The effects of synthetic pheromone exposure on female oviposition and male longevity in *Zygaena filipendulae* (Linnaeus, 1758) (Lepidoptera: Zygaenidae, Zygaeninae)

DAVID THACKERY & JOSEPH BURMAN

Ecology Research Group, School of Human and Life Sciences, Section of Life Science, Canterbury Christ Church University, Canterbury, Kent, ~~postcode~~CT11QU, U.K.

Comment [BJ(1)]: I would hesitate to say this, as the studies were carried out in two back gardens. It would have been difficult to leave the containers in the natural grassland environment, because they would have probably been taken by a member of the public/local farm animals, hence our decision to keep them outdoors, but at a protected location.

Synopsis

The use of pheromone lures for rare insect monitoring and survey is relatively modern since pheromones were primarily developed for the purpose of pest management. As a result of this shift in usage, questions have been raised about the potential implications of powerful synthetic pheromones on fragile wild populations. This study assessed the effect of pheromone exposure on the burnet moth *Zygaena filipendulae* (Linnaeus, 1758). Moths were reared and mated at two separate sites, viz. one of~~at~~ which moths were exposed to synthetic sex pheromone~~s~~, and another site acting as an unexposed control where no synthetic lures were present. Mating experiments were set up at each site in order to assess female oviposition and male longevity. No significant difference was found in oviposition between exposed and control females. A significant difference was recorded between exposed and unexposed male longevity when the males that had a mating opportunity were tested. Males that were exposed to the pheromone blend had a longer lifespan in the pheromone exposed group compared with the control. These findings demonstrate that neither male longevity nor female oviposition is negatively affected by the presence of the pheromone blend. The results of the male longevity experiment suggests synthetic sex pheromones could in fact have an enhancing effect, stimulating males in a local population, and lengthening their period of mating opportunity.

Key words: pheromone, Lepidoptera, Zygaenidae, *Zygaena filipendulae*, longevity, oviposition.

Introduction

Historically the use of insect pheromone attractants was limited to pest monitoring and control (Weatherston & Percy, 1977; Silverstein, 1981; Mitchell, 2002). The use of insect pheromones in an insect conservation setting is a more recent application of the technique (Svensson, Larsson & Hedin, 2004; Franzen & Ranius, 2004). New developments have meant that synthetic pheromone lures can now be used to target indicator species to give an assessment of general biological health of an ecosystem (Bergman *et al.*, 2012). Conservation efforts and land

management planning are aided by the identification of indicator species on site, as bioindicators are often susceptible to small changes in their environment, providing timely information as to the current health of an ecosystem (Sarin & Bergman, 2010; Binzenhöfer *et al.*, 2005). Pheromone attractants (usually synthetic homologues of the insect's mating pheromone) can be particularly effective in surveying or monitoring insect indicator species due to their strong attractive pull on nearby insects, a feature that has proved particularly useful in horticultural applications (Wall & Perry, 1987).

The use of pheromone lures in this setting has already been well demonstrated in conservation of saproxylic insect bioindicators (Musa *et al.*, 2013), and could provide the same benefits for monitoring grassland species. The use of sex pheromone lures reduces the surveying effort while increasing the likelihood of a positive result (Ranius & Jansson, 2002; Tolasch, von Fragstein & Steidle, 2007). The specificity of the pheromone lures can reduce the level of experience required to conduct a survey as the identification process of large assemblages of species is bypassed; as a result experts are not required as frequently in the field (Svensson & Larsson, 2008). As a tool for monitoring of rare and elusive species it is thought to be particularly invaluable due to its accuracy and benefits in efficacy for seeking out discrete or hidden populations (Andersson *et al.*, 2014). It has also been said that use of attractants could be one answer to the 'Wallacean Shortfall' by providing more efficient and accurate surveying techniques (Larsson & Svensson, 2011; Kadej *et al.*, 2014). Despite the potential benefits for conservation, questions have been raised as to the safety of synthetic lures due to their powerful effects on mating and other aspects of physiology (Oleander, Thackery & Burman, 2015).

It is therefore important to determine what effects synthetic pheromones may have on wild populations. One study has used *Zygaena filipendulae* (Linnaeus, 1758) as a model to assess the potential changes to mating behaviour as a result of male desensitisation when the sex pheromone is present (Oleander, Thackery & Burman, 2015). This study showed no evidence of desensitization in male moths, but did not consider effects on male health overall, or indeed effect of exposure on female reproduction. In this experiment we therefore used female oviposition and male longevity as the metrics of reproductive fitness in grassland moths. *Z. ygaena filipendulae* was used in order to draw direct comparisons with the results of Oleander, Thackery & Burman (2015). Additionally, this species provides a good model since the moth is not currently threatened, despite a number of *Zygaena* species being on the IUCN red list (National Biodiversity Network, 2013).

Method

Pheromone blend

Component compounds for the *Z. filipendulae* sex pheromone were purchased from Pherobank B.V., Wageningen, Netherlands. The compounds used were (Z)-7-Dodecenyl acetate, (Z)-9-Tetradecenyl acetate and (Z)-5-Dodecenyl acetate, ~~Z7-12 Acetate, Z9-14 Acetate and Z5-12 Acetate~~ which were diluted with a ratio of 100 : 10 : 3 (Priesner, Naumann & Stertenbrink, 1984) dissolved in HPLC grade hexane. 200 µl of the blend were loaded into the cup of a 20 mm diameter butyl rubber septum (Sigma-Aldrich, U.K.). Each septum therefore contained the following quantities of pheromone components: Z7-12 Acetate 0.1 mg, Z9-14 Acetate 0.01 mg and Z5-12 Acetate 0.003 mg. Septa were wrapped individually in foil and stored at –20°C after all of the solvent had evaporated, then removed from the freezer on the day of use.

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Study location, specimen rearing and conditions

The test specimens of *Z. filipendulae* were acquired from the wild flower gardens at Canterbury Christ Church University at either their larval stage or when pupation had occurred, and were stored in isolation from one another. Individual containers consisted of a plastic food tub with a sealable lid that had been perforated (Fig. 1). In the months prior to the study a culture of *Lotus corniculatus* L. was grown from seed so as to provide a ready food source for the larval stage. The specimens had their food renewed twice daily to ensure the nutrient requirements were met. Once pupation had occurred the containers were cleared of all detritus to maximise successful emergence.

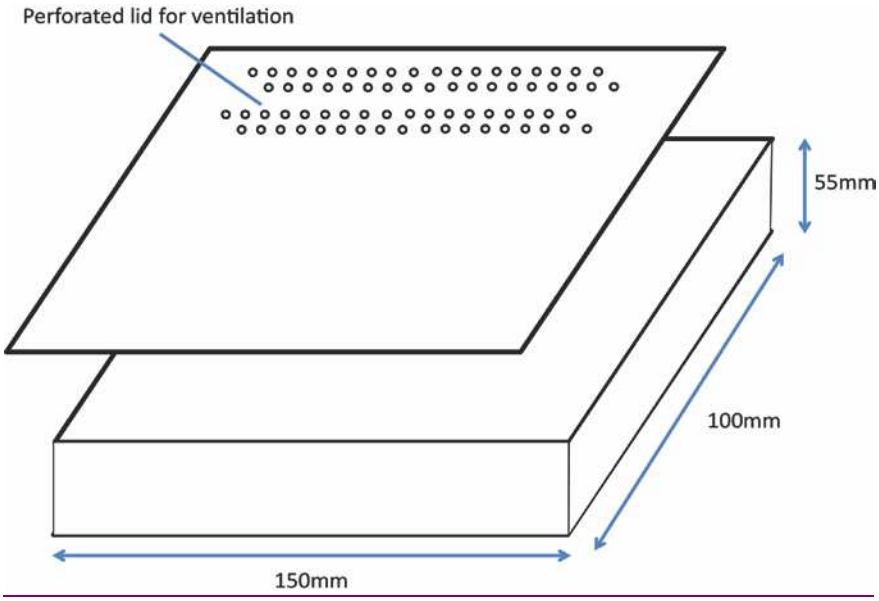

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Fig. 1. Schematic diagram to show dimensions of individual containers for rearing and pheromone exposure of *Zygaena filipendulae* adults.

Two study locations were set up with one being exposed to pheromone lures and the other location without pheromone exposure (unexposed control). Both sites were north facing gardens in Ramsgate, Kent, which experienced a similar amount of shade in the afternoon. The ~~geographical separation~~locations were separated by 1.9 km ~~of the study sites was~~ to ensure that there was no contamination from the pheromone lures on the control site. ~~The two locations used had a separation distance of 1.9 km, and A~~a remote sensor was placed to monitor temperature. The enclosures were positioned so to create similar environmental conditions (no significant differences were noted between logged temperatures at exposed and unexposed sites ($U = 0.249$, $p = 0.249$)).

On emergence the individuals were sexed and marked using a PX-21 Industrial Paint Marker pen (Mitsubishi Pen Company Ltd, U.K.) using the individual coding system set out in Fig. 2. A food source was provided in the form of a sugar solution with a concentration of 1g l^{-1} which was checked daily and replenished as required. The allocation of newly exposed moths to the exposed/unexposed location was decided dependent on the numbers already

allocated to each site so as to keep a balance of individuals. On days when multiple emergences occurred a random number generator was used to determine the site allocation. Each location had a shelving unit which raised the enclosures approximately one metre above the ground; the shelving units were also surrounded with netting in order to prevent interference from wildlife. The locations contained communal containers for the males (maximum of 10 per enclosure) and individual 'mating' containers for the females. At the exposed site, a single pheromone lure was hung amongst the mating cages so that all enclosures experience a similar level of exposure to the synthetic pheromone, whilst the control site had no lure.

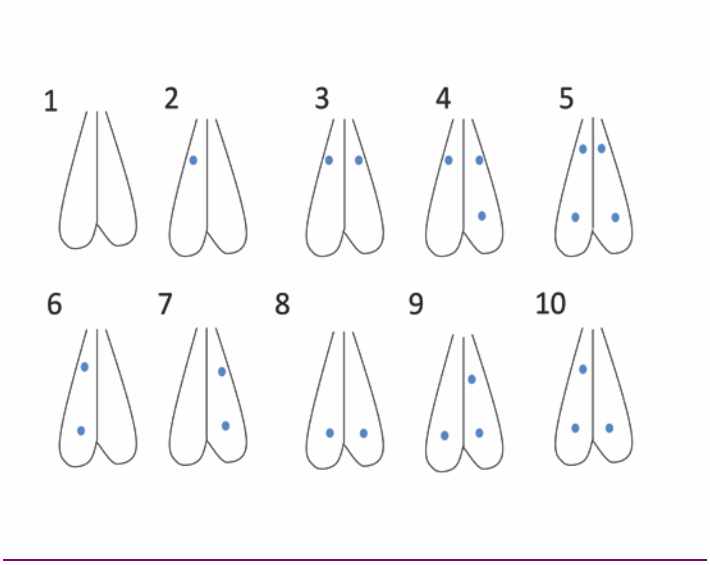

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Fig. 2. Coding system used in order to identify and discriminate between groups of ten individual captive moths. Images represent moth wings marked with dots using a permanent oil-based marker pen.

Fecundity study

Once the females had emerged they were allocated a site (exposed or unexposed) and an identification number. The adult females were held in isolation, each having been placed in a clean individually marked mating container. They were then allowed 24 hours for acclimatisation before a mate was introduced. Once introduced to a mate, pairs were allowed 48 hours to copulate before they were

then separated. A mating opportunity with a single male was provided for each female, and mating rejections/non egg-laying events were not included in the final data analysis. To allow for primary egg laying a period of 72 hours was given. Females were then moved to a clean container and observed for a further 72 hours to allow for secondary egg laying where necessary. All females were given this opportunity regardless of whether they had laid eggs in the initial 72 hours or not. The number of eggs laid in both primary and secondary egg laying events was recorded. These data combined provided the total oviposition for each individual.

Longevity study

On emergence the males were marked and allocated a communal enclosure which contained a food source and either a blank septum (unexposed group) or one that was loaded with the appropriate pheromone blend (exposed group). The males were then given a single mating opportunity after a mandatory 24 hour acclimatisation period in the communal enclosure. Using the identification marks to keep track of specific individuals, the day of emergence and the day of death was recorded in order to establish the longevity of each male.

Hypotheses and statistical tests

Statistical tests of difference were carried out in Minitab 17 in order test the following hypotheses:

1. *Z. filipendulae* males exposed to synthetic sex pheromones will have a shorter lifespan than unexposed males;

2, *Z. filipendulae* females exposed to synthetic sex pheromones will have a lower oviposition than unexposed females.

Anderson Darling tests for normality were used to categorise the type/distribution of data collected. Oviposition data tested negative for normality and therefore a non-parametric Mann-Whitney test was used to test for significant differences between exposed and unexposed female oviposition. The females that failed to reproduce (4 unexposed females and 5 exposed females) were then removed from the dataset in order to take only reproductively active females into account. After removal the data tested positive for normality, but due to the discrete data, a Mann Whitney test was still used to test for significant differences between exposed and unexposed females for reproductively active females only.

The male longevity data were also treated as two groups; one that considered all males, and the other which consisted of only those individuals that had a

mating opportunity. Each group tested positive for normality and a two sample T-test was used to test for significant differences in longevity between the males exposed to the pheromone and those which were not exposed.

Results

Female oviposition. There was no significant difference between total oviposition in exposed and unexposed groups overall (*W = 0.544*, *p = 0.534*, *n = 24*). When comparing only the individuals that laid eggs there was still no significant difference (*W = 69*, *p = 0.603*, *n = 15*) between exposed and unexposed female oviposition (Fig. 3).

Male longevity. There was no significant difference between exposed (n = 16) and unexposed (n = 17) male longevity when all males were considered (*t-value = -1.69*, *df~~DF~~ = 30*, *p = 0.101*). However, a significant difference (*t-value~~T~~ = 2.78*, *d~~.~~f~~.~~ = 21*, *p = 0.011*) was found when comparing only those males that mated (exposed: *n = 11*; unexposed: *n = 15*). Exposed males achieved a greater longevity in this case, as illustrated in Fig. 3.

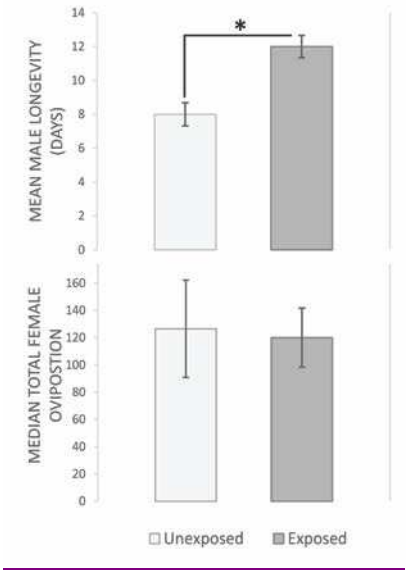

Fig. 3. Male longevity and female oviposition of *Zygaena filipendulae*, comparing pheromone exposed and unexposed treatments. *denotes a statistically significant different between treatments. Error bars show standard error.

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Discussion

The statistical analysis for oviposition data showed that female exposure to the synthetic sex pheromone had no significant effect, either beneficially or detrimentally. This indicates that the presence of the synthetic sex pheromone is unlikely to disrupt the oviposition of the target species, nor enhance it *in situ*. From a conservation perspective this is encouraging, as it adds further support to the notion that pheromones are a safe tool for the monitoring of threatened moths and other insects. Considering there is an exceptional lack of knowledge on the habitat requirements (Cardoso *et al.*, 2011), distribution (Diniz-Filho, de Marco & Hawkins, 2010) and abundance of insect populations through time and space (Gaston & Fuller, 2007), this further emphasises the significant potential for safe synthetic attractants to be used for the monitoring of insects for the purpose of conservation.

Overall the presence of the synthetic pheromone had no significant effect on male longevity. However, when mated males only were considered, exposed males were shown to have a higher average lifespan, living on average 1.5 days longer than unexposed moths. It has been shown in Diptera that mating increases longevity, which may corroborate our observations for this phenomenon (Hicks, Hagenbuch & Meffert, 2004). However, these results also suggest that this enhancement of longevity may be the result of pheromone exposure and not the results of the physical mating event itself. Pre-exposure to synthetic and indeed natural pheromones have previously been shown to 'activate' sexually naïve males (Anderson, Sadek & Hansson, 2003), but are often suggested as having detrimental effects on mating and male health in the longer term. In this study the opposite effect was observed, with mated males living longer after pheromone exposure. This life extending effect of pheromones has been previously observed in nematodes (Kawano *et al.*, 2005), but the present study may represent the first observation of such an occurrence in insects. The implications for these results are again significant when considering conservation applications of synthetic pheromones and go some way to demonstrating their safe use on fragile species, perhaps even demonstrating that exposure may lead to increased longevity of zygaenid moths in their natural habitat.

To increase the understanding of a synthetic pheromone's effect on the target population, further field studies should be conducted to assess the potential for disruption in a wild population rather than captive individuals. The effects noted here are likely to be exaggerated by the 'extreme' level of exposure captive moths would have encountered in the experiment. In this study, moths were held

in close proximity to a high dose of synthetic pheromone for a relatively long period of time. In practise, when pheromone traps are used for monitoring in the field, exposure is likely to occur for much shorter periods of time, and in larger, better ventilated funnel traps (or indeed, without a trap at all). Thus the concentrations encountered by trapped males are likely to be lower in reality, and thus any potential for deleterious/advantageous effects on mating is much lower. Despite this, the fact that extreme exposure causes no negative effect is extremely encouraging from a risk assessment point of view.

Although this study offers evidence for safe usage with Lepidoptera, similar studies would be advantageous to assess the possible effects on other taxonomic groups such as Coleoptera where the potential for pheromone survey is large, but the physiology and chemical ecology of the insects are substantially different.

Conclusion

The presence of a synthetic sex pheromone had no overall significant effect on individual longevity or female life time oviposition of *Z.~~ygaena~~ filipendulae*. However, when combined with a mating opportunity, males lived significantly longer after exposure to a synthetic lure. Although these results give a good indication as to the practical effects of lures used in the field, further studies could investigate effects on total population and alternative target species. These results do, however, add to the growing body of evidence that suggests pheromones to be a safe and highly effective means of monitoring vulnerable insect populations.

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