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Environmental constraints on the physiology of transgenic mosquitoes

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Abstract

Insects have exploited and responded to their environment in a plethora of ways. Environmental changes are used to trigger short- and long-term physiological events and environmental stresses have resulted in evolution of gene families and resistance genes. This highly evolved, tight interaction between organism and environment will be altered in transgenic mosquitoes, and this paper reviews some potential considerations concerning the physiology of transgenic mosquitoes upon release. This papers examines a few of the recent discoveries in *Plasmodium*-mosquito interactions and discusses the impact upon them of the transgenic-mosquito approach. A complex interplay between vector and parasite occurs during transmission, including the exploitation of xanthurenic acid for triggering exflagellation, the induction of a mosquito immune response and its evasion by the invading ookinete, and the ability of the parasite to establish infections when major genes are knocked out. Such functional redundancy in parasite genes is also demonstrable in the immune and detoxification systems of insects. Consequently, where genes can substitute for one another in a given physiological process, there is potentially significant environmental pressure for differential gene expression. In the transgenic context, such compensatory regulation could work to down-regulate and/or select against a transgene. Conversely, additional environmental triggers could be exploited to select positively for a transgenic mosquito. There is potential for heterogeneity at each stage of the transgenic release strategy, and addressing this will be important if such approaches are not to be scuttled by unforeseen factors that could reduce expression of and selection for the beneficial transgenes.

Keywords: Transgenic mosquito; environmental physiology; gene expression; selection; adaptation

Introduction

The tremendous adaptability of insects to respond to cues and perturbations in their environments is well documented. Over evolutionary time, insects have used day length, temperature and aridity to trigger such physiological events as mating, emergence, diapause and aestivation (Nation 2001). Similarly, in a more recent historical context, insects have counteracted the pressures of insecticide control by expression of resistance genes (Ranson et al. 2002) and exploited monocultures of

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plants and animals by emerging as pests in the agricultural setting (Van Emden 1989). This incredible plasticity of insects raises important questions when considering the development and release of mosquitoes expressing transgenic gene products which block pathogen transmission. Such issues must lead us to consider i) whether gene expression will be as expected in transgenic mosquitoes, ii) what the ecological and physiological costs to the mosquito might be of carrying a transgene; and iii) the importance and influence on the success of transgenic strategies of the heterogeneity and complexity of mosquito populations.

Over the last two decades, molecular biology has provided a huge impetus into research upon insect vectors and the diseases they transmit. Most recently, the completion of genome projects has added important tools allowing the study of genes in their evolutionary contexts and the detailed examination of their interactions and physiological roles (Gardner et al. 2002; Hall et al. 2002; Holt et al. 2002; Hyman et al. 2002). However, the recent advances in our understanding of *Plasmodium*-mosquito interactions and the consequent identification of therapeutic targets, some of which will be reviewed below, have necessarily been considered in restricted laboratory settings, and it behoves entomologists to consider the extent to which laboratory models are useful and can be extrapolated into a field context. Similarly, the recent successes in developing transgenic mosquitoes (Catteruccia et al. 2000; Ito et al. 2002; James 2002; Moreira et al. 2002) may have foreseeable and unforeseeable costs to the insect in terms of fitness and effects on expression of non-target genes. Using some of these recent advances as the starting point, this paper will examine the potential for modifications of gene expression by environmental cues, and explore the influence of the heterogeneity of interactions between the environment and the genome of the transformed mosquito.

Laboratory models and common patterns of infection: useful or misleading?

With few exceptions, the major recent advances in mosquito biology have relied upon laboratory model systems. While such models have sometimes been considered as too far removed from the 'real world' to be of direct relevance, genome projects in particular have clearly dispelled such a viewpoint, developing as they have the biology of vectors and pathogens by sustained and systematic comparative approaches (Christophides et al. 2002; Zdobnov et al. 2002). Indeed, patterns of malaria infections in mosquitoes are consistent across various *Plasmodium*-mosquito combinations (Billingsley et al. 1994; Medley et al. 1993), and such patterns point to the possibility of unifying theoretical approaches to transmission systems. Laboratory models also generate data that are often impossible to extract from the field, and with the back-up provided by 'noise-free' cell and molecular approaches, models can now more than ever be considered the springboard into complex and targeted field studies.

Conversely, rarely is full consideration given to the differences in the biology of model organisms compared to their wild counterparts, and this may lead to misinterpretation of results or over-reliance on results that are less relevant to disease transmission. For example, the first description of a malarial chitinase was in ookinetes of *Plasmodium gallinaceum* (Huber, Cabib and Miller 1991), and its expression during ookinete invasion accepted as the model for parasite passage through the peritrophic matrix (PM) (Shahabuddin et al. 1993; Shahabuddin and Kaslow 1994). Indeed, an antibody to *P. gallinaceum* chitinase binds to micronemes of the ookinete, inhibits chitinase activity, and reduces infection to *Aedes aegypti*

(Vinetz et al. 2000). However, the differences in the structure of the midgut and PM between *Aedes* and *Anopheles* mosquitoes (Billingsley and Rudin 1992) infer that parasites transmitted by these different mosquitoes need different adaptations for traversing the PM. This has since proven to be the case; *P. berghei* chitinase is unaffected by allosamidin, and knocking out the chitinase gene does not prevent infection to *An. stephensi* (Dessens et al. 2001). Thus the *P. gallinaceum* model may be less appropriate to the field situation because, although there are clear similarities in chitinase genes and their processing – no pro-sequence and no chitin-binding domain – in *P. gallinaceum* and *P. falciparum* (Tsai et al. 2001), the midgut environments in which they are expressed are quite different.

Furthermore, model systems are inevitably going to under- or differentially represent the full gene complement that is available in wild populations. For example, our understanding of immune responses in mosquitoes to challenge by microorganisms has expanded explosively in recent years (Collins et al. 1997; Dimopoulos et al. 2000; 2002; Christophides et al. 2002), but has relied heavily upon a strain of mosquitoes selected for the encapsulation phenotype (Collins et al. 1986) that is rare in wild anopheline populations (Billingsley and Charlwood, unpub. obs.). Any strain of any organism bred in the laboratory will have undergone bottlenecks in the selection process that will decrease diversity in its genome. For mosquitoes, the potential removal of natural traits important to our understanding of biology and transmission can only be guessed at. There is similar, if not more dramatic, reduction in complexity in malarial parasites studied in the laboratory. Infections by genotypically different parasites are common in many epidemiological settings (Anderson et al. 2000; Babiker, Ranford Cartwright and Walliker 1999), but almost impossible to study in laboratory models where strain and even clonal infections are the norm. It is necessary, therefore, to recognize both the power and limitations of laboratory models, and accept that the move from laboratory to field studies will throw up a broad spectrum of questions related to issues of heterogeneity that are not currently obvious.

Mosquito-malaria interactions: some recent advances

The understanding of the mosquito-*Plasmodium* interactions has advanced considerably and the completion of the genome sequencing projects for both *Anopheles gambiae* and *Plasmodium falciparum*, plus the development of infrastructure to support post-genomic studies will ensure that such advances continue. An elegant adaptation of parasites to the mosquito is the identification of xanthurenic acid (XA) as the major insect factor triggering exflagellation of *Plasmodium* (Billker et al. 1998). High concentrations of XA are present in the mosquito, especially associated with gut and brain tissues and, coupled with the drop in temperature associated with the transfer of blood from the vertebrate host to the mosquito vector, XA activates gametocytes to initiate the exflagellation process. However, the threshold concentration of XA required for gametocyte activation differs between *Plasmodium* species (Arai et al. 2001), such that in the same vectors activation of the gametocyte but not exflagellation will occur. The equivalent factors influencing gametogenesis of the two major human malarial parasites, *P. falciparum* and *P. vivax*, remain unexplored even for clones and strains of these species, and the potential for variation in XA concentrations between individuals mosquitoes within wild populations could be an important component of variation in transmission.

The *Anopheles gambiae* genome project has nurtured the tremendous progress characterizing mosquito immune responses to parasite infections and demonstrates the power of comparative genomics of model systems (Christophides et al. 2002). In *Drosophila*, immune response pathways have been dissected at the molecular level by exploiting genomic databases coupled to the tremendous power of the mutation libraries in this species (Irving et al. 2001). While equivalent mosquito mutants are not available, comparative EST and genomics approaches have allowed the identification and construction of gene families implicated in the encapsulation response by haemocytes. A set of 46 genes in the Pen1 (*Plasmodium* encapsulating 1) region of the *An. gambiae* genome has been described (Christophides et al. 2002) and other families are being identified based upon gene structure, and by advancing from genomics to expression arrays, proteins from different families can be implicated directly in parasite recognition (Dimopoulos et al. 1997; 2000; 2002). With these necessary markers now in place, the final steps – localization and functional expression – in understanding the immune response can be taken.

The potential importance of the immune response at the midgut cellular level and its evasion by the invading ookinete has been demonstrated. Ookinetes migrate across the surface of the midgut before selecting (by mechanisms unknown) and invading a cell, and trigger in that cell immune peptides, nitric oxide and peptidases associated normally with programmed cell death (Zieler and Dvorak 2000; Han et al. 2000). In order to evade this cellular response, the parasite migrates rapidly through the basolateral plasma membrane, moving away from the site of invasion between the basal lamina and plasma membrane of neighbouring cells (Han et al. 2000). In this so-called time-bomb theory, the ookinete survives in the hostile environment of the mosquito midgut by simply migrating away from the immune response of the invaded cell. The apparent lack of pathological effect on the mosquito of high intensity (>100) infections at the oocyst stage remains an unresolved issue (Sinden and Billingsley 2001), but such studies raise intriguing questions concerning both the molecular mechanisms of invasion and infection of the midgut, and the overriding parasite strategies that allow it to have limited cost to the mosquito in the phase of causing cell death at the point of invasion. The low prevalence of high-intensity infections in the field (Billingsley et al. 1994) is suggestive of a selection mechanism, but the whole interplay between the mosquito immune system and the malarial parasite is at an exciting phase with hypothesis-driven research set to exploit the new databases. Not least will be defining the role of haemocytes in older mosquitoes, the variability in immune-response genes and their controlling mechanisms in wild mosquitoes, the cost of cellular damage by the invading ookinete to the mosquito, and the contribution of immune responses to heterogeneity in transmission.

While transgenic-insect production continues apace, recent successes in producing anophelines refractory to *Plasmodium* transmission have identified new interactive mechanisms between vector and parasite. Two successful transgenic mosquito lines have relied upon a construct using the promoter for the midgut secondary hydrolase, carboxypeptidase (Edwards et al. 2000) and the piggyBac transposon (Handler 2002). This promoter will target transgene products into the midgut lumen as a soluble protein, and transformants have been used to express a 12-mer peptide, SMA1 (Ito et al. 2002) or a bee phospholipase (Moreira et al. 2002), both of which substantially reduce transmission of *Plasmodium berghei* through the transgenic *An. stephensi*. The cost in survival terms to the mosquitoes is not well documented, but the leakiness of the system, i.e. the ability of a cloned parasite to be transmitted through the transgenic

mosquito, raises the spectre of evolution of resistance to the transgene products and the eventual abrogation of the transmission-blocking effect.

A rapid but transient approach to understanding the importance of parasite and vector genes in the transmission process is the use of RNAi to knock out specific gene function. While a direct antisense approach has been used to silence a complement-like gene in a mosquito cell line (Levashina et al. 2001) or genetic approaches used to silence the gene coding for the immune peptide, defensin (Blandin et al. 2002), arboviruses have been successfully modified for targeting genes expressed in *Aedes aegypti* and *Anopheles gambiae* (De Lara Capurro et al. 2000; Shiao et al. 2001). The viruses can be presented by injection or in an infective feed, and have the advantage of systemic delivery of the antisense RNA throughout the mosquito. Sindbis virus carrying antisense to the circumsporozoite protein (CSP) of *P. gallinaceum* reduces parasite load in the salivary glands (De Lara Capurro et al. 2000), confirming the important role of CSP in parasite infection of the mosquito (Menard et al. 1997). RNAi will soon become standard, offering exciting possibilities for routine screening of gene function during development and infection, and is a powerful tool in the search for drug and vaccine targets.

Gene-silencing techniques have proven to be as powerful for understanding gene function in *Plasmodium* and has provided novel insights into the interaction of parasites with their vectors. Throughout most of its life cycle, *Plasmodium* is haploid, so knockout of a single gene will remove functionality (Pace et al. 2000). Further, the gene function can be rescued by insertion of homologous or orthologous sequences from the same or different species, thereby allowing careful examination of genes and their regulatory sequences and even the testing of human vaccine candidates expressing the target genes in model parasites (Waters 2002). The first targets for knockout approaches in *Plasmodium* have been those mooted as potential vaccine targets, including transmission blocking antigens. Cytoadhesion TRAP-related protein (CTRP) knockouts of *P. berghei* are unable to infect mosquitoes to the oocyst stage due to reduced ookinete motility (Dessens et al. 1999), and, unexpectedly, the CTRP knockout strain increases expression of defensin in *An. gambiae*, suggesting that there is a subtle interplay between individual parasite molecules and the mosquito immune system.

In *Plasmodium*, one major area of interest resulting from the genome project is the discovery of gene families. The 48/45 kDa protein of *P. falciparum* was detected originally by antibodies that block fertilization of the parasite within the mosquito (Van Dijk et al. 2001). The rational determination of protein and gene sequences from classic molecular-biology approaches has allowed the interrogation of genome databases, such that a large P 48/45 family has now been described with orthologous genes between species and different members of the family expressed at each stage of the life cycle (Waters 2002).

One or more target molecules?

The identification of gene families and the ability to knockout gene function raise important questions concerning the number of target molecules needed for a successful transgenic strategy. In *Plasmodium*, the P25 and P28 proteins have a partial redundancy of shared function. Knockouts of these proteins, identified originally by transmission-blocking antibodies, will significantly reduce but not completely block transmission and the high homology between sequences suggests similarity in function, and that knocking out of one gene is compensated by expression of the other

(Tomas et al. 2001). Such functional redundancy means that single targets are unlikely to be successful, and that the parasite may already have inherent avoidance mechanisms that would be selected for.

There are examples though where apparently single-target control measures are effective over the long term. In more than 10 years of repeated application, mosquitoes remain susceptible to control by *Bacillus thuringiensis* (Charles and Nielsen-Leroux 2000), yet resistance to the closely related *B. sphaericus* has arisen on multiple occasions in several insect species (Nielsen-Leroux et al. 2002). However, *B. thuringiensis* produces multiple forms of the toxin (Wirth, Georghiou and Federici 1997; Wirth et al. 1998) compared to a single form produced by *B. sphaericus*, evidence again that multiple rather than single targets must be factored in at all stages of the transgenic strategy despite their greater fitness costs to the mosquito.

Costs of transgenesis?

Indeed, we have little idea of the true fitness costs of transgenes to the host insect. The transgene constructs will compete for insertion sites in the genome, possibly with other, naturally occurring transposons. Incorporation of the effector sequences – promoters, markers, control agents, selectors – all add to the burden carried by the construct, leading to an inherent competitive disadvantage in terms of integration and possibly stability. The transgenic insect itself will then be required to produce one or more proteins over and above its normal complement, and these may have up- or downstream effects on protein expression in the insect. Further, the current strategies hijack a mosquito promoter that in turn has potential additional burden on the pathways that control it. To date, of the lines of transgenic *An. stephensi* that have been produced, two survived very poorly compared to parental strains, the SM1 strain has reduced fertility, and the PLA2 strain showed negative traits in fecundity, fertility and adult survival (Ghosh, Moreira and Jacobs-Lorena 2002). Similarly, all four strains of *An. stephensi* carrying just reporter constructs, i.e. without additional transmission-blocking genes, survived a maximum of four generations in competition experiments against 'wild-type' mosquitoes, but an important part of this poor fitness was attributed to loss of diversity during the necessary inbreeding that takes place during selection of the transgenic phenotype (Catteruccia, Godfray and Crisanti 2003).

While it is intuitively obvious that there will necessarily be costs to the mosquito of carrying foreign genes, the same may also be true of recessive genes that are selected within laboratory strains. Insecticide-resistant mosquito strains are notoriously difficult to maintain, and the rarity of the melanization phenotype in the wild suggests that it is naturally selected against. Indeed as some enzymes of the melanization cascade are shared between the immune system and other systems, such as cuticle formation and ovarian development (Ferdig et al. 2000), compromising the melanization pathways could lead to systemic effects in the mosquito rather than the targeted modifications that a transgenic approach requires.

Once the premise is accepted that transgenesis has unavoidable costs, then clearly spreading the gene will be at best difficult, at worst impossible in the absence of any driving or selection factor. Inundative release may have transient success depending very much on a list of unknowns (size of target population, scale of release, fitness, assortative mating), but sustainability must be considered an essential part of success. It is also possible that the transgene may be silenced in some way. A large proportion of insect genomes is of transposable-element origin (Holt et al. 2002), and TE's

occurring naturally in the mosquito genome may well compete at the insertion site with the TE carrying the transgene into the mosquito genome. A single point mutation (for example in the promoter) might also be sufficient to silence the transgene, and evidence from gene families suggests this is **not** a rare event (Tijet, Helvig and Feyereisen 2001; Ranson et al. 2002). Further, given that targeting a single gene will lead inevitably to a resistance mechanism, the costs of transgenesis will increase as multiple targets are incorporated into the strategy.

Environment and gene expression: possible effectors

Like all organisms, mosquitoes respond to many environmental triggers for initiating developmental and behavioural processes, and these may work for and against the transgenic strategy. Some of these, such as mating, may be gated and happen just once in the lifetime of the mosquito. Others, such as responses to host odours and subsequent feeding, are cyclical and may initiate a cascade of physiological events such as synthesis and secretion of digestive enzymes or oogenesis. These processes can be further modulated or modified over the life span of the mosquito by such factors as season, short-term weather perturbations or infection by pathogens. Over the past few years, and largely driven by the need to characterize the background in which the transgenes are operating, understanding of mosquito molecular physiology has advanced considerably. However, we have no clear indication of how some of these well-characterized systems are modulated in the natural environment. This is of fundamental importance to transgenic release as there is the potential to up- or down-regulate the transgene due to environmental change.

The digestive process is one that is particularly susceptible to manipulation. When the tomato moth, *Lacanobia oleracea* is fed the cowpea trypsin inhibitor, trypsin expression and activity are inhibited yet the insect compensates for this by up-regulation of other serine proteases (Bell et al. 2001; Gatehouse et al. 1999). *An. gambiae* contains a trypsin gene family expressed in the midgut during digestion, and along with carboxypeptidase, the promoters of some of these enzymes are obvious candidates for transgene expression (Moreira et al. 2000). There remains the possibility that the transgenes could be differentially regulated by diet in the mosquito, and such manipulations could be either a hindrance to expression or exploited to ensure that the appropriate genes are switched on. Similarly, downstream gene expression such as vitellogenesis (Kokoza et al. 2001) may also be affected, potentially dampening the normal physiological processes leading to full egg production. Indeed, the use of vitellogenin promoters for transgenesis (Raikhel et al. 2002) faces similar problems of fitness and of compromising the reproductive potential, but may be less susceptible to unwanted manipulation by external environmental factors.

Understanding the environmental triggers for gene expression could also be a means of promoting spread of transgenics. If transgenes are considered akin to (but something more than) a deleterious mutation, then they will have small, negative impacts on populations that will be amplified over generations and be most obvious in small, isolated populations (Fry and Heinsohn 2002; Caballero et al. 2002). Any over-expression of the transgene would be expected to have negative effects on the fitness of an insect, but this can be reversed if there is environmental selection in its favour. Such is the case with *Drosophila* containing a high copy number of Hsp 70, which, when reared at high temperatures, outgrow the wild type (Roberts and Feder 2000; Minois, Khazaeli and Curtsinger 2001). The tools are now in place for some exciting

studies describing how mosquito genomes respond to environmental triggers and particularly how susceptible transgenic expression will be to environmental effects.

Complexity and heterogeneity: is the devil in the detail?

The plasticity of the insect genome is an important consideration in the expression-environment interaction, and in particular the evolution of gene families offers another element of heterogeneity that must be understood in an environmental context. Gene families can be considered as evolutionary indicators of environmental stress. Detoxification pathways in cockroaches for example are supported by more than 100 related (though not all necessarily functional) gene sequences, showing that the insect has tremendous ability to fine-tune its responses according to the stress received (Tijet, Helvig and Feyereisen 2001). The selection pressures here appear to be on a group of genes rather than on individual sequences, posing further concerns for transgenic release – how will the transgene be compensated for at the genome level, how will it be selected for at the population level, and can it be silenced in favour of another gene exploiting a promoter from the same gene family?

Similar questions can be asked of any gene, but are especially pertinent to the mosquito immune system. Traditionally, mosquito immune responses are considered to be directed primarily at pathogens that have potential to kill it, namely bacteria and fungi. The immune response may play a greater role in mosquito larvae where the aquatic environment is potentially more hazardous. The physiological cost of the immune response (Boëte, Paul and Koella 2002) and the observation that significant numbers of mosquitoes become infected even when the immune response is intact, suggests that its over-activation may be a burden at inappropriate stages of life cycle. More recently, over 200 immune genes have been described resulting from the *An. gambiae* genome project (Christophides et al. 2002) that fall into distinct multigene families and some of which are triggered specifically by *Plasmodium* infection. Thus the immune-response gene families exhibit the high degree of plasticity that makes them both ideal candidates for clearing malaria infections from the mosquito yet high-risk targets for transgenic strategies, as compensatory mechanisms down-regulating the transgene could readily evolve under stress.

There are of course more obvious areas of heterogeneity that can contribute further to environmental modulation of transgene expression. Mosquito-larval nutrition impacts upon size and fitness of the adult (Koella and Lyimo 1996; Koella and Offenberg 1999). Not only will this alter a mosquito's ability to transmit malaria but will also affect its genetic contribution to the next generation. Furthermore, the nutritional status of a larval mosquito also affects the 'first feed' characteristics of the adult. Pre-gravid female *An. gambiae* will often take only a partial blood meal prior or just subsequent to mating, and this blood meal will not result in egg development (Gillies and Wilkes 1965). The partial blood meal will, however, trigger many if not all of the digestive enzymes normally associated with feeding, including those costly transgenes expressed behind a midgut enzyme promoter such as carboxypeptidase. Consequently, even small changes in larval growth conditions can potentially affect early adult gene expression, and this is particularly relevant when gut-enzyme promoters are being exploited for transgenic expression. At the moment, we have no indication how much this detail is important. The genome project is producing the necessary unifying models that will underpin transgenic and other control strategies. The ecological physiologist must then test the models to ensure that control strategies

are not hijacked by the mosquito's proven ability to by-pass expression of one or more genes in response to internal or environmental stressors.

Conclusions

There is growing evidence of the great complexity in the interactions between malaria parasites and the mosquito vectors. The presence of multigene families and of multiple pathways associated with a single response (e.g. the immune response, digestion) is clear indication that both *Plasmodium* and *Anopheles* have evolved complex compensatory mechanisms to overcome internal and external stressors. For evidence of such responsive plasticity one need only consider the selection of drug-resistant parasites and insecticide-resistant mosquitoes. Into such complex genomic backgrounds, the insertion of transgenes blocking pathogen transmission by mosquitoes raises many questions. While transgenic mosquitoes can now be constructed, the cost to the mosquito of single or multiple transgenic approaches remains unknown. Before transgenic strategies progress to far in the laboratory, issues surrounding the environmental influences on gene expression (at gross and fine levels) and heterogeneity and complexity need to be addressed, as these have the potential to hijack the transgenic approach at a late stage, after many years of scientific effort and investment.

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