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Evaluating the sterilizing effect of pyriproxyfen treated mosquito nets against *Anopheles gambiae* at different blood-feeding intervals

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ABSTRACT

Pyrethroid resistant malaria vectors are widespread throughout sub-Saharan Africa and new insecticides with different modes of action are urgently needed. Pyriproxyfen is a juvenile hormone mimic that reduces fecundity and fertility of adult *Anopheles* mosquitoes when used as a contact insecticide. A long-lasting insecticidal net incorporating pyriproxyfen is under development. As wild, host-seeking females may succeed in blood-feeding at different intervals after initial contact with mosquito nets the aim of this study was to determine the effect that age and gonotrophic status (nulliparous or parous) and the interval between initial pyriproxyfen exposure and blood-feeding has in terms of subsequent reduced fecundity and fertility.

Anopheles gambiae s.s. were exposed to pyriproxyfen LLIN for three minutes in WHO cone bioassays. Four regimens were tested with different blood-feeding intervals A-1 hour (nulliparous), B-1 hour (parous), C-24 h (nulliparous), or D-120 h (nulliparous) after pyriproxyfen exposure. Mosquito oviposition rate, fecundity and fertility of eggs were recorded for several days. All four treatment regimens produced levels of mortality similar to unexposed females. The overall reduction in reproductive rate of 99.9% for regimen A relative to the untreated net was primarily due to oviposition inhibition in exposed females (97%). Pyriproxyfen was equally effective against older parous mosquitoes and when blood-feeding was 24 h after exposure. Regimen D produced a reduction in reproductive rate of 60.1% but this was of lesser magnitude than other regimens and was the only regimen that failed to reduce fertility of laid eggs, indicating the effects of pyriproxyfen exposure on reproduction are to some extent reversible as mosquitoes age. In an area of moderate to high mosquito net coverage a host-seeking mosquito is likely to contact a treated mosquito net before: (a) penetrating a holed net and blood-feeding shortly after exposure or, (b) be frustrated by intact nets before succeeding in blood-feeding on an unprotected individual the following night. Mosquito nets are an appropriate delivery system for pyriproxyfen, based on the large reductions in reproductive rate when blood-feeding between 1 h and 24 h after exposure. Combining with a pyrethroid should be an effective approach if susceptible mosquitoes are killed and resistant mosquitoes sterilized.

1. Background

Pyriproxyfen is a juvenile hormone mimic which inhibits eclosion of agricultural, domestic, and aquatic insect pests and was first registered by the United States Environment Protection Agency (US EPA) in 1995 (EPA, 2011). Pyriproxyfen is safe to use in the context of human habitations, with an LD_{50} oral toxicity in rats of >5,000 mg/kg body weight and World Health Organization (WHO) toxicological classification U (unlikely to present acute hazard in normal use) (WHO, 2002). Pyriproxyfen has been recommended by WHO Pesticide Evaluation Scheme (WHOPES) for control of

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mosquito larvae since 2000 (WHOPES, 2001) and is particularly effective for long-lasting treatment of water storage containers for control of dengue vectors *Aedes aegypti* (Marcombe et al., 2011; Sihuincha et al., 2005) and *Ae. albopictus* (Vythilingam et al., 2005) but can also be used for field control of *Culex quinquefasciatus* and *Anopheles* species (Yapabandara et al., 2001). Laboratory and experimental hut trials have shown that pyriproxyfen treated mosquito nets are efficacious against adult female *Anopheles gambiae* (Ohashi et al., 2012; Ngufor et al., 2014). Unlike conventional neurotoxic pyrethroid insecticides, pyriproxyfen is not an effective adulticide, but has been shown to reduce the longevity of cohorts tested in laboratory bioassays (Ohashi et al., 2012). While the mode of action is not known, pyriproxyfen does affect juvenile hormone and ecdysteroid titers, which appear to inhibit fecundity of adult mosquitoes and subsequent fertility of any eggs which are laid (Sihuincha et al., 2005). Ohashi et al. showed that exposure to polyethylene netting treated with 0.1% (35 mg/ai/m^2) and 0.01% pyriproxyfen resulted in complete sterilization of *An. gambiae* for at least one gonotrophic cycle (Ohashi et al., 2012).

When determining the efficacy of pyriproxyfen treated netting it is important to consider the interval between pyriproxyfen exposure and blood-feeding that may occur in the natural environment. WHO recommends full coverage of all people at risk of malaria in areas targeted for malaria prevention with LLIN (WHO, 2008). Several African countries have conducted mass distribution campaigns with a target of universal coverage (UC) (WHO, 2014a). However, there is strong evidence that mass distribution campaigns have failed to meet targets of UC. ITN usage rose to only 55.7%, 59.1% and 84.8% respectively in Tanzania, Kenya, and Benin following UC distribution campaigns in 2011 (West et al., 2012; Zhou et al., 2014; Tokponnon et al., 2013). In this scenario of moderate to high LLIN coverage it is feasible that a host-seeking mosquito may be exposed to a pyriproxyfen treated net while being prevented from blood-feeding, before successfully blood-feeding the following night (24 h after exposure) on an unprotected individual. In the study of Ohashi et al. there was 100% reduction of fecundity of *An. gambiae* exposed to pyriproxyfen treated netting (0.1 and 0.01%) in standard WHO three minutes cone bioassay either when unfed (24 h before blood-feeding) or blood-fed (24 h after blood meal) (Ohashi et al., 2012).

Harris et al. showed that Centers for Disease Control and Prevention (CDC) bottle bioassays with pyriproxyfen tested against *An. arabiensis* that were blood-fed 24 h before exposure resulted in a significant reduction in fecundity (Caroline Harris et al., 2013). However, there was no reduction of fecundity for *An. arabiensis* that were blood-fed 24 or 72 h after exposure, or 72 h before exposure (Caroline Harris et al., 2013). Based on these findings it was concluded that pyriproxyfen would be most effectively deployed against mosquitoes that were recently blood-fed, for example as a residual spray to target freshly fed mosquitoes resting on sprayed walls (Caroline Harris et al., 2013). Mbare et al. conducted similar trials with *An. gambiae* and showed that exposure up to 24 h before or after blood-feeding resulted in a moderate reduction in egg laying of between 56 and 62% (Mbare et al., 2014). Exposure to pyriproxyfen 48 h before blood-feeding had only a 32% reduction in oviposition, while exposure 48 or 72 h after blood-feeding had no impact (Mbare et al., 2014).

Olyset Duo® is a factory produced long-lasting insecticidal net (LLIN), consisting of polyethylene netting incorporated with a mixture of 1% pyriproxyfen and 2% permethrin, that is currently undergoing WHOPES evaluation (WHO, 2014b). The primary aim of this study was to determine the efficacy of pyriproxyfen treated mosquito nets in terms of reduced reproductive capacity with different intervals between exposure and blood-feeding, that are representative of moderate/high mosquito net coverage. The secondary aim was to determine whether exposure to pyriproxyfen is equally effective at reducing the fecundity of nulliparous and older, parous *An. gambiae*.

2. Methods

2.1. Definition of key terms

Oviposition = proportion of female mosquitoes that laid eggs.
Fecundity = measured by the number of eggs laid.
Fertility = proportion of laid eggs which hatched into L2 larvae.
Reproductive rate = number of L2 larvae produced per adult female tested (i.e. a combined measure of oviposition, fecundity and fertility).
Sterilization = complete suppression of offspring production.

2.2. Mosquito net samples

Factory produced mosquito nets consisted of 195 denier polyethylene monofilament incorporated with 1% pyriproxyfen and a mesh size of 75 holes per sq/m (Sumitomo Chemical Co., Ltd., Tokyo, Japan). Mosquito nets were equivalent to Olyset Duo® but did not contain permethrin, in order to determine the contribution of pyriproxyfen. Netting samples were cut from each side of the mosquito net after removal from the packaging and were not washed before testing. Untreated netting of the same specifications was used as a negative control.

2.3. Test insects

Adult female mosquitoes of the *An. gambiae* RSP strain were obtained from the insectary at Kilimanjaro Christian Medical University College (KCMUCo). The RSP (reduced susceptibility to permethrin) strain was originally colonized in 1992 from field collections near Kisumu, Kenya and has since been reared at KCMUCo since 2009 and selected with permethrin to 100% frequency for the kdr mutation L1014S, known as kdr-east (Vulule et al., 1999).

3. Experimental design

The following intervals between exposure and blood-feeding were tested with *An. gambiae* RSP:

A-1 h (nulliparous),
B-1 h (parous),
C-24 h (nulliparous),
D-120 h (nulliparous).

These testing regimens were chosen to represent potential scenarios that may occur in the natural environment. Regimens A and B represent a host-seeking mosquito that is temporarily frustrated by a pyriproxyfen treated net before penetrating a hole and blood-feeding within 1 h of contacting the net. Regimen C represents an area of moderate/high mosquito net coverage where a mosquito is prevented from blood-feeding by pyriproxyfen treated nets before blood-feeding the following evening on an unprotected person. The longer five days interval (regimen D) was included to determine whether the effect of pyriproxyfen diminished over a longer interval and would not normally occur in the wild. Standard WHO 3 min cone bioassays were conducted using sugar-fed, 2–4 days old, nulliparous *An. gambiae* RSP. For experiments using parous mosquitoes, blood-fed *An. gambiae* RSP (age 2–4 days) were separated into individual cups and oviposition observed for five days prior to inclusion in bioassays when aged 9–11 days. Bioassays and subsequent holding of test mosquitoes for recording fecundity and fertility were conducted at 27 °C (±2 °C) and 75% (±15%) relative humidity. Conditions were monitored using data loggers (Gemini TinyTag TV-4500, UK). Following bioassay, mosquitoes were