel 1999; Walker 2008)). From this, we conclude that our experimental treatments did not produce a detectable response in lipid or glycogen levels.

From other studies, we expected size and reserves to be linearly related for this species (Briegel 1990; Walker 2008). Our inability to detect such a relationship led us to question the quality of the data and investigate potential sources of error in the procedure we used to quantify metabolic constituents. However, we have not uncovered any deficiencies that would explain our unexpected results. The person who conducted the quantification of lipids and glycogen was well-practiced with the procedure, equipment, and the experimental system (Walker 2008). Standard curves were generated with the spectrophotometer immediately before absorbances of our samples were measured. And while we detected a strong effect of processing date on mean lipid levels, variance was equal between the two days and we suspect the effect was from a recording error in sample dilution prior to measurement of absorbance, which we dealt with by either including date in the statistical model or by simple arithmetic adjustment (see *Methods*). Furthermore, our measures of lipids and glycogen were comparable to other studies. Briegel (1990) found calories from teneral lab-reared *Anopheles* in the range of 0.1-0.5. Walker (2008) found calories from lipids in the range of 0.2-2.0 for field-collected *An. gambiae*. Our measures ranged from 0.5-2.5. Breigel found total calories from all carbohydrates in the range of 0.01-0.2. Walker found calories from glycogen (a major carbohydrate) in the range of 0-0.17. And we found calories from glycogen in the range of 0-0.09. See Table 5.3 for mean lipid and glycogen measurements. Finally, it is also important to note that the treatments themselves appear to have been properly applied; we were able to demonstrate treatment effects and interactions on size at emergence based upon our factorial design (Chapter 4).

Assuming the lipid and glycogen estimates were not compromised in some unknown way, our results suggest that our experimental treatments have decoupled reserve levels from body size. To our knowledge, this is the first study to test the combined effects of multiple variables in the larval environment upon metabolic constituents of newly emerged mosquitoes. Measures of lipids and glycogen in mosquitoes are typically variable (Briegel 1990a), and it is possible that the sample size of 80 used for this study was inadequate to detect effects from our treatments, especially if there were interaction effects among our three factors. However, all else being equal, for a sample of this size we would expect to see a relationship between winglength and the constituents we measured (Briegel 1990a; Briegel 1990b; Blanckenhorn et al. 2007; Walker 2008), but we did not. That we did not see such a relationship suggests that our treatments decoupled reserve levels from size. Such a decoupling is consistent with effects on metabolic constituents that are specific to different variables in the larval environment of *An. gambiae*.

It is reasonable that food, water depth, and temperature could each have distinct effects on body composition. Food availability determines growth rate, and, all else being equal, individuals with more food will both become large and accumulate large reserves. Greater water depth, however, may affect diving activity for foraging (see Chapter 4), which would not only adjust both rates of energy usage and acquisition, but could conceivably affect how resources are allocated between muscle tissue and lipids, either to facilitate swimming or to influence buoyancy. Finally, temperature affects both developmental rate and energy usage, which could affect body composition if changes in either of these processes affect usage or accumulation of different metabolic substances unequally.

Conclusion

Although we were unable to detect effects of larval food, water depth, and temperature upon lipids and glycogen levels of teneral *Anopheles gambaie*, absence of the expected relationship between body size and these two metabolic constituents suggests that our experimental treatments decoupled size and metabolic reserves. Further work is needed to evaluate how important environmental factors in the larval habitat influence teneral body condition and subsequent long-term adult traits for this species.

Acknowledgements

Kelly walker directed and helped conduct the procedure for measuring lipids and glycogen.

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Tables

Table 5.1. Output from MANOVA for lipid and glycogen content of experimentally-reared teneral An. gambiae.

Source of variation	Df	Pillai	approx F	num Df	den Df	Pr(>F)
(Intercept)	1	0.96549	923.26	2	66	< 2.2e-16 ***
Food	1	0.15355	5.99	2	66	0.004082 **
Depth	1	0.05119	1.78	2	66	0.176597
Temperature	1	0.06703	2.37	2	66	0.101306
Sex	1	0.01146	0.38	2	66	0.683516
Date	1	0.70289	78.07	2	66	< 2.2e-16 ***
WL^3	1	0.03603	1.23	2	66	0.297931
Residuals	67					

**p < 0.01; ***p < 0.001. Date refers to processing date and is included to account for a procedural artifact that strongly affected mean lipid values. See *Methods*. WL^3 stands for cubed wing length

Table 5.2. Linear model output for size-specific caloric content from lipids (SSCCl).

Source of variation	Df	Sum of Squares	Mean Squares	F value	Pr(>F)
Food	1	0.3327	0.3327	4.6409	0.0351 *
Depth	1	0.0416	0.0416	0.5797	0.4493
Temp	1	0.0220	0.0220	0.3063	0.5819
Sex	1	0.3092	0.3092	4.3120	0.0419 *
Date	1	5.9019	5.9019	82.3179	< 0.0001 ***
Food×Depth	1	0.4310	0.4310	6.0110	0.0170 *
Food×Temp	1	0.3462	0.3462	4.8282	0.0317 *
Depth×Temp	1	0.1089	0.1089	1.5187	0.2224
Depth×Sex	1	0.2092	0.2092	2.9173	0.0926 .
Food×Depth×Temp	1	0.2350	0.2350	3.2775	0.0750 .
Residuals	63	4.5168	0.0717		

"."$p < 0.1$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 5.3. Mean measures of lipid and glycogen content in An. gambiae with 95% confidence intervals.

Body constituent	Mean	95% confidence interval
Total lipids	181.50	± 12.23
Calories from lipids	1.63	± 0.11
SSCC from lipids	0.67	± 0.07
Glycogen	5.79	± 0.89
SSCC from glycogen	0.009	± 0.002

SSCC = size-specific caloric content, a size-adjusted measure.

Figures

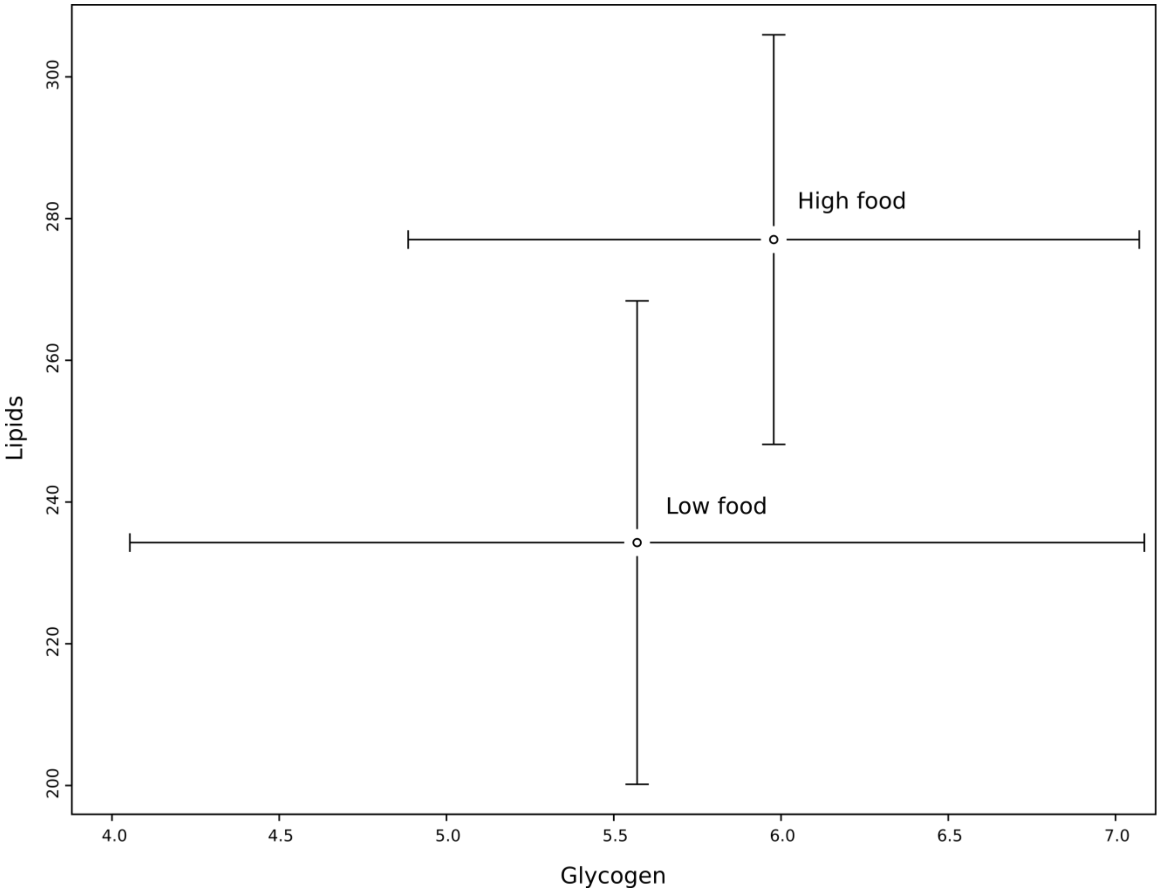

Figure 5.1. Mean lipids and glycogen in µg plotted as a bivariate response to food level.

Low food corresponds to 0.2 mg/larva/day and high food to 1.4 mg/larva/day. Note that data were adjusted to remove an effect of processing date on lipids (see *Methods*).

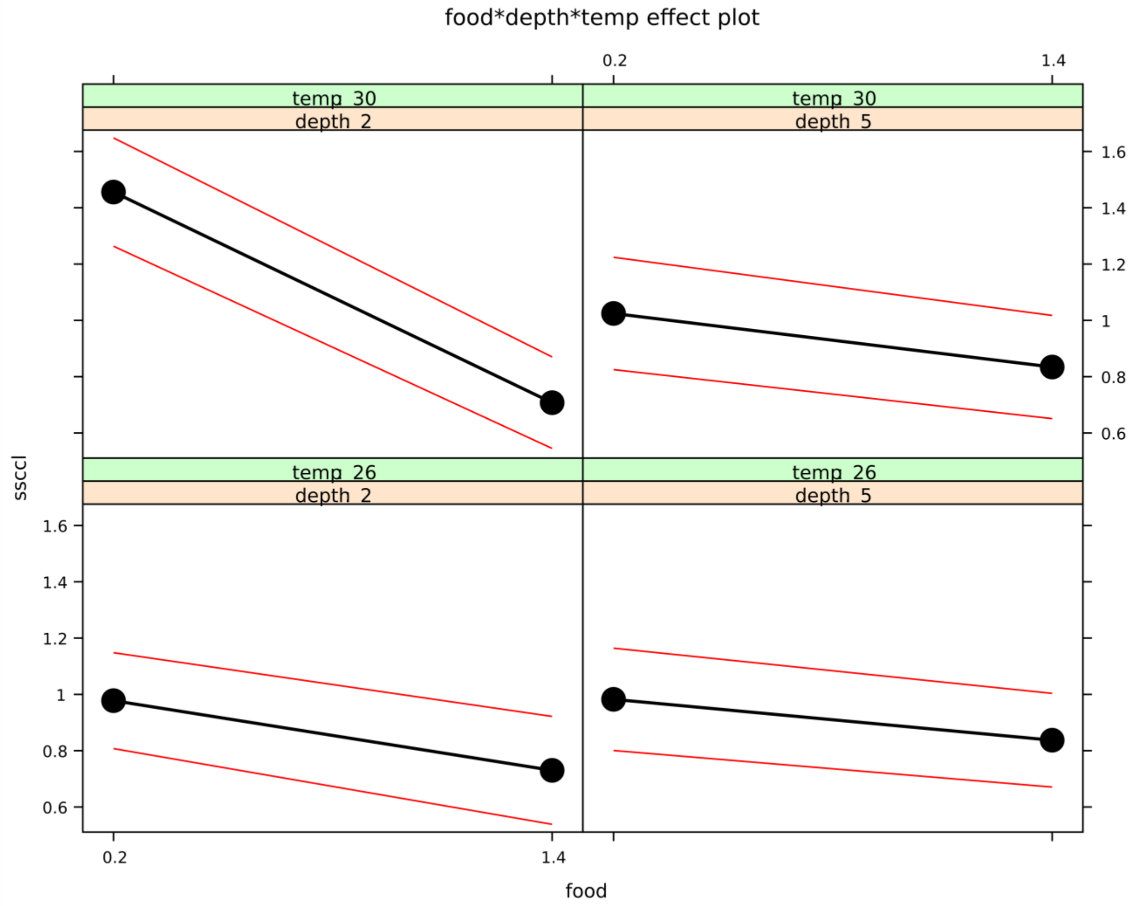

Figure 5.2. A three-way interaction between food, water depth, and temperature.

Figure shows adjusted means with 95% confidence intervals for size-specific caloric content from lipids of newly emerged *An. gambiae*. Units are mg for food, cm for depth, and °C for temperature.

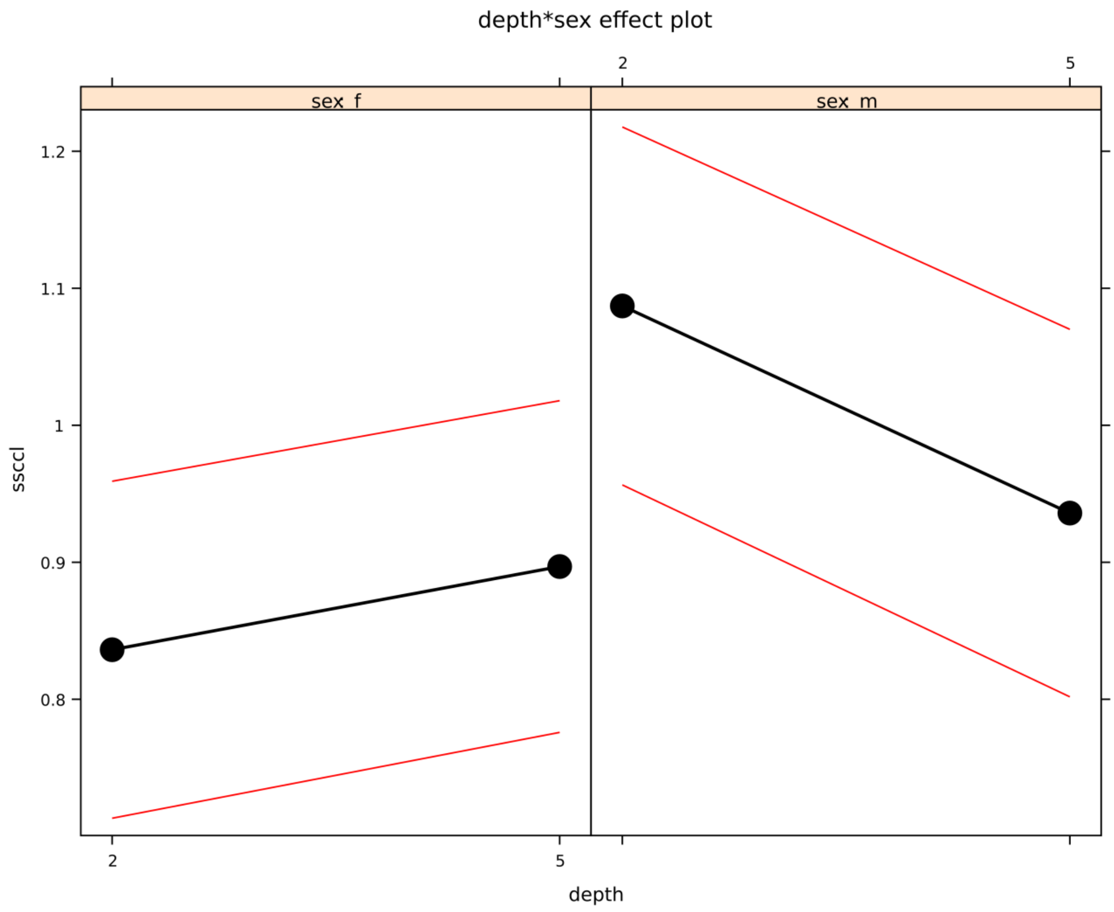

Figure 5.3. Effect plot of two-way interaction between depth and sex for size-specific caloric content from lipids of newly emerged An. gambiae.

The figure shows adjusted means with 95% confidence intervals (Fox, 2003). Units for depth are cm.

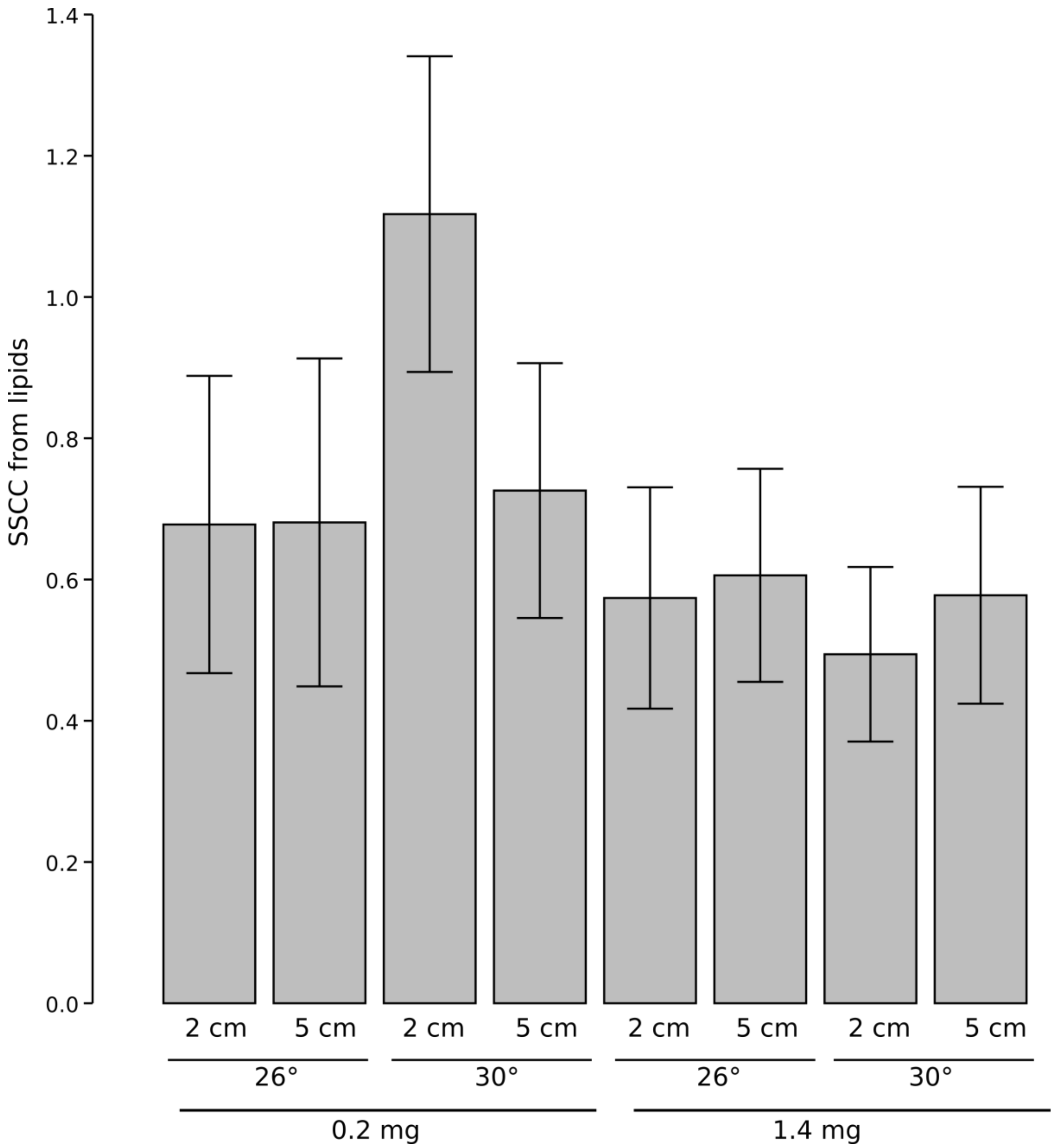

Figure 5.4. ***Mean size-specific caloric content from lipids by treatment. Bars indicate 95% confidence intervals.***

Note that lipid values were adjusted to account for an artificial effect of processing date.

Connecting statement

In this next, final data chapter the focus moves away from plasticity of life history traits to briefly consider how environmental conditions affect larval diving behaviour. Conditional behaviour may be thought of as the final word in plasticity, allowing organisms to adjust immediately to new conditions; by modifying their activity *An. gambiae* larvae may effectively alter their environment.

Chapter 6

Effects of food, water depth, and temperature on diving activity of larval *Anopheles gambiae sensu stricto*: evidence it dives to forage

Abstract

Anopheles gambiae larvae have frequently been observed to dive, but the ecology of this behaviour has not been extensively examined. We manipulated food level, water depth, and temperature for individually-reared larvae and observed diving activity. Larvae dived more often under low food, which suggests that they dive to forage. There was only weak evidence for effects of water depth or temperature on diving activity. Our analysis suggested that these two factors influence dive duration and residence time at the container bottom and we briefly discuss potential causes for such effects in the context of energy budgets. Our evidence that larvae of this important vector of human malaria adjust their behaviour to better acquire resources underscores the idea that understanding larval ecology of this species is important for predicting how it will respond to environmental change.

Introduction

Performance of the aquatic larval stage of the malaria vector *Anopheles gambiae sensu stricto* (herafter *An. gambiae*) affects how abundance and distribution of this medically important species will respond to changes in climate and land-use (Gimnig et al. 2001; Yasuoka & Levins 2007; Chaves & Koenraadt 2010). An aspect of larval ecology that has not been extensively studied for this species is the behavioural ecology of diving (but see Tuno et al. 2004, 2007; Futami et al. 2008). *An. gambiae* larvae have often been observed to dive but this behaviour is poorly understood. One possibility is that they dive to forage (Tuno et al. 2007). *An. gambiae* has often been assumed to feed exclusively at the water surface (Wotton et al. 1997; Gimnig et al. 2002), but this notion is largely inferred from characteristics of other species of *Anopheles* (Walker & Merritt 1993; Wotton et al. 1997). Furthermore, a 1992 review on the feeding ecology of larval mosquitoes has cautioned against classifying species into narrow feeding categories (Merritt et al. 1992). There is evidence that suggests bottom-feeding is important for *An. gambiae*; it was found to dive to the bottom of water columns much more frequently than *Anopheles funestus* and to crawl on the bottom more actively than *Anopheles arabiensus* in a study that compared diving activity among these three species (Tuno et al. 2007). *An. gambiae* larvae have also been shown to generally perform better in shallow water; Timmermann and Briegel (1993) found in a laboratory study that *An. gambiae* showed highest rates of survival in water less than 1 cm deep and did not survive to maturity in water deeper than 5 cm. This species tends to occupy shallow, ephemeral habitats best described as puddles (Ndenga et al. 2011). It has been speculated that *An. gambiae* prefers such habitats because they harbour few, if any, predators (Minakawa et al. 2004; although there is evidence for cannibalism in this species (Koenraadt et al. 2004)). Another potential benefit provided by shallow water is ready access to the bottom for foraging.

The environmental variables food availability, water depth, and water temperature have all been shown to influence larval growth and development and are all potentially relevant to both diving behaviour and climate change. Food availability is germane to whether larval diving is a foraging activity for the obvious reason that the purpose of foraging it to acquire food. In general, as food becomes more available less activity is needed to acquire it (Anholt et al. 2000). Thus if diving by *An. gambiae* reflects foraging activity we would expect greater food availability to reduce levels of diving. Water depth is relevant to diving because it directly determines swimming distance to food deposited at the habitat bottom and will affect energetic costs and benefits of diving. Temperature determines metabolic rate in poikilotherms such that higher temperature generally increases both rate of energy consumption and activity levels (Kooijman 2009). Temperature is potentially relevant to diving activity because the effects it has on metabolism influence costs and benefits of foraging, which may in turn influence activity levels in a way that is reflected in behaviour. Finally, since each of these three factors affects the energy budgets of individuals it is interesting to consider whether their impacts on diving behaviour are independent of one another.

We conducted a factorial experiment in which we manipulated food level, water depth, and water temperature for larval *An. gambiae*. We made video recordings of larvae and examined how these three factors influenced diving activity.

Methods

Larval *An. gambiae* were reared individually in vials in a factorial experiment using food level, water depth, and water temperature. Larvae were exposed to two levels of each factor for a 2×2×2 design. The two food levels were 0.2 and 1.4 mg/larva/day (Nutrafin basix staple for tropical fish, Hagen Inc). Water depths were 2 and 5 cm. Water temperatures were maintained using a system of water baths at either 26 or 30°C. Refer to Chapter 4 for greater detail of the experimental set-up.

Video recordings of larvae were taken to assess diving activity. For recording, vials were temporarily moved from their temperature-controlled water baths to an observation bath of the same temperature that had a transparent wall. Larvae were allowed to recover from this possible disturbance for one minute and then recorded for 10 minutes. Multiple response variables were extracted from the video. We measured frequency and duration of dives because we expected that an increase in foraging activity would be reflected by more frequent or by longer dives. To deal with zero-inflation of the dive frequency data we further considered the binary response of whether or not larvae were observed to dive in a recording (see *Statistical analysis* below). We also measured the number of times larvae made contact with the container bottom and how long they stayed on the bottom because we expected the food resource to sink and accumulate. Recordings were taken over a one week period corresponding to days five through 12 of larval development.

Statistical analysis

Generalized linear models (GLM) were used to analyze five different response variables, 1) whether larvae were observed to dive, 2) the frequency larvae dived, 3) duration spent below the water surface, 4) the frequency larvae contacted the container bottom, and 5) how long they remained on the container bottom. Analyses were conducted using R version 2.12.1 for Linux (R-Development-Core-Team 2010). Backward selection was used achieve reduced models; beginning with highest-order interactions we sequentially removed terms with the greatest p-value and compared each resulting model to the previous model with a likelihood-ratio test or Chi-square test (using R's anova function) at $\alpha = 0.05$ (Quinn & Keough 2002; Crawley 2007). We supplemented this process with information theoretic model comparisons based on AICc using the MuMIn package in R for instances where p-values from our various model comparisons and estimates of factor significance were near 0.05 (Anderson & Burnham 2002; Johnson & Omland 2004). Once relevant factors were identified with model selection, R's effects package was used to generate adjusted means and confidence intervals to assess the effect of individual factors (Fox 2003).

The data for larval dive frequency and duration were zero-inflated. To deal with this, we conducted an analysis in which we considered diving a binomial response, coding dive durations into dived (1) and didn't dive (0). For these binomial data we used the lmer function from R's lme4 package and applied a generalized linear mixed effects model with the Laplace approximation (Crawley 2007). The random component of our model was vial id to account for multiple videos taken of the same individuals.

In the GLM for dive frequency, the number of dives made per recording, we specified a Poisson error distribution and included larval id as random factor to deal with repeated measures (Crawley 2007). We excluded records in which there were no dives (zero counts) from our analysis because our model for the binary data had already identified factors relevant to the presence and absence of dives.

To analyze the dive duration data we calculated mean duration within recordings as well as within individuals to avoid pseudoreplication and analytical complications arising from specifying both a gamma distribution and a random effect of repeated measures on individuals (Bolker 2011). We applied a GLM specifying a gamma distribution which is appropriate for time-to-event or duration data (Crawley 2007).

For our analysis of the number of times larvae contacted the container bottom we used a GLM with a Poisson error distribution, a square-root link function, and larval id as a random factor. Records in which larvae did not dive were excluded — this response variable measured touches per dive rather than per recording.

Finally, we examined how long larvae remained on the vial bottom. We used a GLM assuming a gamma distribution. As for dive duration, we averaged bottom residence time by both recording and individual to avoid pseudoreplication and complications from repeated measures.

Note that sexed individuals represented a subset of the individuals that were observed to dive because not all larvae survived to adulthood, the point at which they could be sexed. This meant we could not use information theoretic tools to compare models that included sex to those that used the larger data set in which sex was excluded. We dealt with this by limiting our initial analyses to subsets of the data for which individuals were sexed. If sex dropped out during the model selection process we restarted the analysis using the full data set excluding sex. Sex was dropped from all of our analyses.

Results

The best model for whether or not larvae were observed to dive included only food (Table 6.1). A higher proportion of larvae were observed to dive under low food than high food (23 and 80% respectively; 95% CI for the difference between these two proportions = 39-75%).

For larvae that were observed to dive at least once, the number of dives per recording was also found to be affected only by food such that dive frequency was greater under low food (Table 6.1, Figure 6.1). The adjusted mean number of dives per recording was 1.21 under high food and 2.68 under low food.

When we considered the duration of dives, our stepwise model selection process resulted in the null model indicating that our experimental factors did not explain dive duration. However, model comparison based on AICc identified the best model included food, depth, temperature, and food-by-depth interaction (Table 6.1, Figure 6.2 and Figure 6.3). A Chi-square test indicated that this model was significantly better than null ($p < 0.02$).

The number of bottom contacts per dive was not influenced by the experimental factors.

The best model for bottom residence (how much time larvae spent on the vial bottom per dive) retained water depth and temperature as explanatory variables (Table 6.1, Figure 6.4 and Figure 6.5).

Discussion

Broadly, our results indicate that diving activity of *An. gambaie* larvae was affected by our three experimental factors, food availability, water depth, and water temperature. We briefly discuss the various responses we detected in the context of fitness associated with forage diving. Recall that our predictions were based on foraging theory that assumes a trade-off between increased risk from foraging activity and growth and survival benefits of acquiring food.

Larvae dived more frequently under low food. This effect of food on the presence and frequency of diving activity (Table 6.1, Figure 6.1) supports speculation that *An. gambiae* larvae dive to forage (Tuno et al. 2004, 2007). Models that assume a trade-off between growth and mortality generally predict that greater activity levels increase rates of food uptake (Anholt et al. 2000). The benefit of increasing activity levels is that more resources are acquired. However, the costs of increased activity are twofold; first the act of diving uses up metabolic energy, and second, the probability of encountering and being killed by a predator increases with activity level. Thus if larvae are diving to forage we expect them to exhibit greater diving activity under low food. While we found that larvae dived more often under low food, duration per dive was not found to be greater (Table 6.1, Figure 6.2). This lack of effect on duration suggests that if larvae are diving to forage they are not taking more time in the water column or on the container bottom to capture or ingest food.

Deeper water increases the distance that bottom-foraging mosquitoes must swim to obtain food and is expected to increase costs in terms of both energy and time. If diving represents a foraging activity for *An. gambiae*, and oxygen limitation is not relevant (which it may be, see Reiter 1978), we would expect larvae to respond to increased water depth by taking fewer dives of longer duration to increase food uptake per dive and offset increased costs. We found that depth did not affect dive frequency but that it may have increased bottom residence time (Table 6.1, Figure 6.4, p = 0.090). Depth was also identified as a factor potentially affecting dive duration (Table 6.1, p = 0.067), but here depth decreased duration under low food and increased it under high food (Figure 6.2, p = 0.092). Since we expected deeper water to increase dive duration it is interesting that our data suggest that dive duration decreases with depth under low food. However, it seems plausible that reduced energetic condition or reduced benefits of foraging from low food could produce such an effect. We would generally expect larvae to spend less time diving at the point where the return from foraging activity becomes less than or equal to its cost.

Water temperature was found to potentially affect two of the five responses we considered, dive duration and bottom residence time. We expect higher ambient temperatures to generally increase both metabolic rate and activity level in mosquitoes. Metabolic rates in poikilothermic organisms are affected by temperature such that raising the temperature is analogous to speeding up time (Kooijman 2009). So we expected higher temperature to increase the amount of food that larvae use per unit time and consequently larvae would forage more intensively, either engaging in more frequent dives or spending more time foraging at the container bottom. We found neither. Diving frequency was unaffected by temperature and both dive duration and bottom residence time were lower under the higher temperature (Figure 6.5 and Figure 6.3). A potential explanation for these observed decreases in diving activity at higher temperature is that activity rate was increased causing larvae to complete foraging tasks more quickly. Another possibility is that smaller body size caused by higher developmental rate resulted in a relative reduction of energy needed for maintenance, thus reducing the need to forage.

Mechanistic models that explicitly track energy acquisition and consumption may be useful for predicting how larval mosquitoes will respond to their environment (Buckley et al. 2010), and diving behaviour may provide useful insights into how larvae use and manage resources and metabolic energy. Lucas and Romoser (2001) found that *Aedes aegypti* and *Aedes albopictus* pupae that were stimulated to dive frequently used substantially more energy than unstimulated ones (had ~ 10% less energy than controls). We have previously observed that the effect of increased water depth on development of *An. gambiae* is similar to that of reduced food (see Chapter 4). Our current results show increased rates of diving under low food (Figure 6.1). And food and water depth do not act independently upon age and size at emergence (see Chapter 4). Because our three experimental factors are all relevant to larval energy budgets we were curious whether they would affect diving behaviour independently. The final models for our measures of diving activity only contained one interaction term for one measure of diving activity, depth-by-food for dive duration (Table 6.1). While it is reasonable that food and depth would be non-independent with respect to dive duration, we do not have an obvious explanation for the relationship we observed (Figure 6.2). Diving is well-studied in mammals and birds but theories from this arena have not been widely applied to insects. Time-energy budgets estimating energy expenditure and requirements might improve understanding of how environmental factors affect diving activity and performance of larval mosquitoes.

It is possible that the effect of food level on diving activity we have observed was not relevant to natural systems – our experimental culture has been maintained in a lab for many years and fish-food may differ substantially from this species' natural foods both in nutritional composition and spatial distribution. Alternate or supplemental explanations for *An. gambiae* diving behaviour include avoiding predation (Futami et al. 2008) or physical disturbances (Paaijmans et al. 2007), or regulating temperature (Bayoh & Lindsay 2004; Paaijmans et al. 2008), oxygen (Reiter 1978), and exposure to sunlight, including UV radiation (Tuno et al. 2005); further research is needed to properly assess the ecological role of bottom feeding for *An. gambiae*.

Conclusion

Our results provide support for the idea that bottom foraging is ecologically important for *An. gambiae* larvae. We found a clear pattern of increased frequency of diving under lower food, which is consistent with increased foraging effort. However, evidence for effects of water depth and temperature were comparatively weak and it is unclear these two factors are relevant to diving behaviour for this species.

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Tables

Table 6.1. Generalized linear models for significant response variables (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\dagger}p < 0.1$).

Response	Fixed effects	Estimate	S.E.	*z*-value	*p*-value
Dived[a]	intercept	0.450	0.216	2.081	0.038 *
	food	-2.140	0.494	-4.336	<0.001 ***
Dive frequency[b]	intercept	1.638	0.060	27.263	<0.001 ***
	food	-0.537	0.190	-2.825	0.005 *
Dive duration[c‡]	intercept	0.041	0.012	3.501	<0.001 ***
	water depth	-0.023	0.012	-1.864	0.067 †
	temperature	0.022	0.011	2.016	0.048 *
	food	-0.020	0.020	-1.016	0.314
	depth*food	0.066	0.039	1.709	0.092 †
Bottom residence[d]	intercept	0.038	0.014	2.725	0.009 **
	water depth	-0.025	0.015	-1.733	0.090 .
	temperature	0.026	0.013	1.950	0.058 .

[a] whether or not individual larvae were observed to dive in a recording, assuming binary error distribution, larval id as random factor.

[b] number of dives per recording for larvae that were observed to dive, assuming Poisson error distribution with square-root link, larval id as random factor.

[c] average duration of separate dives by recording and individual, assuming gamma error distribution.

[‡] stepwise model selection converged on the null model for this response variable. The model reported here was identified using information theoretic methods.

[d] average duration spent on the vial bottom per dive by recording and individual, assuming gamma error distribution.

Figures

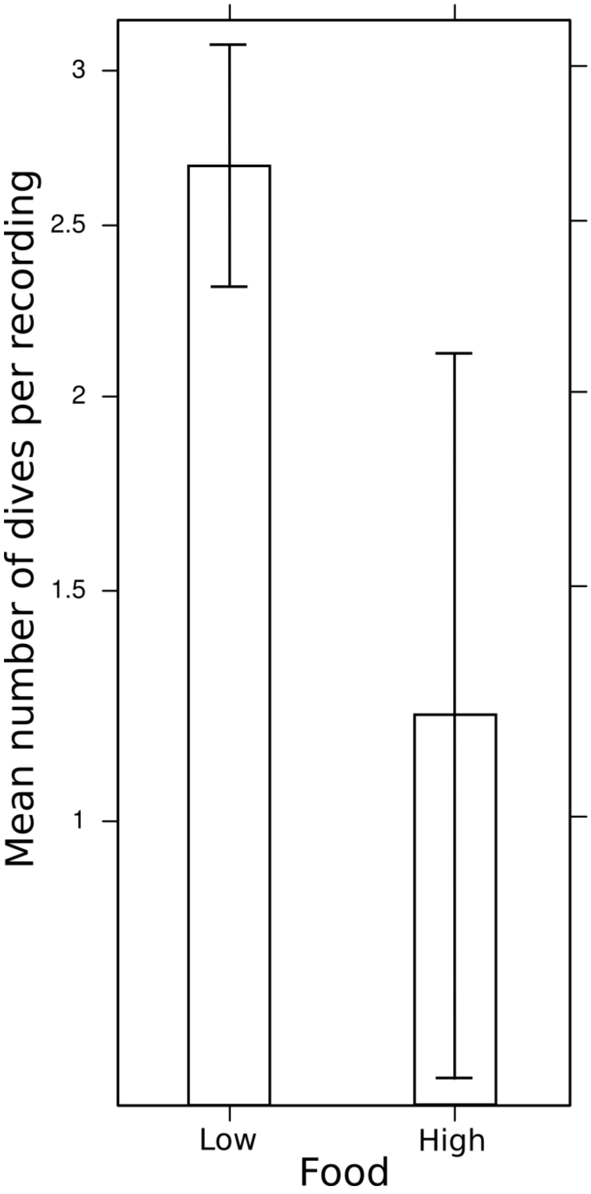

Figure 6.1. Adjusted mean number of dives under low and high food for recordings in which there was at least one dive.

Confidence intervals = 95%. The adjusted mean number of dives was 1.21 under high food and 2.68 under low food. Levels and axes were determined with R using the effects package (Fox 2003; R-Development-Core-Team 2010). Vertical axis is labeled on the scale of the response variable but plotted on the scale of the linear predictor.

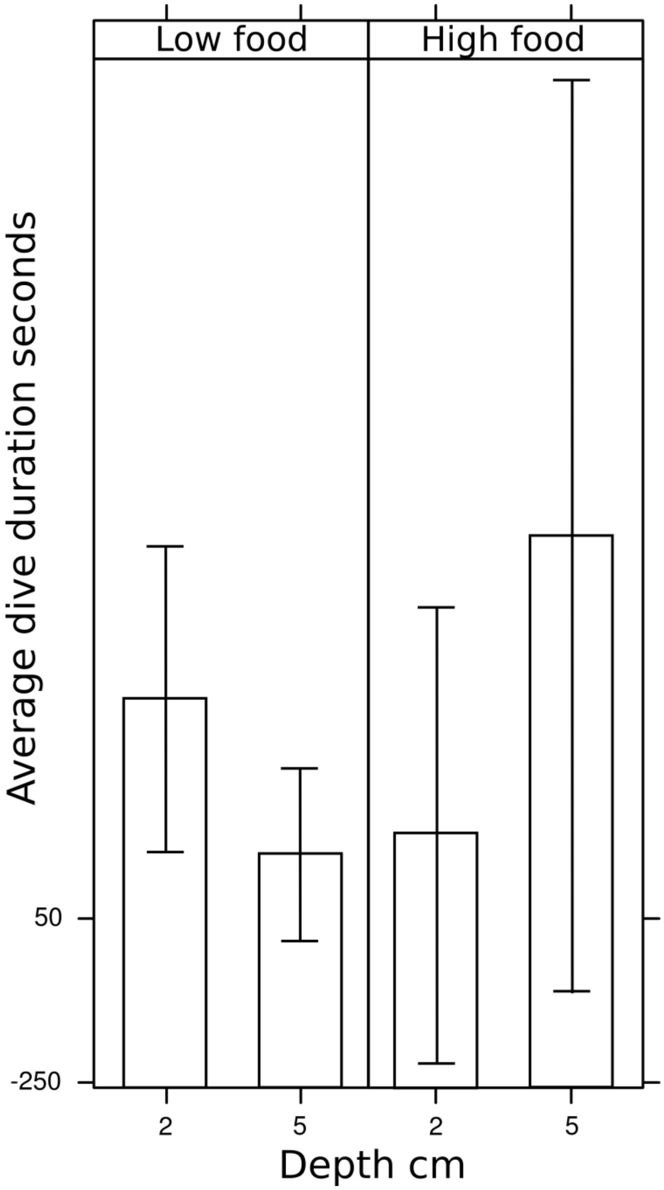

Figure 6.2. Adjusted mean dive duration in response to depth and food, with 95% confidence intervals.

Low and high food were 0.2 and 1.4 mg/larva/day respectively. Levels and axes were determined with R using the effects package (Fox 2003; R-Development-Core-Team 2010). Vertical axis is labeled on the scale of the response variable but plotted on the scale of the linear predictor.

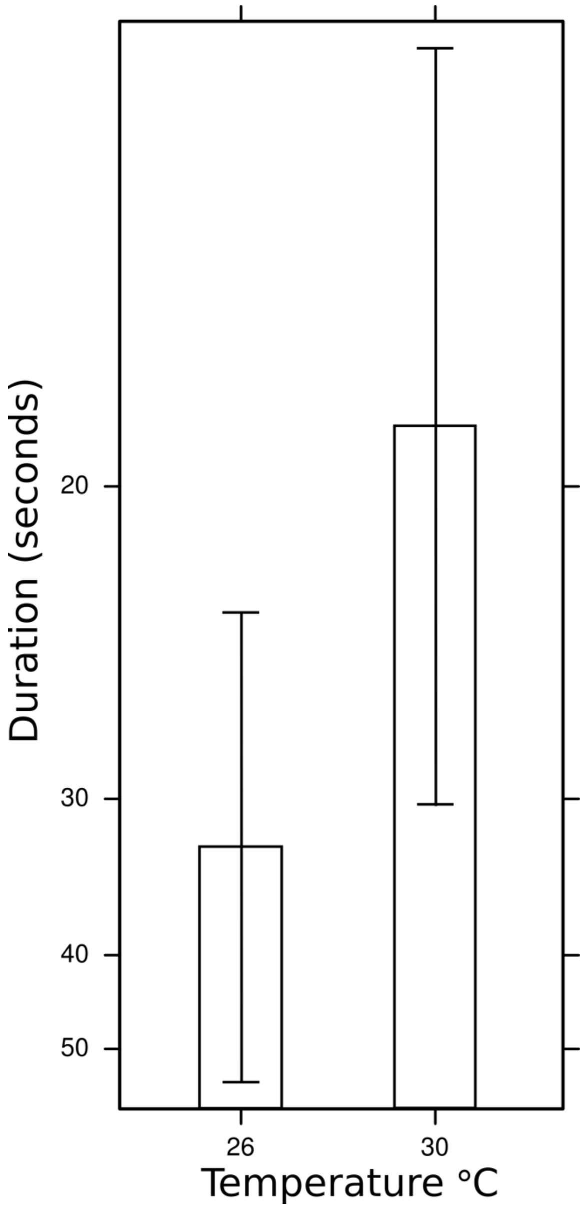

Figure 6.3. ***Adjusted mean dive duration in response to temperature, with 95% confidence intervals.***

Levels and axes were determined with R using the effects package (Fox 2003; R-Development-Core-Team 2010). The y-axis is non-linear and descending because it is labeled on the scale of the response variable but plotted on the scale of the linear predictor.

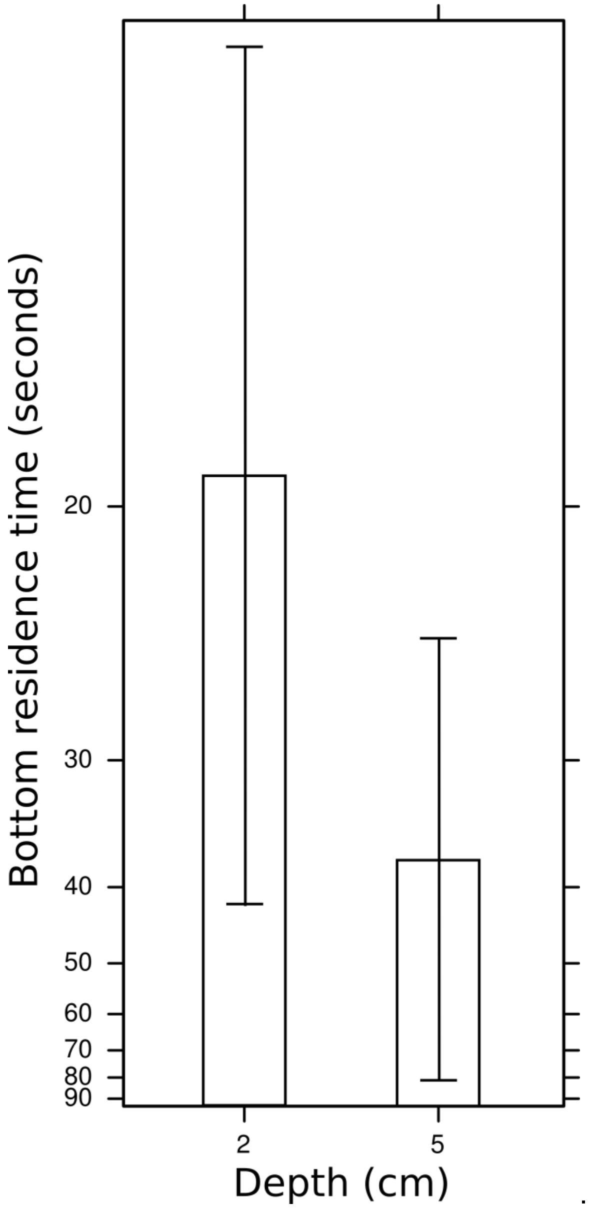

Figure 6.4. Adjusted mean bottom residence time in response to water depth, with 95% confidence intervals.

Levels and axes were determined with R using the effects package (Fox 2003; R-Development-Core-Team 2010). The y-axis is non-linear and descending because it is labeled on the scale of the response variable but plotted on the scale of the linear predictor.

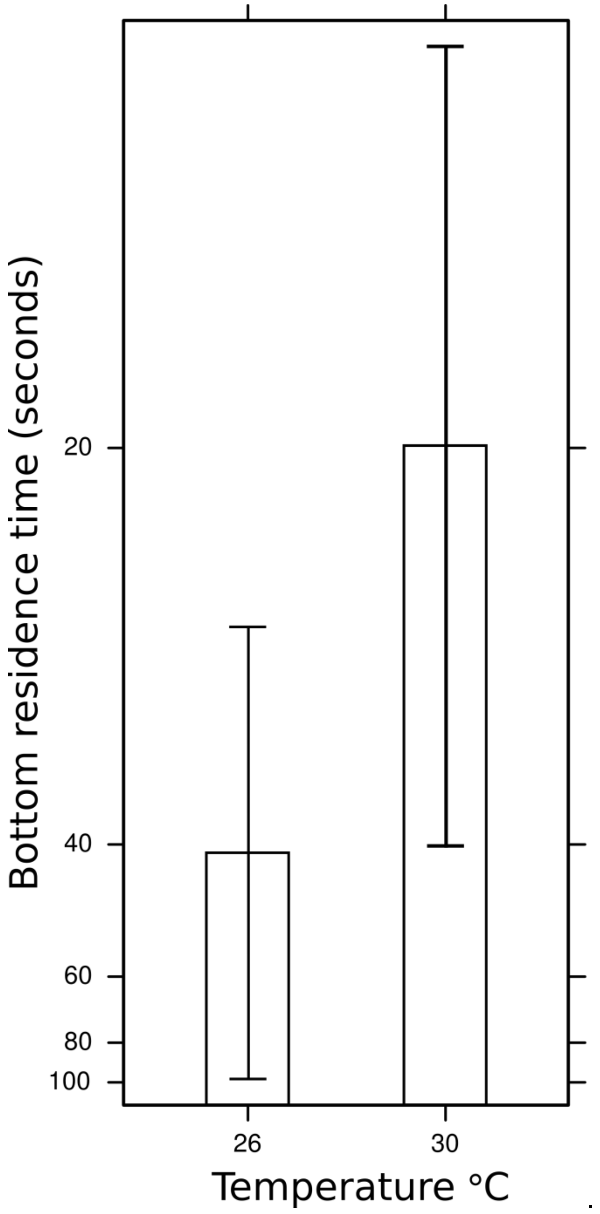

Figure 6.5. Adjusted mean bottom residence time in response to water temperature, with 95% confidence intervals.

Levels and axes were determined with R using the effects package (Fox 2003; R-Development-Core-Team 2010). The y-axis is non-linear and descending because it is labeled on the scale of the response variable but plotted on the scale of the linear predictor.

Chapter 7

Conclusion

I have applied theory of optimal age and size at emergence to understanding how complexities associated with the aquatic larval growing environment influence larval growth and development of *Anopheles gambiae sensu stricto*. There are two major, non-independent components that make up the larval ecology of mosquitoes. First, there is the multifaceted, often randomly variable, environment. And second, there is the evolved non-random, yet flexible and complex, biology of the mosquito. I have attempted to explore the interface between these two parts. A general conclusion that may be taken from this effort is that both a growth-mortality model of optimal age and size at emergence as well as an explicit consideration of the energetic effects of different environmental factors are useful for understanding why and how *An. gambiae* responds the ways it does to various larval conditions.

Chapter 2 described a dynamic state variable model that was developed to assess the general importance of variable resource availability for predictions of optimal age and size. It was found that random variation of food around a mean level did not strongly affect predictions of optimal growth and development for individuals. Introducing risk of starvation caused growth to occur submaximally in order to allow for a reservoir of metabolic energy that could be used in the event of a temporary shortfall in food. This tactic mitigated potential costs of uncertain food availability to the degree that optimal emergence differed little between a constant food scenario and a variable one with an equivalent mean food level. Interestingly, introducing the element of uncertainty caused individuals to delay the larval-to-adult life history transition because fitness benefits from encountering a large amount of food by chance were non-linear, making it worth the risk of staying in the growing environment a little longer. The overall effect of this delay upon the pattern of age and size was, however, not very pronounced. This investigation required a mechanistic account of energy acquisition and allocation. Under the growth submodel that was used, the relative energetic value of becoming larger changed under different resource levels, and remaining small and accumulating energy for future growth was the best thing to do under low resources, even though becoming larger allowed an individual to acquire more food.

Chapter 3 described a larval rearing experiment in which mean daily food levels for *An. gambiae* were manipulated to switch either upward or downward at either earlier or later points during the larval period. Predictions made using a general optimality model grounded in the trade-off between growth and mortality accounted well for observed size at emergence and less-well for observed age. From the experiment, final size was determined by the food level that larvae experienced during the latter portion of their development (Figure 3.2). That is, larvae that were exposed to a switch from low to high ended up becoming the same size as larvae that experienced high food for their entire development. And conversely, larvae that went from high to low had the same final size as larvae under constant low food. For age at pupation, the effects of the treatments differed between low-to-high and high-to-low switches. Going from low to high, there was only a small effect on observed age at pupation, such that pupation was slightly delayed. While this effect occurred in the same direction as predicted by the model, it differed substantially in magnitude. In contrast, under a high-to-low switch pupation occurred earlier than under constant low food, which was as predicted. However, there was again a large difference in magnitude between observed and predicted age at pupation. There are a few potential biological causes for these discrepancies between prediction and observation. In the case of a low-to-high switch, larvae appear to have increased their rate of growth in response to the change in food availability, a well-known phenomenon called compensatory growth (Metcalfe & Monaghan 2001). Such growth is generally expected to come with fitness costs, but these costs have often proven to not be readily apparent. In the case of the high-to-low switch, larvae took longer than expected to mature. One possible explanation for such a delay is that the developmental machinery of the mosquito is not plastic enough to respond immediately to such a change in environmental conditions. Another possibility is that the relatively large size that the larvae had achieved under high food suddenly becomes relatively costly to maintain under low food, resulting in fewer available resources for the process of maturation. Yet another possible explanation for the discrepancies between observed and predicted age at pupation under a switch from high to low lies in the mosquito's imperfect knowledge of its environment; from the mosquito's perspective, environmental conditions have switched downward once and it is possible they could switch back upward. Remaining in larval form increases opportunity to take advantage of improvements in growth conditions.

In Chapter 4, I described a factorial experiment in which individual larvae were reared at either high or low levels of three environmental factors, food level, water depth, and temperature. This chapter considered the effects of these factors upon larval survival, and age and size at emergence. Only food was found to affect survival, with more food generally improving the chances for developing larvae. All three of the experimental factors, as well as sex, affected the life history traits age and size at emergence. Moreover, there were several statistically significant interactions among the various factors (refer to the chapter itself for specific results). An important outcome of this effort was the finding that while several of the observed effects were context-dependent (i.e., the influence of one factor depends on the level of another), some of these interaction effects could be understood by considering the energetic influence of individual factors in the context of an L-shaped relationship between age and size across different environments (Day & Rowe 2002). The vertical part of the L reflects individuals that have experienced growth conditions that were generally favourable energetically. And within this group, factors that affect development will generally influence *size* but not age at emergence, which will be minimal. Conversely, the horizontal part of the L shows individuals that have experienced a growing environment that was generally poor energetically. Within this group, factors affecting development will influence *age* at emergence but not size, which will now be minimal. This insight into how and why age and size at emergence respond to factors differently depending on the environmental context may be valuable for understanding how populations will respond to new environments. These insights are especially important in light of the fact that age and size each have different implications for population level processes. Another interesting outcome of Chapter 4 is that larval rearing temperature was found to be fundamentally different from the two other experimental factors, food level and water depth.

Temperature appears to effectively shift the minimum size (and perhaps age) threshold for the L-shaped age-size curve.

Chapter 5 was an extension of Chapter 4. Levels of lipids and glycogen were measured for newly emerged adults from the factorial rearing experiment. This topic is of interest because energetic condition has important implications for both population level processes of *An. gambiae*, as well as epidemiology of malaria (Ma & Roitberg 2008). However, no effects of the experimental treatments upon the measures of lipids and glycogen were detected. This lack of relationship may have resulted from a combination of limited sample size and the typically high variability of such measurements for mosquitoes. Surprisingly, there was also no relationship between body size (measured as wing length) and the estimates of metabolic constituents. This lack of relationship was surprising because other studies have consistently found size to be correlated with lipids and glycogen (Blanckenhorn et al. 2007), and the sample size should have been adequate to detect this simple relationship. A possible explanation for this unexpected result is that the eight experimental treatments did affect levels of metabolic constituents such that lipid and glycogen levels became decoupled from size.

Chapter 6 was yet another outcome of the rearing experiment described in chapter 4. Here, video data were used to assess effects of food, water depth, and temperature upon diving behaviour of *An. gambiae*. The main finding of this work was that larvae dived substantially more under low food. Such an effect is consistent with increased foraging activity (Anholt & Werner 1995), suggesting that larvae dive to acquire food from the bottom of the water column. This species is commonly assumed to feed at the water surface and this result points toward an alternate conception for its larval ecology. The idea that *An. gambiae* acquires its food from the habitat bottom is supported by observations from other studies that it does not survive in water more than a few centimetres deep (Timmermann & Briegel 1993). Also, diving for food introduces questions about how water depth and levels of dissolved oxygen influence energy budgets of developing larvae. Understanding how and whether this species will be able to exploit available aquatic habitats in new environmental contexts may benefit from development of time-energy budgets similar to those applied to other classes of diving animals.

An accurate model of individual larval growth and development that could be applied to predicting changes in populations of *An. gambiae* would be highly desirable for applications in public health. However, rather than attempting to develop such a model, this body of work has examined some of the ecological complexities that typically make development of useful models challenging. Chapters 2 and 3 were concerned with environmental variability, while Chapters 4 through 6 addressed complexity that arises from the presence of multiple environmental variables. A prominent theme throughout this work has been the application of the growth-mortality framework to understanding developmental outcomes. In Chapter 3 it became clear that this simple framework, while useful for understanding broad patterns in age and size at emergence, was not alone sufficient to accurately predict *An. gambiae's* responses to particular ecological scenarios. Similarly, in Chapter 4 the growth-mortality framework provided a useful context for understanding statistical interactions among environmental factors that influenced age and size at emergence but did not offer a means of accurately predicting how developing mosquitoes will respond to new combinations of factors. The growth mortality framework provides better understanding of how larval *An. gambiae* react to their environment, but it does not provide the detailed mechanistic understanding of development that would be needed for applications in public health. This body of work has, however, touched upon an approach that shows some promise in this respect.

Explicit consideration of energy budgets offers a potentially valuable approach to understanding responses by larval mosquitoes to a complex world. During the development of the dynamic state variable model presented in Chapter 2 it was necessary to consider energy budgets in order to understand how individuals respond to environmental variation. Similarly, in Chapter 4, considering how different experimental factors influenced energy budgets of individual larvae provided a means of interpreting complex observed effects upon age and size at emergence in the context of the L-shaped age-size relationship. Understanding an organism's biological responses to multiple independent environmental factors becomes much simpler if many or all of those factors can be reduced to their influence upon the energy of an individual. And as demonstrated in Chapter 2, such an explicit energetic approach can be couched within a broader fitness-based framework. This idea is not novel. Kooijman (2010) has been developing a system of models that uses this approach for over 30 years. The work presented in this thesis suggests that there is potential benefit to applying this existing set of tools, called Dynamic Energy Budget theory, to understanding dynamics of important vectors of human disease such as *An. gambiae*.

Considering that malaria is transmitted by adult mosquitoes, it may seem frivolous to focus on the ecological complexities of the larval rearing environment. However, as discussed in Chapter 1, models grounded in biological mechanisms rather than strictly empirical measures offer the potential to predict outside of the narrow range in which such measures have been made. Using biological information to predict the impact of climate on population growth rates of mosquitoes will likely require building age-structured population models that make use of the vital rates for different age classes (Stone et al. 2009). I have attempted to provide insight into how these rates are affected by environmental context.

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