APPROPRIATE SEX-SEPARATION TECHNOLOGY

FOR *ANOPHELES* MOSQUITOES

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It is imprecise to say that mosquitoes transmit agents of disease: female mosquitoes transmit agents of disease. Therefore, the male is an accessory to the transmission process whose numbers generally have little affect on transmission. If sufficient males are available to mate a sufficient number of females to produce sufficient progeny to exploit all available larval habitats, any excess males above this contribute primarily to the degree of competition for virgin females. It is safe to assume that male availability seldom limits mosquito populations, therefore competition for virgins is the male *raison d'être*. Consequently, from a genetic control standpoint (including RIDL, SIT, translocations, CI), males can be released in large numbers and no increase in disease transmission will result. This makes male mosquitoes the ideal - indeed the only feasible - sex appropriate for release.

Sex separation in the context of fertile transgenic release.

Most discussions of release of fertile mosquitoes have considered effectors and drive mechanisms that will be expected to increase the frequency of an allele in a population. Because of the aforementioned considerations, it is apparent that whatever effectors and drive mechanisms may be used, only males will be released to introduce the gene into the population. Further, regardless of the drive of spread accomplished by a drive mechanism, its rate of spread will be in proportion to the initial 'innoculum' of foreign genetic material in the population(s). This means that releases of large numbers of males over large areas are more likely to achieve successful reductions in transmission than lesser efforts.

Sex separation in the context of SIT

Similarly – and more conventionally – it is apparent that the sterile insect technique (SIT) has been most effectively implemented as a male-only release. Because females are usually more easily radiation-sterilized than males, the release of some females is often tolerated when an agricultural pest is the target. In the context of malaria transmission, tolerance for females in releases will be lower – maybe *much* lower.

Pre-transgenic biological sex separation

Several inventive pre-transgenesis methods for sex separation of mosquitoes that were based solely on biological differences of males and females have been used. These had varying degrees of success and were implemented at different scales.

Blood-feeding differences. Insecticide blood-feeding citrated bovine blood containing 0.5% malathion was fed to adults in condoms during the coastal El Salvador *Anopheles albimanus* SIT program (Lowe et al. 1981). Cage contamination with malathion killed some of the non-blood-feeding males and eliminated only 95% of females occurred before release. Moreover, the method required holding the adults to a blood-feeding age.

Mosquito pupa sexual dimorphism. In addition to having a larger diameter than larvae, pupae are sexually dimorphic in size to varying extents with males being smaller than females. Size separation has therefore been a useful pupa collection and sexing technique in the laboratory and in small factory settings. Pupae can be separated from larvae regardless of the species, but the stringency for sex separation of pupae is determined by the intrinsic differences in the degree of size difference. This in turn is species-specific, and to a lesser extent a result of culture conditions. In its simplest form, visual separation has been used to hand-select male *Culex quniquefasciatus* pupae (Krishnamurthy et al. 1962). Two semi-automated types of devices have been invented: the purpose-designed Fay and Morlan device (1959) which utilizes adjustable glass plates to perform size selection . This was used used very effectively for *Culex pipiens*, but the efficiency for *Anopheles albimanus* (Dame et al. 1974) was much lower. It was subsequently improved, and this model is still available ((Focks 1980). While it is probable that better separation could be achieved if more consistent culture conditions *and* optimisation of culture conditions for sex separation existed, this method at best still requires cultivating both males and females to the pupal stage. A device using precisely positioned plates over a sluiceway has also been used for *Culex pipiens* (Gerberg et al. 1969). To my knowledge, none of the size methods has every been truly automated, however this is quite feasible and the limitations on its usefulness are likely to be practical and biological.

Pre-transgenic genetic methods for sex separation

Interspecific genetic sex separation. Sterile hybrid males result when *Anopheles arabiensis* males are mated to *A. melas* females. This method was used in a rather unconventional sterile insect technique attempt against *A. gambiae* (Davidson et al. 1970). On the face of it, this appears to be a useful approach since two objectives of SIT – sterilization and sex separation – are achieved without manipulation of progeny. Reality was not cooperative: males competed well in cage studies, but behavioral differences resulted in failure of the effort in a small-scale field trial.

Y chromosome translocations pseudo-linked to dominant selectable markers

- *Anopheles culicifacies*, dieldrin resistance (Baker et al. 1981)
- *A. albimanus*, propoxur resistance (Kaiser et al. 1978)
- *A. gambiae*, dieldrin resistance (Curtis 1976)
- *A. arabiensis*, dieldrin resistance (Curtis 1978, Lines and Curtis 1985)
- *A. stephensi*, dieldrin resistance (Robinson and Lap 1987)
- *A. quadrimaculatus*, malathion resistance (Kim et al. 1987)
- *Culex quinquefasciatus*, malathion resistance (Shetty 2003)
- *Cx. tarsalis*, malathion resistance (McDonald and Asman 1982)

Y chromosome translocations and visible markers

These kinds of systems could be used in automated sorters such as are used for pupa sex and in the COPAS system for separation of labeled *Drosophila* and nematodes.

- *A. stephensi*, *Black* mutation allows visible sex selection(Malcolm and Mali 1986)

- *A. albimanus*, *stripe* character allows visible sex selection (Mukiama 1985)

Other

The fortuitous isolation of a dominant sex-linked temperature sensitive lethal allowed the development of sex separation. This approach deserves consideration, but a good genetic screen would be necessary to efficiently isolate useful mutations.

Culex tritaeniorhynchus, dominant sex-linked tsl (Baker et al. 1978, Sakai and Baker 1974)

Transgenic methods for sex separation

Several methods of transgenic sex-separation are being actively studied, and one or more is certain to be successful in the laboratory (see Alphey 2002). Implementation is likely to be determined by public acceptability and competitiveness considerations.

Differential splicing with non-sex-specific effectors

The doublesex gene, originally studies in D. melanogaster(insert ref), has the capability to be re-engineered to express sex-specific transcripts in both males and females. The *A. gambiae dsx* gene has been defined and is under active study.

Sex-specific promoters with non-sex-specific effectors

The vitellogenin promoter is an attractive promoter for female-specific expression. However, in keeping with the need to express a female-eliminating gene as early in development as possible, an early stage sex-specific promter is necessary.

Sex-specific effectors with non-sex-specific promoters

##male specific lethal###

Outcomes for sex-separation technology –7 S

Small

Elimination of females early in development frees resources for production of more males and cost savings per male released.

Simple

Factory production methods are most robust when simple methods are applied. Selections such as heat and brief chemical exposures will likely win out over methods involving inter-strain crosses, use of prolonged labile chemical treatments etc.

Switchable

Because a factory producer colony must be maintained for production of males for release, the mechanism that eliminates females must allow female development and reproduction.

Stringent

How much female contamination is acceptable? In an SIT program, nuisance biting and disease transmission of released females will determine stringency. In a fertile release involving a transmission-defective effector, only nuisance biting must be considered. Only flawless effectors are likely to ever be implemented, therefore it seems unlikely

that this a significant contribution to transmission from released females will occur. Nonetheless, if sex-separation methods to eliminate females are available or can be developed with relative ease, it seems likely that support only for fertile male mosquito releases will exist.

Stable

Unstable sex separation methods require frequent stock repurification and multi-stage production to eliminate undesirable breakdown genomes. For this reason, simple systems that consist of few vital parts will be most successful. Conversely, complex systems with numerous vital components – effectors, promoters, and environmental conditions for separation will be more likely to fail.

Salable

Sudan: Our experience thus far has indicated that transgenic systems will be more acceptable in disease-endemic areas. Esoteric risk scenarios are less weighty in the overall scheme when real hazard of continued disease transmission is considered. Furthermore, use of more sophisticated technology and building necessary expertise and infrastructure is more attractive in developing countries.

Reunion: During the development of an SIT project on Reunion Island, consideration of release of transgenic insect releases would end national and local support for research and development before the launching. Given the vocal European opposition to transgenic products, government representatives are not receptive to developing support for a project that would bring headaches to their desk. Other options to manage malaria and vector control – for Reunion at least – are available.

Sexy

Males must arrive in the field able to compete for wild females. This means that the selection method must not create inordinate reductions in male competitiveness. If introgression of genetic material into the sexing strain is necessary to increase competitiveness, vital sexing componenets within a large chromosome tract that suppresses recombination will be less amenable to such an effort than a single locus.

Where do we begin?

The beginning point is to realize the importance of sex-separation technology regardless of which particular flavor of mosquito release is envisioned. Its development is critical to eventual implementation of any conceivable release of mosquitoes - both sterile males and fertile transgenics. Priority should also be given to developing measures of male competitiveness and determining the genetic, behavioral, and physiological components that control it.

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