Chordotonal organ mechanosensory pathways as potential targets for the control of mosquito borne diseases

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Abstract

Mosquito-borne infectious diseases are the cause of millions of deaths every year, with disease transmission inseparably linked with mosquito physiology and biology. Mechanosensory signalling plays an essential role in multiple parts of the mosquito life cycle, making it a potential target for insecticides. In spite of this, and the existence of several 'mechanotoxins', these pathways have so far not been exploited. My thesis examined the various aspects of mechanosensation-dependent insect behaviour, as well as the effect the chordotonal organ-specific compound pymetrozine had on these behaviours.

Behavioural studies using *Drosophila melanogaster*, including competition assays and flight tests, were completed to investigate the impact of pymetrozine exposure. Exposure produced significant decreases in flight ability and male reproductive fitness. *Drosophila melanogaster* lines previously reported to have insecticidal resistance were exposed to pymetrozine to check for potential cross-resistance, with no pymetrozine resistance being found in any case tested.

Three mosquito species were then studied in depth - *Aedes aegypti, Anopheles gambiae* and *Culex quinquefasciatus*. Using an already existing *Drosophila* auditory model, electrophysiological experiments were conducted to compare between different species and sexes. A considerable level of sexual dimorphism was found throughout, especially in the auditory nonlinearities associated with transducer gating. Energy and displacement gains with regards to the mechanical auditory system were approximated and the nerve response to different stimuli was investigated. Male and female responses to compound injection were also calculated.

Pymetrozine exposure was found to lead to complete loss of ChO mechanosensory function in these three species, as well as two additional insecticidal resistant *Anopheles gambiae* lines. Mathematical modelling of pymetrozine was discussed, particularly with regards to control programmes. 'Mechanotoxins' have so far been vastly underutilised as both insecticides and as a tool to explore auditory systems. These compounds hold great potential as methods of insect control.

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CAP = Compound Action Potential

ChO = chordotonal organ

DD = Dark/Dark

IPF = Intrapulse frequency

IPI = Interpulse interval

JO = Johnston's organ

LDV = Laser Doppler Vibrometry

LD = Light/Dark

Nan - Iav = Nanchung/Inactive

ppm = parts per million

TTX = Tetrodotoxin

TeNT = Tetanus neurotoxin

WBF = Wing Beat Frequency

WHO = World Health Organisation

1. Introduction

Significant decreases in mortality and morbidity rates associated with some mosquito borne diseases have been seen worldwide over the past decade, particularly with regards to malaria and lymphatic filariasis (1, 2). These reductions have been possible as a result of control programmes involving indoor residual spraying of insecticides, the distribution of insecticidal mosquito nets and advances in therapeutic and preventative treatment of the diseases (for example, using artemisinin combination therapy to combat malaria) (3-6).

Increasingly, however, these significant advances are being placed under pressure through a combination of insecticidal and antibiotic resistance from the vector and parasite respectively (7-9). These dangers are further enhanced by the relatively limited number of insecticides that are available for distribution, some of which share similar mechanisms of action thus making resistance development to a single insecticide class highly problematic (10-12). Insecticidal resistance has not yet become sufficiently prevalent as to render continued usage of currently available insecticides ineffective, with insecticide treated bed nets still being reported to be more effective than untreated nets regardless of resistance levels for example (13). However, the serious problems that emerged during previous disease control attempts which relied too heavily on too few control measures (such as DDT) can serve as a reminder of the importance of using multiple methods of control (14, 15).

Alongside these growing concerns of resistance, the increasing spread of some mosquito species to previously uninhabited sections of the world could lead to increased disease transmission – *Aedes aegypti* and *Aedes albopictus*, which act as vectors of a number of different diseases such as dengue, have already been identified in Southern Europe and are predicted to increase in range considerably over the coming decades (16, 17). These changes in distribution are driven partly by climate change (18, 19) but also by the unprecedented scale of urbanisation and globalisation taking place worldwide (20, 21).

These twin sets of pressures place increasing demands on mosquito control programmes – insecticides and antibiotics are becoming more and more essential at a time when their effectiveness is decreasing constantly. It is clear therefore that new insecticides, with preferably novel mechanisms of action, are required to reduce the burden placed on the compounds that have currently been approved for use. Given the substantial delays that can occur between the identification of a promising compound and the actual introduction of that substance to the field, insecticides that have already been demonstrated as efficacious as well as safe for use alongside humans would be ideal for this purpose.

Slow acting feeding inhibitors (referred to here as mechanotoxins) for example are currently used as insecticides for crop pests but are also a potentially promising class of compounds for adaptation to combatting mosquito populations. One member of this class is pymetrozine (Syngenta, Switzerland), which has been shown to eliminate chordotonal organ (ChO) function in several insect species (22, 23). ChOs are ciliated stretch receptors which act as external sensory organs in many insect, but not mammalian, species; in *Drosophila melanogaster* ChOs are involved in both proprioceptive and auditory sensing (24, 25). Abolishing ChO function in plant-sucking insects has been reported to cause starvation (26).

The ablation of ChO function that follows pymetrozine exposure occurs specifically in these mechanoreceptors because the compound targets the Nanchung/ Inactive (Nan-Iav) ion channel heterodimeric complex, which appears to be solely confined to these units (27), and as such at low concentrations pymetrozine has been declared safe for use as a generic pesticide. This mechanism of action renders the drug distinct from other insecticides already in widespread use and makes it an interesting candidate for investigation.

Proper insect mechanosensory function is reliant on ChOs (28). Mechanosensation and mechanosensory feedback has been identified as being involved in flight (29), courtship rituals (30, 31), host identification by female mosquitoes for blood feeding attempts (32) and circadian rhythm regulation (33). Mosquito courtship in particular has been studied in depth because of the unusual phenomenon of frequency matching within couples at higher order harmonics of the fundamental flight tone (34). Given that this process necessitates both flight and auditory function before successful copulation can occur, it is a promising target for compounds which could theoretically deprive mosquitoes of mechanosensory capabilities.

Whilst much more is known of mechanosensation in *Drosophila melanogaster* than in mosquito species, there are still gaps in the general knowledge of the mechanosensory system. Pymetrozine therefore offers two different topics of interest; first as a proven pesticidal compound against crop pests that has the potential to be effective against mosquitoes and could therefore relieve some of the pressure felt by the currently used toxins; second as a tool to pharmacologically ablate mechanosensory function in insects and thus allow for experimentation and investigation into a relatively unexplored area of biological importance. That a compound with this mechanism of action has not yet been tested in mosquitoes increases its potential relevance as both a toxin and a laboratory tool.

This project therefore seeks to integrate these two possible usages by attempting to assess the potential of pymetrozine (and thus also other mechanotoxins that act in a similar manner) for use in mosquito control programmes whilst also utilising the drug to explore the relative importance of mechanosensation at different stages of the insect life cycle.

2. Literature review

2.1. Major vector species of mosquito borne diseases

There has been a significant shift in the relative contributions of communicable and non-communicable diseases towards the global burden of disease over the past century, with non-communicable diseases now being responsible for approximately 60% of the total burden (35-37). This burden is unequally distributed however, with many low- and middle-income countries tending to suffer inordinately more from communicable diseases than high-income countries– malaria for example was the sixth most common cause of death in low-income countries in 2012 but was not present in any other income-bracket list (38). Whilst malaria is responsible for the majority of the morbidity and mortality caused by mosquito-borne diseases worldwide, other pathogens (in particular, lymphatic filariasis (39) and dengue (20)) also significantly contribute to this global problem; mosquito transmitted diseases clearly therefore remain a serious issue in certain areas of the world (35, 40).

Whilst there are currently over 3500 different identified mosquito species worldwide (41), the vast majority of these are not necessarily relevant when considering disease transmission – only 70 of the 460 formally recognised *Anopheles* species have been proven to be able to transmit malarial pathogens for example (42). Further than this several major mosquito species play a disproportionally large role in the transmission process of many of the most epidemiologically important mosquito borne diseases. These include *Anopheles gambiae, Ae. aegypti* and *Culex quinquefasciatus,* which together can be considered as the major vectors of malaria, dengue, Chikungunya, yellow fever and lymphatic filariasis (43-46).

As such, whilst the role other mosquito species play in the transmission of various diseases should not be neglected (such as *Anopheles arabiensis* and *Ae. albopictus* with regards to malaria and dengue respectively), these three species remain the major targets of many vector control programmes; there are significant differences between these species however that prevent all-encompassing control measures from being applied (47-49).

Differences in the biology of the various mosquito-borne pathogens can also result in enforced changes to disease control programmes. For example whilst the incubation periods of many mosquito borne diseases are temperature dependent they can also differ significantly between pathogens - the average incubation period for dengue has been approximated between 4.7 and 6.5 days whilst for lymphatic filariasis (when considering the *Wuncheria bancrofti* pathogen) this period can be between 10 and 20 days (50-53).

2.1.1. *Aedes aegypti*

The global expansion of *Ae. aegypti* has proceeded at a significant rate since the 1980's as urbanisation and climate change has increased this mosquito species' survival range to include even southern Europe (16, 54). This expansion has fuelled a similarly significant increase in the number of cases of dengue and the Chikungunya virus, with dengue in particular being the most prevalent human arbovirus worldwide (20).

Transmission of these two diseases occurs predominantly in urban environments as a result of the strong preference of *Ae. aegypti* mosquitoes for such living habitats, with egg-laying and thus development of larval and pupal stages tending to occur in small man-made containers around human habitats (17, 44, 53). With the aforementioned increased rate of urbanisation that has occurred over the past 50 years (which shows no signs of halting) this has placed almost half of the world's population at risk for *Ae. aegypti* borne diseases (55). Females deposit single, unfloated eggs from which larvae, which rely on a siphon for respiration and lie at an angle to the water surface, emerge (56).

Female *Ae. aegypti* are highly anthropophilic (with reports of over 90% of blood meals coming from humans) and will generally only feed from other food sources in the absence of humans (which is uncommon in densely populated urban environments) (47, 57). Females also often take multiple blood meals within a single gonotrophic cycle (which lasts typically 3 to 4 days), greatly increasing the risk of disease transmission should a female become infectious (58-60).

In addition to this, dengue transmission tends to occur during the daytime and outdoors, thus placing the mosquito outside the range of the insecticidal nets and indoor insecticidal spraying that have proven highly effective against *An. gambiae* in some locations (61). As such control of *Ae. aegypti* borne diseases has proven particularly challenging and requires a variety of different control mechanisms, which tend to target egg laying sites as well as the immature stages of adult development (62).

2.1.2. *Anopheles gambiae*

In contrast to the preference shown by *Ae. aegypti* and *Ae. albopictus* for urban environments *An. gambiae* has overall demonstrated a strong inclination towards more rural areas of sub-Saharan Africa, where it remains the dominant malaria vector (43). As malaria was responsible for approximately 429,000 mortalities in 2015, with 92% of these deaths in the African region, malaria control programmes have heavily targeted this species in order to reduce the burden of disease (1, 63).

Female *An. gambiae* act as highly effective vectors of malaria because of their strong anthropophilic tendencies – a reported 90% of blood meals are taken from humans and females from this species are able to identify and target individual humans within a crowd of other animals (64). Females are capable of taking multiple blood meals every gonotrophic cycle (which lasts approximately 2 days) but this is highly dependent on the size of the mosquito, with smaller females requiring more blood feeds (65, 66). Although female *An. gambiae* feed from sources of sugar throughout their life-cycles this does not seem to significantly alter their blood feeding habits with regards to humans (67, 68).

An. gambiae females have a strong propensity for indoor biting at night and such vector control programmes have found significant success by distributing mosquito nets sprayed with insecticides as well as indoor spraying of houses with insecticides (1, 69, 70). Females from this species also tend to lay their eggs on the surface of calm, clean bodies of water which can then be targeted using larval source management; these eggs are laid singly on floats, with the emerging larvae lacking a siphon and lying parallel to the water surface (56, 71). Whilst extensive control programmes have produced significant reductions in mortality and morbidity, malaria control has not yet been completely successful and future elimination attempts require even greater efforts to be made.

2.1.3. *Culex quinquefasciatus*

Similar to *Ae. aegypti, Cx. quinquefasciatus* mosquitoes tend to prefer urban environments in tropical and sub-tropical regions - *Cx. quinquefasciatus* larvae are able to survive in heavily polluted water, such as sewage tanks, however whilst *Ae aegypti* larvae can only survive in relatively cleaner environments (56, 72). *Cx. quinquefasciatus* is the major vector species for West Nile Virus, Saint Louis encephalitis and the nematode *Wuncheria bancrofti* (73).

Although *Cx. quinquefasciatus* eggs are deposited on the surface of the water as large rafts of more than 100 eggs, the larvae and pupae which emerge and develop from these eggs act in a similar manner to *Ae. aegypti* of a comparative developmental stage – adult mosquitoes from these two species also rest parallel to the ground in an identical manner (56).

Cx. quinquefasciatus females are highly opportunistic and will acquire blood meals from multiple sources depending on nearby availability, meaning that estimates of anthropophilic behaviour can vary significantly depending on the environment of the region studied (74, 75). There is evident plasticity in female feeding habits as well, with an overall tendency to attempt to acquire blood meals indoors during the night but also a significant amount of biting attempts outdoors in some locations - this high level of variation extends to the percentage of females which take multiple blood meals per gonotrophic cycle, which lasts for around 3 days on average (72, 76, 77).

As a result of the flexibility demonstrated in *Cx. quinquefasciatus* biting habits, control programmes have been forced to utilise multiple methods of vector control, ranging from attractive toxic sugar bait stations in key locations for egg laying to the controlled introduction of bacterium targeting mosquito larvae (78, 79).

2.2. Mosquito-borne disease transmission

As is clear from section 2.1, there are significant differences between the major mosquito species associated with disease transmission. There are some similarities that are maintained across the three species however, with frequency matching of males and females flight tones at higher order harmonics of the fundamental wing beat tone being reported as an essential part of courtship in all of these species - females from all three species must also consume blood meals in order to produce viable offspring, with this process directly linked to disease transmission (34, 56).

Transmission of a mosquito-borne disease can occur when a female mosquito that is infected with a disease takes a blood meal from a human and transfers a pathogen in the process. Beyond this fundamental step in the process, tremendous variation is observable even in mosquito biting habits (47, 80, 81), pathogen development (82-84) and tendency towards anthropophilic behaviour (64, 85, 86) (as noted in section 2.1). These differences are seen not only between species but also between members of the same species in different areas (87). This has made the biting process itself a key target for insecticidal control measures because of its ubiquity amongst all mosquito species of interest.

Species specific preferences for human blood meals (as opposed to blood from other animals) can play an important role in disease transmission (88, 89). Mosquito attraction to humans is mediated by a number of different factors including mosquito genetic predisposition as a result of odorant receptor evolutionary pathways (90) and human genetics (91). There are potentially many physical and chemical cues that females use to identify suitable hosts (92), such CO_2, temperature and body odour (93-96) – female mosquitoes may then integrate these separate sensory cues during host seeking (97).

Olfactory regulation in females, particularly with respect to odorant receptors in *An. gambiae*, has therefore become a potential target for control measures that seek to prevent biting events from occurring (98-100). The increasing capability to produce transgenic lines in laboratory settings (such as those created to study the odorant receptor co-receptor gene *Orco*) has also provided greater levels of insight into olfactory centre organisation (101).

Pathogen infection can create significant changes to insect blood-feeding behaviour; for example, *Anopheles gambiae* mosquitoes infected with the *Plasmodium* parasite are far more attracted to human odorants and are more likely to attempt to obtain blood meals than their uninfected counterparts (102-104).

In addition to this, mice infected with *Plasmodium chabaudii* (the rodent malarial parasite) display enhanced attractiveness to mosquitoes during key periods of the pathogen cycle in which the mammals are highly infectious (105).

The blood feeding process itself follows a reasonably standard pattern; after a female mosquito has located a potential source of blood and landed upon it, she will skim her labellar tip over the surface of the skin in order to identify promising insertion points. Once she found a satisfactory position she will separate her forelegs symmetrically around her entire proboscis so as to generate as much force as possible whilst her fascicle penetrates the skin in a series of rapid thrusts. The labium and maxilla will then follow through the puncture (106). Both the labium and fascicle have been suggested to be essential to the blood feeding process, with females lacking a labium unable to penetrate human skin (107) whilst those without a fascicle have drastically reduced blood sensory capability (108).

The flexibility of the fascicle tip allows it explore fully the freshly created perforation in search of a blood vessel; once one has been located and entered, the maxillary palps vibrate in phase with the entire mouthparts of the female to stimulate blood flow and begin the feeding process (109). There are several steps therefore throughout the feeding procedure in which mechanosensation could play an important role, with the reported presence of ChOs in the female mosquito labellar lobes also suggestive of a mechanosensory component of the biting procedure (110, 111).

2.3. Current control programmes

The significant variation observable between mosquito species in terms of biting habits, breeding locations and global distributions (as discussed in section 2.1) necessitates targeted control programmes that change focus according to the vector and disease in question. Certain situations require specific control tactics; for example indoor residual spraying may be effective against *An. gambiae* females that prefer to bite indoors but could be relatively ineffective against other *Anopheles* species that tend to blood feed outside the confines of the home (112, 113).

The tendency for *Ae. aegypti* and *Ae. albopictus* females to thrive in urban environments whilst *An. gambiae* prefer more rural areas is also indicative of the unsuitability of generic control programmes as different control measures are required for the different environments (114, 115). Vectors of the same disease, such as lymphatic filariasis, may even require different insecticidal strategies depending on the continent that they are found upon (116).

At their most general therefore, most current mosquito control programmes rely on a mixture of targeting the disease vector via some manner of insecticide application (117) whilst also combatting the pathogen with either prophylactic mass drug administration (118) or rapid treatment of individuals presenting signs of illness with antibiotics (or similar medical compounds), which not only help to reduce morbidity associated with the disease but also decrease the risk of transmission events occurring (119). Some prophylactics can also have an effect on the mortality rate of biting mosquitoes, further enhancing their usefulness (120). These generic programmes are then tailored to specific situations in order to be as effective as possible.

Further than this, vaccines for certain diseases are already available, such as yellow fever (121) and dengue (122). Although a malaria vaccine will hopefully become available for inclusion in control programmes within the foreseeable future the precise timelines for its potential deployment are unclear at this time (123).

2.3.1. Vector control

2.3.1.1. Insecticidal nets and spraying

There are four major categories of insecticide classified as suitable for use in order to combat mosquitoes, all of which have distinct mechanisms of action and can be used in various combinations; these are the pyrethroids, the organophosphates, the organochlorines and the carbamates (12). These can then be further classified as targeting either voltage-gated sodium channel proteins (with both the pyrethroids and the organochlorides preventing the closure of these channels (124, 125)) or the enzyme acetylcholinesterase, which is essential for the termination of synaptic transmission - the carbamates reversibly inactivate this enzyme whilst the organophosphates permanently inactivate it (126, 127).

The two most common methods of introducing one of these insecticide types to the mosquito vector are indoor residual spraying of pesticides and long lasting insecticidal bed nets (128). Both have proven highly effective in reducing disease prevalence in a variety of circumstances by decreasing the number of disease transmission events – both by eliminating female mosquitoes that are about to take a blood meal and by removing females within the mosquito population that are able to successfully produce offspring (129-131).

These two control mechanisms can be used in conjunction with one another in order to maximise coverage, though the effectiveness of such a control programme is dependent on many different variables including location, spraying frequency and bed net coverage rates (132-134). Both of these methods of control are far less effective against mosquito species that tend to take blood meals outdoors or are less anthropophilic than other vector species and so require supplementation as part of an integrated programme (135, 136).

Preventing biting events from occurring in regions where biting and disease transmission tend to occur outdoors (and as such outside the effective range of indoor pesticide spraying) requires different strategies. The effectiveness of topical insecticidal repellents to combat mosquitoes attempting to bite in such places is uncertain, with large variation between the results of different studies (137). Low compliance rates, inappropriate usage of repellents and suboptimal coverage compound coverage can all play a significant role in this however rather than there necessarily being fundamental issues with the use of repellents (138, 139). Insecticide treated clothing has shown more effective results in terms of biting protection but requires repeated treatment of insecticides to counteract the removal of compounds when the clothing is washed (140).

2.3.1.2. Larval management and oviposition traps

Another method that allows for targeting of mosquitoes species that do not bite indoors (and as such could be considered beyond the reach of bed nets and indoor spraying), as well as for the utilisation of different pesticides than those used to target adult insects, is the targeting of the larval stages of the mosquito developmental cycle (141). This has shown some positive results in certain situations (142-144) and has thus led to the trial of many different types of control compounds (145, 146) as well as unique mechanisms of compound dissemination (147).

Identification of areas containing high larval densities can be difficult for mosquito species that tend to prefer urban environments (such as *Ae.* aegypti) and can result in significant costs associated with door-to-door searching efforts (62, 148). Targeting specific locations, such as junkyards, which are likely to contain suitable locations for mosquito egg-laying to occur can help to make larvicidal applications more time and cost efficient (149, 150). Area-wide low volume insecticide dispersal using commercially available sprayers has also been trialled and found to be effective in reducing larval emergence, though a corresponding decrease in adult population size was unfortunately not observed (151, 152).

This seeming disparity between significant reductions in apparent larval populations and a seemingly steady population size for adults has been reported for several studies that targeted the larval stages of mosquitoes using a variety of methods and could be due to inadequate distribution of the larvicidal compound or potential effects of larval density on adult survival rates (150, 153, 154). Regardless of the precise cause, the lack of a decrease in the adult mosquito population size raises some doubts over the effectiveness of larval management programmes (62).

One method that could be therefore be used as a supplementary measure alongside larvicidal applications is the introduction of oviposition traps; small pots of water containing both oviposition stimulants and an insecticide so that when a female mosquito attempts to lay eggs she is exposed to the compound (62). These traps are particularly suitable for targeting *Aedes* species in urban environments and have shown positive results in field trials, though they are likely to be less effective if used to target *Anopheles* species, whose egg laying habits are significantly different (155, 156).

2.3.1.3. Attractive toxic sugar bait

Another control method that can be utilised against mosquito species that tend to bite outdoors is the usage of attractive toxic sugar baits, which involve doping a sugar-based source of mosquito food with a pesticide and have proven successful as well in specific circumstances, provided the mosquito species that is being targeted tends to feed regularly from specific sugar sources (157, 158).

Utilising insecticidal ingestion as the method of exposure can circumvent issues involved with some cuticular resistance mechanisms and can lead to increased compound efficacy but does still require the compounds involved to be safe for use around humans (159).

There are two separate versions of the attractive toxic sugar bait measure, with one entailing spraying the toxic solution onto vegetation near ponds and other water sources known to contain mosquito larvae whilst the other involves setting up toxic sugar bait stations inside residences – field trials utilising both methods have noted reductions in estimated adult population sizes of greater than 80%, with the female population who had completed multiple gonotrophic cycles being particularly effected (160, 161).

This specific population is of great interest for control programmes as it is these females which are the most likely to be infected with a pathogen (given the necessary disease incubation period) and as such reduction in the size of group can lead to a disproportionate decline in disease incidence. Treating livestock with similar insecticides, alongside other methods utilising zooprophylaxis, has also shown some potential (162, 163).

2.3.1.4. Proposed control methods utilising fungal, bacterial and genetic mechanisms

Trials of fungal and bacterial (principally *Wolbachia*) pathogens have demonstrated significant potential as alternative methods for targeting mosquito species (164-166). Fungal pathogens can be sprayed indoors in a comparable manner to that of traditional insecticides in indoor residual spraying programmes and have the benefit that mosquito mortality schedules can be pre-determined; this in theory should decrease the possibility of resistance developing as well as reducing the number of infectious bites that occur (167-169).

Wolbachia pathogens have been utilised in a completely different manner however; *Wolbachia* infected *Ae. aegypti* that have been released in semi-field trials have been reported to invade existing mosquito populations and block dengue transmission because of pathogen-mediated changes to mosquito host biology (such as increased feminisation of males and cytoplasmic incompatibility) (170, 171). Whilst this technique appears highly promising and could be extended to the control of many mosquito borne diseases, there are some concerns over the impact that *Wolbachia* could have on localised biodiversity (172).

A variety of genetic methodologies also seek to disrupt disease transmission via the controlled release of laboratory reared mosquitoes into the field – these include the sterile insect technique and the release of mosquitoes carrying a dominant lethality (173, 174). The first technique involves the release of artificially sterilised males who are thus unable to produce offspring despite mating with females with (theoretically) the same likelihood as non-sterilised wild males, which should result in a decrease in population size (although this has not always been the case in field tests) (175, 176). Male mosquitoes released utilising the second technique are mutants carrying dominant lethal mutations which result in their female offspring suffering from a drastic reduction in competitive fitness (177, 178). Genetic techniques have a significant level of potential with regards to mosquito control but have yet to become fully integrated in vector control programmes.

There are therefore a large variety of potential vector control tools that can be used – unfortunately however several of these measures (such as the genetic techniques) have not been widely implemented whilst others (such as larval source management) require extremely high levels of coverage and expenditure to have any observable effect. As such, insecticidal nets and indoor residual spraying continue to provide the backbone of vector control efforts. Whilst they have so far done so successfully, this could cause problems in the future if insecticidal resistance reaches a critical threshold; they are also unable to substantially help control efforts targeting female mosquitoes that bite outdoors.

2.3.2. Pathogen control

Preventative treatment reduces both disease incidence and transmission by stopping disease progression should an protected individual be bitten by an infected mosquito, with mass drug administration of compounds such as ivermectin having been reported to lead to significant decreases in the prevalence of diseases like lymphatic filariasis (179, 180). Chemoprevention is only possible for diseases which have appropriate antibiotic treatments available however and so is not suitable to combat certain diseases spread by *Ae. aegypti* for example (181, 182).

The successes observed for such programmes targeting lymphatic filariasis and onchocerciasis have resulted in such control measures being suggested for expansion to malaria control programmes, whilst recognising certain caveats regarding the possibility of increased antibiotic resistance prevalence (183-185). Seasonal malaria chemoprevention using a combination of amodiaquine and sulfadoxine-pyrimethamine has already been recommended in some regions of sub-Saharan Africa and has the potential for significant decreases in malaria-associated morbidity (186, 187).

In addition to preventative treatment programmes, immediately treating patients who present any signs of illness can also help to decrease disease transmission as well as reduce key morbidity and mortality factors (188, 189). Unfortunately whilst this is possible for malaria because of the existence of artemisinin combination therapy, which has rapidly become the gold-standard antibiotic course for malaria in spite of increasing reports of antibiotic resistance (189, 190), there are no specific remedies available for treating infection with dengue or the chikungunya virus. This means that only treatment of the disease symptoms is possible, as well as attempting to reduce the possibility of further mosquito biting events (by transferring infectious individuals to hospitals for treatment and continually keeping them under insecticidal nets for example) (191).

2.3.3. Vaccination

Vaccination has repeatedly been demonstrated as among the most effective methods of combatting infectious diseases, with pathogens such as smallpox, polio and measles either being greatly reduced in incidence or completely eradicated due to sustained vaccination programmes (192). Unfortunately vaccine development can be a drawn-out and arduous process with no guarantee of success – the development of an effective vaccine to combat malaria for example has been particularly challenging (193-195).

As a result of this, much has been made of, and many hopes attached to, the RTS,S/AS01 malaria vaccine. Trials so far conducted have produced mixed results – there is published evidence of infection prevention over a four year period (196) but this has been found to strongly wane over time, which can cause significant issues as the average age of infection increases (197). In addition to this, the original goal of 50% efficacy has not been met during any of the later stage trials and the vaccine is not effective against *Plasmodium vivax* strains (198). Whilst other vaccines targeting malaria are being researched, they remain well behind the RTS,S/AS01 vaccine in terms of the developmental pipeline, meaning that for the foreseeable future at least vaccines will play at best a supporting (rather than a major) role in malaria control initiatives (195, 199).

Vaccines are available for certain mosquito-borne diseases however, with the yellow fever vaccine in particular proving to be safe, long-lasting and highly efficacious (200). A dengue vaccine, CYD-TDV, has been licensed for use in some countries and although it has encountered some issues regarding serotype susceptibility it could be become an effective tool in regions suffering from a high dengue burden (122, 201). Early stage trials for potential Zika virus vaccines have been initiated as well, though given the experiences of such trials for malaria vaccines early stage results should be treated with extreme caution (202, 203).

Whilst suitable vaccines are not available to combat every infectious disease, where they are obtainable they form a key part of control strategies. Nevertheless it must be stressed that even the most effective vaccines acting alone will not lead to the elimination of many mosquito borne diseases and as such should be utilised as part of a wider ranging programme of control measures that also feature different mechanisms of disease control.

The use of vaccines as a control measure does hold a significant advantage over the other two methods of control however in that it alone is not subject to the growing concerns regarding insecticidal and antibiotic resistance that could cause severe problems for disease control initiatives in the future (7, 190, 204).

2.4. Insecticidal and antibiotic resistance

Evolutionary pressures have led to the development of high levels of insecticidal resistance in many mosquito species (205-207). The extent of the selective pressures faced by mosquitoes can be judged by the rapidity in which susceptible populations develop resistance – for example the mosquito mortality rate in some locations after insecticide exposure (at currently advised levels) has fallen from 100%, when the toxin was first introduced, to less than 50% after several years of constant use (208, 209). In particular, within three years of spraying in Mexico (210) and after less than a decade of interventions in Tanzania mosquito populations with almost no discernible resistance levels became highly resistant to pesticides (211).

Resistance to the pyrethroid class of insecticides is of particularly significant importance because insecticidal bed nets can only be treated with members of this category of compounds and a significant proportion of toxins used for indoor spraying are pyrethroid based (12, 212). This has made reports of the development and globalisation of pyrethroid resistance all the more concerning (213-215) especially when taken in conjunction with the evolution of resistance to other insecticides classes (as well as resistance to multiple insecticides, which is often linked to cytochrome P450 enzyme mutations) (216-219).

Resistance can be classified into one of four types; cuticular (absorption of the compound is reduced due to cuticular modification (220)), metabolic (enzyme activity increases prevent toxin effectivity (221)), target-site (change in compound binding-site structure reduces probability of successful insecticide binding (222)) or behavioural (behavioural adaptations allow for toxin avoidance (223)). Behavioural resistance is the least well categorised and understood of the four but has been suggested as having a sizeable impact on vector control (224, 225).

Resistance mechanisms can occasionally have indirect consequences on important aspects of mosquito behaviour. *ace-1*^{R} mutants for example display resistance to both carbamate and organophosphate based toxins but also demonstrate altered biting frequency and rhythmicity as result of changes to their salivary protein expression patterns (226). The *ace-1*^{R} mutation confers a fitness cost on mutants (227), which is not uncommon for mutant alleles. This reduction in competence can then lead to a reduced response to pathogen infection in the mosquito, causing a relative increase in infectivity potential in these resistant individuals and thus potentially increasing the likelihood of infectious bites occurring as population resistance grows (228, 229).

Insecticidal resistance does not however necessarily have to mean total resistance to all concentrations of a specific compound, meaning that insecticide spraying and bed nets can retain some usefulness even in resistant areas (230). In addition to this, testing the youngest mosquito age bracket could artificially inflate the apparent level of population resistance as insecticide resistance has been suggested to decrease as mosquitoes grow older (231, 232) - no complete causal link has been definitively identified yet however and the confounding influence of other factors (such as natural selective pressures) may also play a significant role. The general overall increases observed in resistance levels however help to force the case that new insecticides are required for use in the field.

The increasing reports of insecticidal resistance have been matched by a similarly growing number of accounts of antibiotic and drug resistance within various pathogens. For example, artemisinin resistance has been reported for almost a decade (8) and has become substantially more prevalent since then (7, 233), whilst ivermectin resistance has been noted more and more as the compound is distributed to wider populations (234). This pattern of isolated outbreaks of resistant pathogens becoming widespread has been seen previously, with previous frontline antibiotics such as chloroquine becoming useless within the span of a decade (235).

Resistance can emerge as a result of non-completion of antibiotic treatment or due to inadequate doses of the treatment being administered to patients because of the distribution of counterfeit, substandard drugs, with the considerable selective pressures placed on pathogens by mass drug administration being particularly important as well (236, 237).

There are few novel antibiotics sufficiently advanced along the developmental pipeline to have the potential for a significant impact in the next few years (238), with neglected tropical diseases in particular suffering from a lack of research funding in this area (239). Most new treatments proposed in the near future involve repurposing compounds already in use against one disease, such as using the anti-filarial drug ivermectin against malaria (240, 241) – this is a common occurrence within the sphere of helminth infections for example (242). These approaches can yield some successes and help to maximise the effectiveness of the repurposed compounds by targeting multiple diseases, but can also increase the likelihood of resistance developing.

The development of resistance has repeatedly prevented global eradication of several diseases over the past century (243-246), though this has not been helped by the overreliance of control efforts on a limited number of vector control mechanisms (247). The expansion of the available tool set can only help the disease control effort.

2.5. Use of *Drosophila melanogaster* as a model organism

Drosophila melanogaster is widely used as a model organism because of a number of major advantages associated with the insect; they are relatively cheap to maintain, reproduce quickly and efficiently and their genomes are well mapped and open to manipulation (248). *Drosophila* genetics have been at the forefront of the overall field of genetics for decades (249, 250), with the recent development of the CRISPR-Cas9 system in *Drosophila melanogaster* opening even more pathways for exploration (251, 252). The CRISPR-Cas9 system allows for targeted deletions of specific coding sequences of the genomes, enabling efficient investigation of gene knockouts – perhaps most interestingly with regards to this project it also enables the introduction of mutations that lead to insecticidal resistance, the consequences of which can then be explored (253, 254).

Most pertinently for this thesis, *Drosophila melanogaster* has been used not only as a model of hearing loss (255, 256) but also of both insecticidal resistance (257, 258) and mosquitoes – in terms of their genomes (259, 260), parasite development within a host (261) and disease transmission (262). Both types of insect also rely on ChOs for proprioception and auditory function (28, 263) and as such mechanotoxins which are able to successfully target *Drosophila melanogaster* could potentially be included in anti-mosquito programmes.

Whilst there remain significant differences between the two fly types, certain key sections of the *Drosophila melanogaster* life cycle can be considered as simplified versions of the corresponding mosquito behaviour – *Drosophila melanogaster* auditory communication during courtship for example does not require the complex harmonic interaction and exploitation of distortion products that is necessary in mosquitoes species (34) but both courtship processes involve sensory location of sexual partners and the active hearing of wing beats (264, 265).

It seems plausible therefore that if a mechanotoxin was able to significantly impair *Drosophila melanogaster* habits, especially copulatory functions, then the corresponding effect on the relatively more complex and delicate mosquito systems would be even greater. Taken in conjunction with the increased simplicity of arranging *Drosophila* species based experiments, as well as the available depth of literature and knowledge regarding *Drosophila melanogaster* mechanosensation as compared to mosquitoes, this provides a powerful argument in favour of using *Drosophila melanogaster* as a basis for behavioural tests which can then be extended to mosquito species when desired.

2.6. Sensory organs and mechanotransduction

There are two major classes of mechanosensory organ found within *Drosophila melanogaster*, with Type I sense organs being ciliated whilst Type II organs are non-ciliated – Type I organs can be further distinguished into bristles, campaniform sensillae and ChOs (25, 266, 267). There is some overlap between the general roles played by each of the receptor classes – this is shown in figure 1 below (which illustrates in detail the stimuli each sensory organ is receptive to) with regards to proprioception in particular, with several different receptor types being involved in this important sensory activity. There also appears to be some overlap in the mechanotransduction channels themselves (for example the ion channel NompC is present throughout the Type I organs and appears to be linked to the mechanotransduction process in each receptor (268-270)). There still exists a great deal of specificity in organ function however, with this thesis being specifically focussed on the role of ChOs with regards to auditory and proprioceptive mechanotransduction.

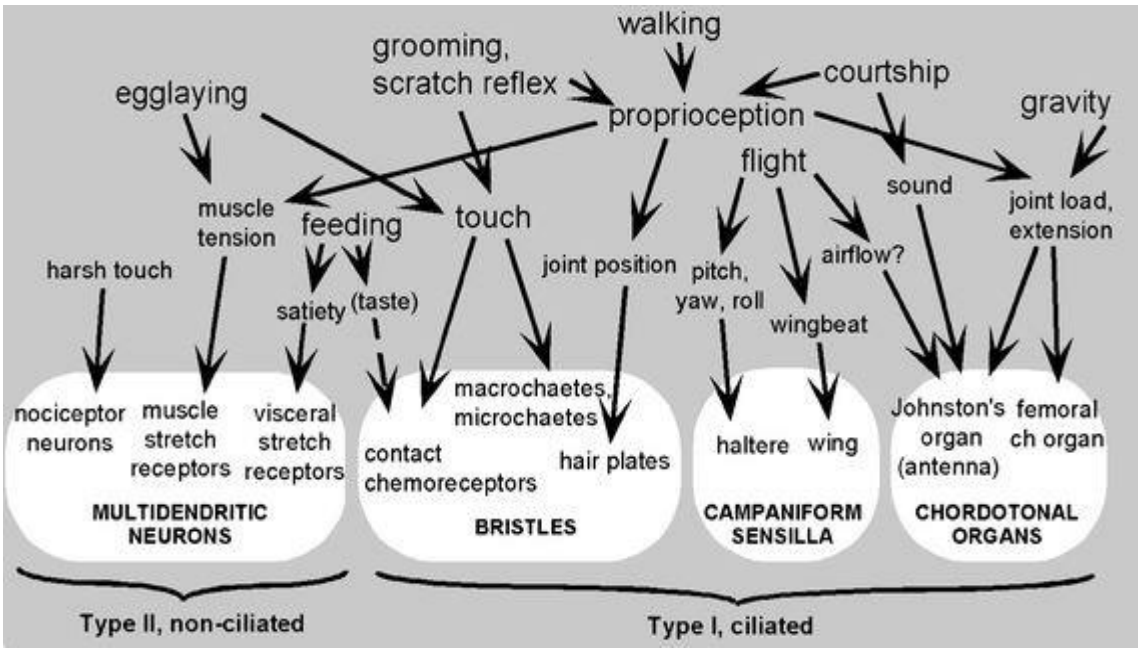


Figure 1: Overview of the major sense organs within Drosophila melanogaster, with arrows linking behaviours and stimuli to the appropriate sensory organ (adapted from (25)).

ChOs are the primary substrate sites for graviception and auditory mechanosensation and mechanotransduction in insect species (25, 271). These sensory organs are ciliated stretch receptors present throughout the insect body which transduce stimulated displacements into electrical signals, and are comprised of repeated sub-units known as scolopidia (267). Each scolopidia can contain between one and four sensory neurons - figure 2 contains both a physiological outline of the structure of the organ and the relative locations of some of the most important ion channels found within ChOs (24). NompC and the heterodimeric ion channel formed by Nanchung and Inactive are of particular interest, with both being involved in the mechanotransduction process and the Nan-Iav heterodimer being reported as the site of action for pymetrozine (all of which are discussed in sections 2.7, 2.8 and 2.9).

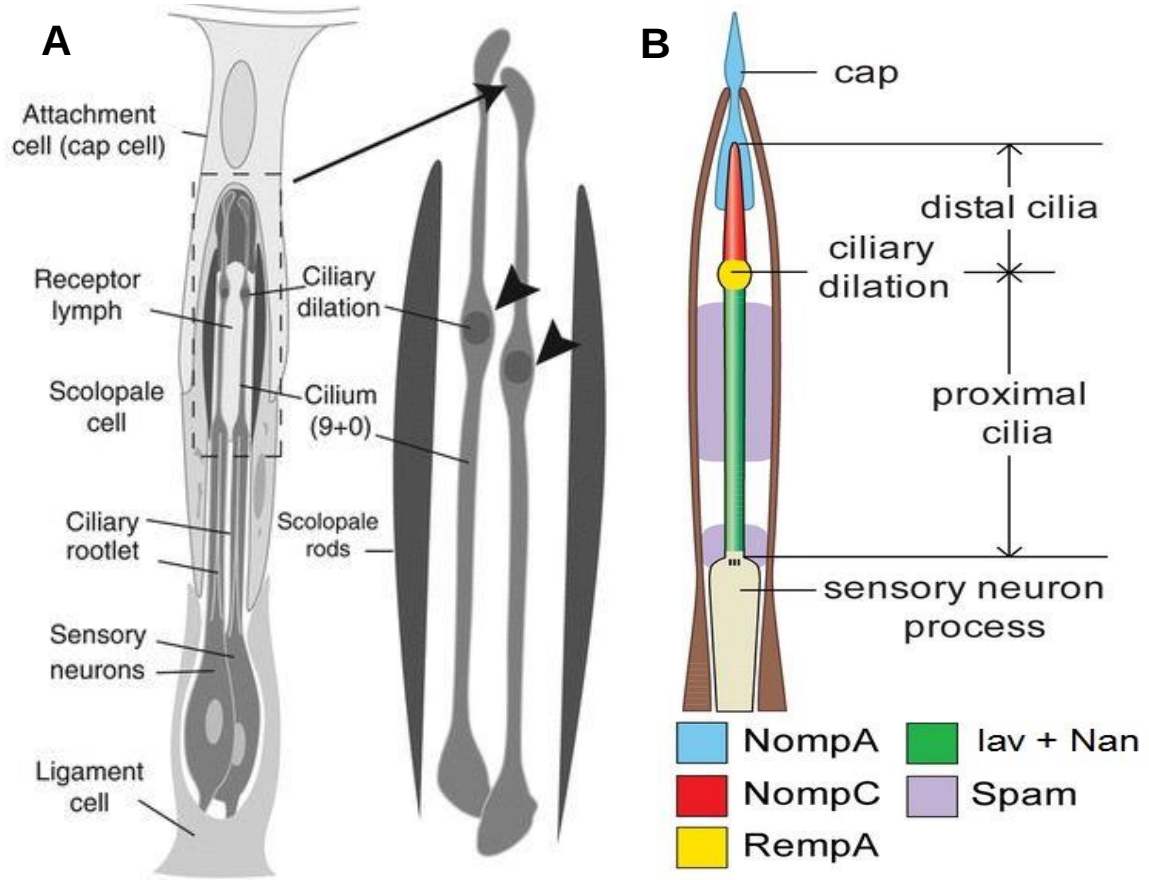


Figure 2. A) Physiological outline of a standard ChO showing key components of the organ (adapted from (272))

B) Relative locations in a ChO of several ion channels considered important for proper ChO function (adapted from (273)).

Whilst the site of transduction within ChOs has been widely agreed to be the distal cilium, the molecular origin has yet to be determined. There are two competing models currently proposed; a NompC model in which the ion channel NompC (=TRPN1) plays the role of transduction mediator whilst the Nan-Iav heterodimeric ion channel complex acts as amplifier and a Nan-Iav model in which the places are essentially reversed and NOMPC now acts as a pre-amplifier (274). In both models NompC is not involved in wind/gravity sensation, which is instead exclusively associated with Nan-Iav (274).

Whilst there is no overwhelming evidence in support of either model, NompC's confirmation as a certified transduction channel subunit directly gated by mechanical stimuli lends that theory extra credence (269), though there remains the possibility that another channel, such as Piezo (275), is in fact the site of auditory mechanotransduction – it could also be the case that the basic, single mechanotransducer channel model is not sufficient complex to capture the full process. Irrespective of the precise transduction machinery however, ChO function is essential for (ChO mediated) proprioception, audition and at least some aspects of temperature sensation (28).

2.7. Auditory systems in *Drosophila melanogaster* and mosquito species

Both mosquito species and *Drosophila melanogaster* have antennal ears that are utilised as auditory receivers, with transduction taking place within the Johnston's organ (JO) of these insect species (see figures 3 and 4 for organ illustrations) (76, 276). The JO is housed in the pedicel or second antennal segment of the antennal receiver and is the largest ChO within the insect body; indeed the male *Ae. aegypti* JO is the largest ChO in the insect world as it is comprised of approximately 7300 scolopidia and 16000 sensory neurons (277). In contrast to this, female *Ae. aegypti* have only half this number (278) and the *Drosophila melanogaster* JO encloses merely 500 neurons (279).

The *Drosophila melanogaster* antennal ear is comprised of three separate parts. These consist of a feathery arista which is directly attached to the third antennal segment (known as A3 or the funiculus), with movement of A3 resulting in the stretching and compression of the two opposing JO neuronal populations housed within the second antennal segment (referred to as A2 or the pedicellus) – figure 3 contains a schematic of how the rotation of A3 causes this stretching of mechanoreceptors. A3 is capable of one dimensional rotation only in response to stimulation (274).

Of the aforementioned 500 *Drosophila melanogaster* JO neurons there are five recognized neuronal subgroups, denoted types A – E (271). These subgroups were classified using UAS-GAL4 lines which expressed GFP in JO neurons with images of the JO from these strains then being used for classification purposes, alongside electrophysiological experiments, calcium imaging and gravitaxis behavioural assays - classes A and B have been thus identified as involved in auditory transduction whilst C and E are essential for wind/ gravity sensation (with type D's exact function currently unclear) (24, 280).

This apparent specification of transduction, with NompC only expressed in type A and B neurons whilst Nan-Iav is expressed across almost all neurons, further complicates the proposed transduction models discussed previously in section 2.6 and has led to the classification of two transducer populations; an auditory 'sensitive' population (comprising classes A and B) and an auditory 'insensitive' population (comprised of the other classes).

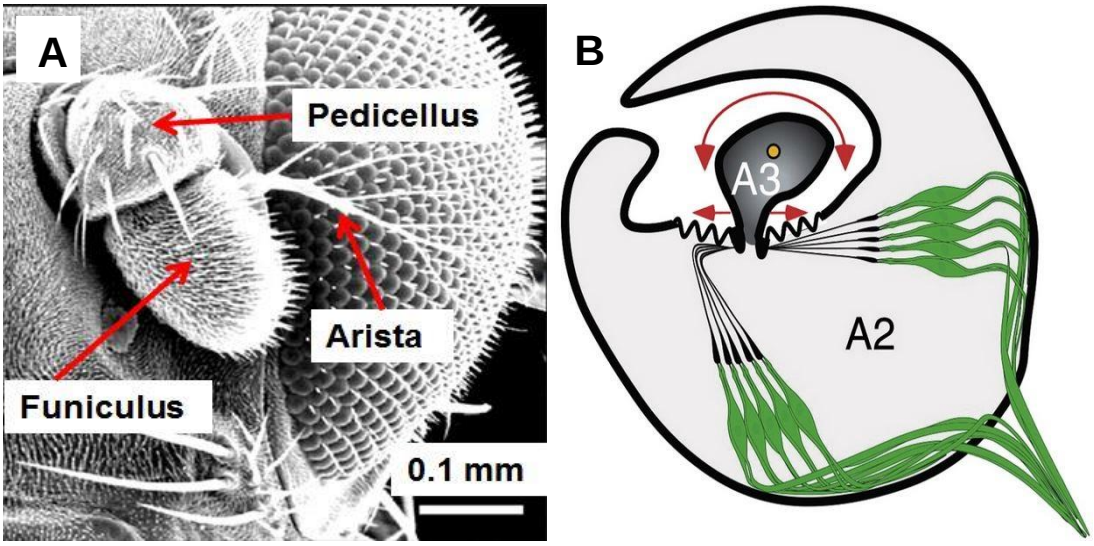


Figure 3. A) The antennal ear of Drosophila melanogaster (adapted from (281)) B) The internal distribution of scolopidia, as well as the effect of rotations of A3 on these neuronal populations (adapted from (282)).

As A3 has only a single mechanical degree of freedom with regards to its rotational movement, the *Drosophila melanogaster* antennal system can be modelled as a damped harmonic oscillator – Laser Doppler Vibrometry (LDV) allows for extremely sensitive, unstimulated free fluctuation measurements of the antennal ear to be recorded, from which assessments of the level of auditory function of the individual can be made (283, 284). The antennal frequency tuning of different *Drosophila species* for both males and females is set to match the spectral composition of the species-specific male courtship songs (274, 284).

The transducer machinery within A2 has been described by a gating spring model of transduction, in which elastic elements open ion channel gates in response to stimulus forcing - the major hallmarks of transducer gating and active hearing have been well documented and demonstrated in this species (and are discussed in section 6.1) and include energy injection into the auditory system, self-sustained optoacoustic emissions, frequency selectivity and sensitivity and a compressive non-linearity that results in an inverse relationship between sound intensity and auditory sensitivity (282, 283, 285).

Drosophila melanogaster courtship requires only the male to produce wing beats, which the female is capable of detecting at the fundamental harmonic (279). Mosquito auditory systems by comparison contain a significantly greater level of complexity because of the higher order of intricacy involved in acoustic communication for many mosquito species; whilst *Toxorhynchites brevipalpis* are able to communicate during courtship using first harmonic wing beats (264), species such as *Ae. aegypti* are forced to harmonically converge on higher order wing beat harmonics for courtship to occur (34).

This frequency range is typically above 1000Hz, although neither male nor female appear to show interest in frequencies greater than 400Hz (34). That the two sexes can still communicate is due to the manipulation of auditory distortion products generated by the direct interaction of the wing beats produced during regular flight in both males and females (276, 286).

This type of communication process requires complicated auditory machinery, including a flagellum capable of multi-dimensional movement (with the mosquito flagellum being modelled as an inverted pendulum with two degrees of mechanical freedom) as well as a considerably larger JO than in *Drosophila* species. Significant progress has still been made in comprehending the system however, with investigations into frequency tuning mechanisms and the presence of amplificatory machinery being conducted in addition to reports of mathematical modelling of the system as a force-damped oscillator (287-290).

In both male and female mosquitoes the auditory system is comprised of a flagellum (composed of 13 subunits known as flagellomeres) which is attached to two basal components, the pedicel and the scape (76) – figure 4 contains images of both the flagellum and the pedicel for reference. In contrast to *Drosophila melanogaster* however, there is a significant level of sexual dimorphism in the auditory system of many mosquito species (276); in those species where there is a low level of dimorphism such as *Opifex fuscus* copulation is completely different from that observed otherwise (291, 292).

This sexual dimorphism is evident not only in the dense fibrillae which extend from the male flagellomeres (which are more structurally variable than in females) but also in the size and composition of the JO (293-295). The male auditory system in particular is incredibly sensitive and finely tuned, although the female is also in possession of a far more sensitive system than most other insect species (288). Auditory sensitivity across the different species may vary greatly but in general female auditory systems tend to be more sensitive than males at lower frequency values (below 200Hz) and individual components of the female auditory system have been reported to be tuned to different frequencies (296).

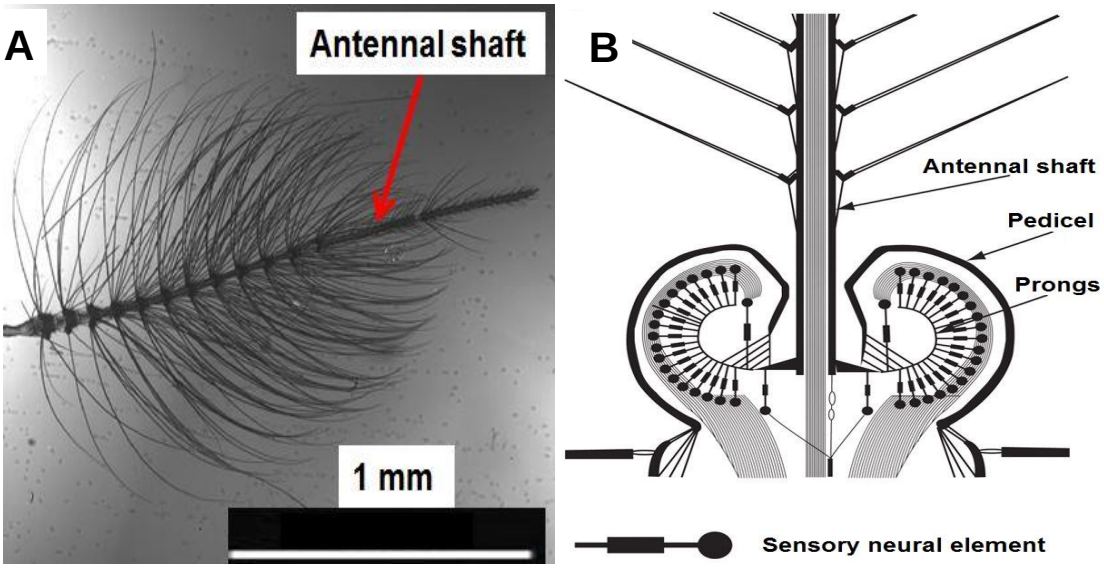


Figure 4. A) The antennal shaft of a male Culex quinquefasciatus mosquito B) Generalised male mosquito antennal system (adapted from (287).

As compared to the five subsets of scolopidia identified in the *Drosophila melanogaster* JO, four different scolopidia types (labelled A – D) have been reported in the equivalent organ in mosquito species (277, 278). The vast majority of these JO scolopidia are type A, which comprise over 90% of the total neuronal population in both males and females and are inserted radially along the inner surface of the pedicel's prongs (76).

Whilst males and female *Aedes aegypti* have approximately equal numbers of both Type B and C scolopidia as well as similar numbers of prongs, only the male JO contains a Type D scolopidium – prongs found in the male *Ae. aegypti* pedicel are also far longer and thinner than those of the female (76). In contrast to this however the pedicel of female *Anopheles stephensi* contain a single Type D scolopidium, though females of this species have only 47 prongs as compared to 78 for males (297, 298).

Antennal fibrillae extension does not occur in male *Ae. aegypti* and *Cx. quinquefasciatus*, with fibrillae remaining constantly erect in both of these species (299-301). Male *An. gambiae* do demonstrate fibrillae erection however during time periods associated with copulatory activity (302, 303). The mechanism by which this erection is modulated is not fully understood but is thought to be related to the release of specific flagellar neurotransmitters which then result in secondary messenger systems being released (304).

Such complex auditory machinery is essential because, unlike in *Drosophila* species where both male and female antennal systems are broadly tuned to the same stimulus, male and female mosquitoes have separate flagellar and JO frequency tuning (which is discussed in more detail in sections 2.10.2 and 7) (34). Male auditory function has tended to be the primary focus of investigations because of the significantly larger JO present in males. This emphasis has for example allowed for the detection and confirmation of nonlinearities in their auditory systems (305), the source of which is likely to be active transducer modules within the auditory neurons (276).

This large JO, in addition to dense antennal fibrillae to increase flagellar surface area, is necessary for males because they are required to locate females for courtship to be possible (305). Male mosquitoes not only seem to place significantly greater energy resources into locating females but also appear to exert more effort in order to induce copulation, with modulation of the fundamental WBF of male *Ae. aegypti* for example being greater than for females of that species (306).

2.8. Auditory mechanotransduction and efferent feedback in mosquito species

Auditory mechanotransduction per se has not yet been directly studied for mosquitoes as it has for *Drosophila* species and its role in mosquito auditory function is not fully clear. This is partly because of evidence concerning spontaneous oscillations, a phenomenon unique to male mosquitoes in natural situations which results in relatively large flagellar oscillations (on the order of 1 μm) in the absence of stimulation.

Spontaneous oscillations have only been elicited in female mosquitoes (as well as in *Drosophila melanogaster*) via DMSO injection (283, 289) – such injections also result in oscillations in male mosquitoes. This is likely the result of decreases in the stiffness of the system following injection, possibly as the result of DMSO's potential effect on membrane stiffness (307). Within the gating-spring model of mechanotransduction (as previously described for *Drosophila melanogaster* for example (285)) this could imply that the steady state stiffness has decreased and as a result the gating spring stiffness has been reduced to a negative value (i.e. the system has become so compliant that active forcing is no longer necessary for self-sustained oscillations to occur).

In male mosquitoes these spontaneous oscillations have been hypothesised as arising in the absence of compound injection as a result of dynein–tubulin motors present in ciliated sensillae, a process that should thus not involve mechanotransduction (308). The reasoning behind this exclusion is based on the results of colchicine injection, an alkaloid which should disrupt microtubules in dendritic sensillae, into the JO of a male mosquito whose flagellum was spontaneously oscillating. The compound caused the cessation of the oscillations whilst theoretically leaving mechanotransduction intact (though the significant reduction in JO nerve response following injection suggests this assumption may not be reasonable).

This does not necessarily prove the independence of the two systems however, as that would require spontaneous oscillations to occur in a system that no longer retained mechanotransducer functionality, whereas here colchicine may act to prevent oscillations whilst leaving the basic mechanotransduction apparatus intact. In either case, the role of mechanotransduction in mosquito auditory systems has not yet been completely defined.

The existence and possible importance of efferent modulation of auditory function within several insect species (including mosquitoes) is also presently undecided. Whilst the auditory systems of *Drosophila* species display many of the hallmarks of active hearing and share remarkable similarities with vertebrate hair cells and ears, previous experiments in *Drosophila melanogaster* found no evidence of efferent feedback within the auditory system.

This was determined by injection with tetrodotoxin (TTX), a powerful toxin that targets voltage gated sodium channels and prevents stimulus conduction along neurons effected (309). TTX should therefore be able to sever any efferent feedback loops present within the *Drosophila melanogaster* auditory system. Whilst TTX injections resulted in an almost total loss of action potential production, there was no observable effect on the mechanics of the auditory system (laboratory data, unpublished).

Another test for the existence of efferent feedback in *Drosophila melanogaster* utilised the conditional expression of tetanus neurotoxin (TeNT) in a pan-neuronal manner (310). TeNT acts at neuromuscular junctions by blocking presynaptic membranes, thus preventing the release of neurotransmitters in this region (311, 312). Obstructing synaptic transmission across all neurons did not prevent mechanical feedback gain in *Drosophila melanogaster* and as such this compound, like TTX, was judged to have no effect on the auditory system. This result was interpreted as meaning that no efferent control system exists within the *Drosophila melanogaster* auditory system, a conclusion which is in agreement with previously published literature (313). It is important to stress however that thus far only evidence of the absence of an efferent system has been presented rather than proof that such a system does not exist.

Efferent modulation of audition had long been suspected for mosquitoes without any real confirmation of such systems being reported (290). Male *Cx. quinquefasciatus* mosquitoes however have recently been found to contain such an efferent system within their auditory framework, possibly to allow for modulation of sensory neurons (314). The presence of efferent systems within male mosquitoes could indicate a greater complexity necessary for proper auditory function within mosquito species. Males from other mosquito species seem highly likely to also contain efferent auditory networks as a result of this and could help to further explain some of the systems of auditory control within male mosquitoes.

2.9. Pymetrozine

Pymetrozine is a slow acting feeding inhibitor currently distributed as a crop spraying insecticide for use against use against pests such as aphids which feed by inserting their stylus into crops and imbibing phloem sap – the compound is sprayed onto crops that require protection from such pests, which then ingest pymetrozine if they attempt to feed (26). Pymetrozine exposure does not directly result in insect mortality as mechanosensory function is not a directly essential requirement for insect survival. It does however cause starvation in insects that can no longer feed naturally and are not provided with a replacement source of nutrition; it is also possible that in the field a lack of proprioceptive and auditory capabilities leads to a decrease in survival probability as predators become more difficult to avoid (23).

Pymetrozine has been confirmed as safe for use in its current application method and has shown high levels of efficacy at concentrations as low as 10^{-7} M during injection tests (315). These concentration estimates are misleading however as the usual mechanism of exposure is ingestion, which therefore requires greater concentrations to be effective especially when applying the toxin in the field.

Previous studies using locusts identified ChOs are the target of pymetrozine, though the mechanism of action and ion channel target site was not reported at that time (22). Since then the major target of pymetrozine in *Drosophila melanogaster* has been confirmed as the Nan-Iav heterodimeric ion channel complex, which is involved in the conduction of calcium (316, 317). All other channels tested requiring at least a hundred fold increase in concentration for any effect to be seen (27).

Further than this, the compound only affects these transient receptor ion channels when they form a single heterodimer and otherwise leaves them undisturbed. As it is only in ChOs that this heterodimer is found within insects, pymetrozine can be considered to be a ChO specific mechanotoxin. Pymetrozine acts as an agonist to this channel complex and compound exposure results in ablation of ChO mechanosensory function as mediated by this complex by promoting an increased cellular calcium influx that functionally deteriorates ChO neurons (27).

TRPV homologs of Nanchung and Inactive have been identified in *Ae. aegypti* male ChOs (318). This provides evidence for pymetrozine being able to affect mosquitoes in a similar manner as for other species – this is also supported by reports of strong effects of pymetrozine on a variety of species (26, 319).

There are no published reports of the impact of pymetrozine exposure on mosquito species and as such nothing is currently reported as to how long the effect of pymetrozine exposure would last for mosquitoes or whether such exposure would affect feeding from sugar sources, searching for hosts or even fecundity.

These important topics have begun to be investigated in other insect species - for example there are reports of a decrease in aphid fertility after pymetrozine consumption and pymetrozine exposed *Drosophila melanogaster* are able to successfully feed from sugar sources in laboratory conditions (although the *Drosophila melanogaster* mechanism of feeding is distinctly different from that of insects that require stylus insertion to feed) (27, 320). Pymetrozine injection experiments in *Drosophila melanogaster* have demonstrated a reduction in aristal sensitivity following exposure and an increase in overall aristal effective stiffness (27). This is also the case if the insect has ingested the compound either as an adult or during the larval stage; this reduction insensitivity has been identified as well if *Drosophila melanogaster* are exposed to pymetrozine solely during the larval stage and are not exposed as adults (unpublished data, Jörg Albert lab).

As would unfortunately be expected in a widely used insecticide, resistance has been documented in several insect species, though the basis of this resistance has not yet been reported (321-323). This resistance has developed over prolonged exposure to the toxin however and could potentially be mitigated by using combinations of insecticides together.

Pymetrozine exposure could have a negative impact in various ways on mosquito species because any behaviour mediated by ChOs could be affected by compound exposure. For example, pymetrozine could alter blood feeding behaviour, either directly by worsening flight ability or indirectly by altering circadian rhythm regulation. It could also decrease copulation success by damaging auditory capabilities (as well as the previously mentioned potential impact on flight) or it could simply decrease mosquito fitness to the extent that that the mosquito is outcompeted by a competitor or consumed by a predator.

2.10. Key components of insect life cycles that pymetrozine could target

2.10.1. Flight

Flight initiation and regulation in *Drosophila melanogaster* is the product of multiple sensory feedback mechanisms including mechanosensation (324). Mechanosensory neurons within the JO itself play a key role in flight maintenance by controlling wing motor reflexes (325) and also by maintaining constant flight speeds (326). A specific sub-class of neurons (denoted as class D) has been proposed as the site of this maintenance activity and has been identified as wind sensitive (327), supported by the significant antennal deflections required to produce activation of these neurons (274).

Aristaless *Drosophila melanogaster* demonstrate significant increases in groundspeed variability compared to *Drosophila melanogaster* which have functional aristae (29), which is most likely due to sensorimotor delays associated with flight maintenance solely mediated via visual feedback (328). The usual regulatory feedback loops become increasingly unstable as instabilities gradually build up throughout the entire system, eventually preventing proper flight control. Proprioceptive feedback loops in particular have been described as essential to regular flight pattern establishment (329) and mutants for mechanosensory genes expressed in the JO are demonstrably worse at flight initiation than wildtype controls (272).

Whilst there is a high level of variability in the level of flight ability necessary for survival (with female urban *Ae. aegypti* being reported to require much shorter flights between blood meal sources and oviposition sites than rural *An. gambiae* females, which are forced to cover longer distances (330, 331)), successful flight initiation and maintenance is in general essential across all species and both sexes, particularly with regards to predator avoidance and the localization of sources of glucose (56, 76).

The processes which underlie successful flight are regulated by a variety of mechanisms, but of particular interest to this project is the reported role that the antennal systems of mosquito species seem to perform in flight maintenance – graviception has also been suggested to be localised to the JO in mosquito species and air speed modulation is presumed to be a result of drag on the antenna (76, 332, 333).

Even partial removal of the flagellum results in significant if slight changes to wing beat amplitude, though interestingly removing equal amounts of both flagella minimises these changes (76). This suggests that the halteres (modified hindwings involved with flight control and mainly populated with campaniform sensillae (334, 335)) are more central to flight maintenance for mosquitoes than the antennal system (332).

The importance of the maintenance of proper flight mechanisms in mosquitoes with regards to not only copulation but also for females to find suitable blood meals should not be underestimated and bears repeating (336, 337). Assuming that mosquito flight regulation is mediated in a similar manner to *Drosophila melanogaster*, it seems possible that ablation of ChO mechanosensory function using pymetrozine would lead to significant decreases in overall flight capability.

2.10.2. Courtship

Many insect species display intricate courtship rituals designed to communicate mating suitability and availability (amongst other factors), with courtship events in both *Drosophila* and mosquito species tending to follow specific, identifiable processes (34, 338).

Drosophila melanogaster courtship rituals follow a well-recognised pattern (339), depicted in Figure 5 below; the male first uses sensory stimuli to locate a suitable female before using a foreleg to tap the female's abdomen. Provided the female has remained receptive up until this point, the male will extend a wing and begin to produce two distinct types of song, denoted as either pulse or sine songs. Pulse songs are comprised of rhythmic downward strokes of the wing at an interval and frequency specific to different *Drosophila* species (and which is closely correlated with the frequency tuning of the antennal system (284)) whilst sine songs consist of the constant production of sine waves (with figure 2 containing examples of each of these song types). The dynamic frequency range of both types of song is relatively small, which taken in conjunction with the one-dimensionality of the *Drosophila melanogaster* sound receiver requires the male to get as close to the female as possible during song production (340).

If the female judges the song to be of acceptable quality she will allow the male to proceed with genital licking and finally copulation. The entire process is dependent on the female's willingness to proceed, without which the male will be unable to successfully copulate (341). After impregnation, the female generally remains unreceptive to further courtship attempts for at least three days (342).

Whilst pulse songs have been identified as crucial to successful copulation attempts due to its' proposed role in species identification, sine songs have been considered as inessential (343) and are thought to be mainly present to enhance female receptivity to mating (344). The distribution of song type seems to be non-random and the result of the males' judgement of the courtship stage as well as the state of the female (345).

Two key components in determining the quality of a pulse song are the time interval between separate pulses within a pulse train (commonly referred to as the interpulse interval (IPI)) and the frequency of a pulse itself (known as the intrapulse frequency (IPF)) (346) - a diagrammatic representation of both the IPI and the IPF is included in Figure 5 part B. For *Drosophila melanogaster* typical IPI values of about 35ms have been reported, alongside IPF estimates of between 150 and 250Hz and sine song frequency values of approximately 150Hz (281, 284).

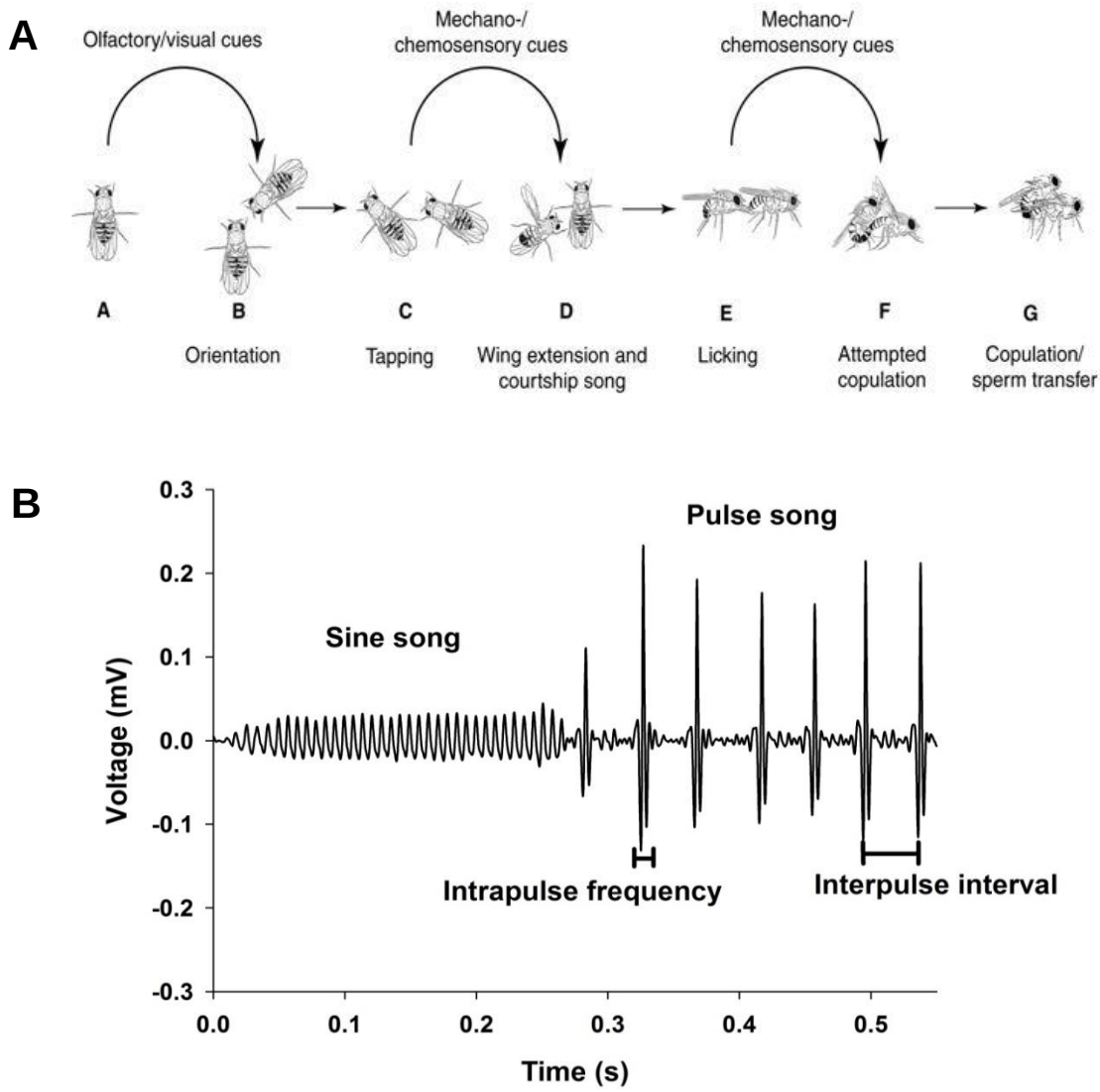


Figure 5. A) The reported courtship pattern identified in Drosophila melanogaster mating attempts (adapted from (339)).
B) Section of a courtship song produced by a Drosophila melanogaster male with important segments highlighted.

The roles that auditory feedback and proprioception play with regard to song production should theoretically be significant – ChOs are distributed throughout the bodies of *Drosophila* species, including their wings, and could therefore be involved with both the males' auditory perception of the songs he is creating and with the proprioceptive sensation that his wings are moving. These feedback loops could then allow the male to constantly control and optimise the intrinsic characteristics of his wing beats and thus improve his chances of reproductive success (340, 347).

In spite of this the majority of reports have not found compelling evidence of any major auditory requirements for the production of quality songs. *Drosophila melanogaster* male mutants without aristae or antennae (*aristaless* and *antennaless*) were able to create songs statistically indistinguishable from control flies (329, 348, 349) whilst physical removal of the entire antenna did not prevent copulation from occurring (350). On the other hand, other *atonal* mutants have been found to produce significantly different songs from wildtype males –this could however be the result of developmental pleiotropic effects that were not properly controlled for within the experimental design (340).

Previous work within the Albert lab found that flies with ablated antennal function had a significantly greater IPF than control flies, though the IPI and sine song frequency remained constant throughout (unpublished data, Jörg Albert lab). This ablation of function was brought about by gluing either one or both of the antennae to the head, with both cases producing similar results. As such it remains unclear whether auditory and proprioceptive feedback mechanisms (and therefore ChO mechanosensory function) are important for male *Drosophila melanogaster* during courtship attempts.

None of the previous attempts at investigating this topic have used a pharmacological method of eliminating antennal function. Given this, and the current lack of clarity on the topic, pymetrozine's possible effect on courtship behaviour overall (and song production specifically) is worthy of assessment.

In contrast to *Drosophila melanogaster*, female mosquitoes can play a much greater role in mosquito courtship behaviour – this is more similar in some ways to the duetting seen in *Drosophila virilis*, which has been found to rely on auditory sensation to succeed (351). Harmonic convergence of male and female flight tones on shared higher order harmonics (typically the third female and second male harmonic) has been reported for *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus*, with an example of such convergence shown in figure 6 part A (352-354).

This frequency convergence has been suggested as conveying information about the genetic quality (i.e. greatest fitness benefits associated with mating) of the male to the female in a similar manner as the pulse song in *Drosophila* species (355, 356). Whilst it seems highly likely that harmonic flight tone convergence is an active process as both male and female mosquitoes change their flight tones in response to one another (with deafened mosquitoes being reported as unable to harmonically converge with mosquitoes of the opposite sex (352)) , this frequency matching may be less important than the auditory distortion products created by this convergence (357, 358).

For example, whilst the mechanical tuning of the male flagellum is theorised to be focussed on the female fundamental wing beat frequency (WBF), the female flagellum is tuned to the cubic distortion product of both her own and her prospective partner's wing beats i.e. the difference of the male WBF and twice the female WBF (as highlighted in figure 6 part B) (358).

The JO of male mosquitoes on the other hand is tuned to the difference tone (the difference between the male and female WBFs); the tuning of the female JO is not presently completely understood and could be more related to identifying predators or blood meal sources than courtship, with female *Culex territans mosquitoes* for example displaying a high degree of phonotaxis towards amphibian auditory cues (358-360). If the female JO is indeed tuned to specific frequencies for this reason, it provides an interesting point of comparison for the relative importance each sex places on copulation in contrast to other activities.

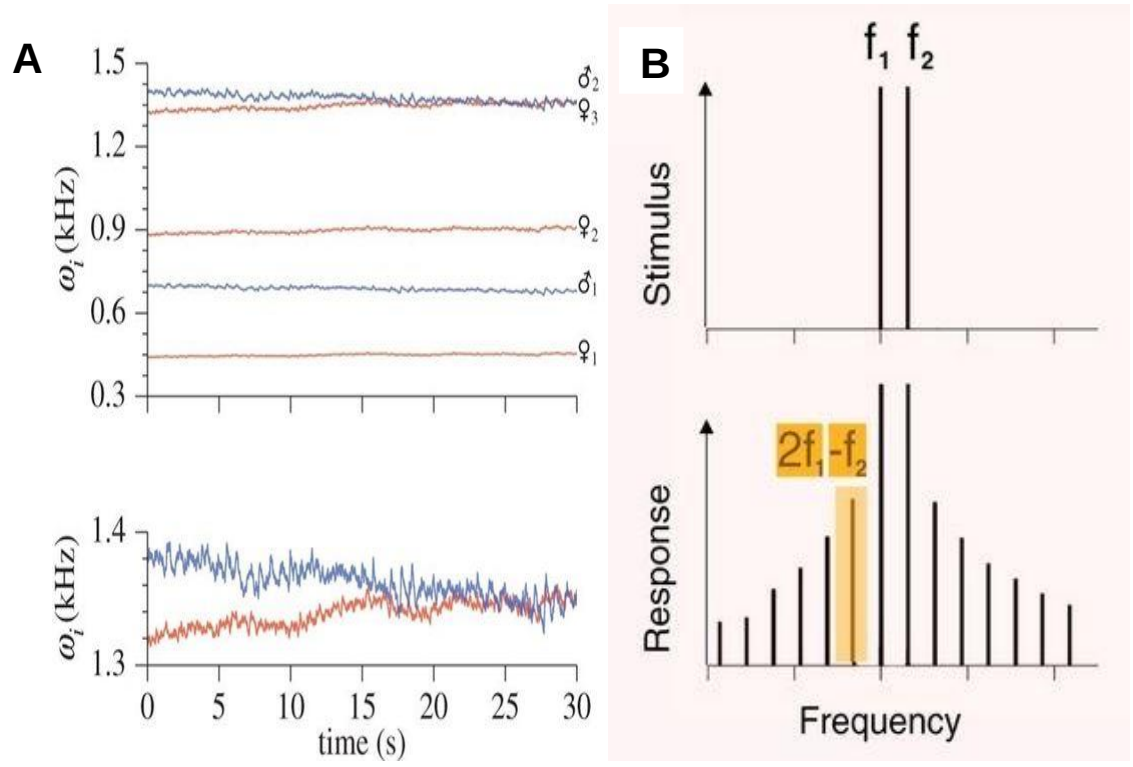


Figure 6. A) Harmonic convergence of individual male and female mosquitoes as demonstrated by changes in wing beat frequency over time; red and blue lines show female and male wing beat frequencies respectively for different harmonics, with the bottom image showing a better resolution of changes to the third female and second male harmonic (adapted from (357)).

B) Distortion product formation from the interaction of two pure tone stimuli, f_1 and f_2, with the potentially relevant 2 f_1-f_2 distortion product highlighted (taken from (276)).

The male auditory system may require a significant level of sensitivity because of the behavioural mating patterns of mosquitoes – swarms of males form normally around dusk and these males are required to identify and locate female wing beats from the overwhelming noise surrounding them (which includes the sound generated by their own wing beat) (361, 362). The WBFs of different mosquito genera are identifiable and measurable using velocity-sensitive pressure gradient microphones to record flight attempts by individual mosquitoes which can then be later analysed (353, 363).

In the field, copulatory events for *Aedes*, *Anopheles* and *Culex* species tend to occur mid-flight, though copulation for *Ae. aegypti* can occur occasionally on the ground (56, 76). Males have a short period of time in which to transfer his sperm or adjust his position once the mosquitoes are attached to each other so that both partners can sustain constant flight (303, 364). Within caged laboratory conditions, mating during flight is still possible but the later stages of copulation (particularly insemination) are more commonly observed for couples attached to the sides of the cage (76, 365).

Female mosquitoes generally mate once per lifetime but there is evidence across multiple species that a small yet significant proportion of the female population that undergoes multiple insemination events (366, 367). This polyandry appears to depend on the post mating interval between copulation attempts by separate males and could have a significant impact on control programmes that utilise sterilised or modified male mosquitoes in order to reduce mosquito population sizes (by decreasing the number of females that can produce viable offspring), as females would then become more likely to mate with wildtype, non-sterilised males (368).

2.10.3. Circadian rhythm regulation

The maintenance of an internal circadian clock bestows numerous benefits on any organism that develops such a system, with circadian rhythms of activity having been reported in a wide variety of different species (369, 370). Understanding the processes behind the maintenance of these rhythms, as well as the effect such rhythms can have on behaviour, is of particular interest for anthropophilic mosquito species because of the strong rhythmicity of female biting habits and male copulatory events (76, 371).

Circadian rhythms in *Drosophila melanogaster* have been well documented using a variety of systems, with robust entrainment being shown using a variety of different stimuli (or Zeitgeber) such as light, temperature and vibration (372). Typically such circadian activity is examined using monitoring systems which quantify the time spent in different activity phases during and after periods of entrainment to stimulation, either using simple activity counts or more complex video analyses (373, 374).

In mosquito species, circadian activity can be quantified using either laboratory experiments or biting catches in the field (in which female mosquitoes are captured during biting attempts and a count is kept of which times captures most commonly occur). Laboratory experiments were previously conducted by keeping individual mosquitoes in glass containers alongside auditory detection equipment so that flight activity could be detected. These mosquitoes were then scored for each minute during each hour spent flying (and thus received a score of between a minimum of 0 and a maximum of 60) which could be aggregated across groups of interest (76). Recently this system has in general been superseded by techniques more similar to those seen for *Drosophila melanogaster* (375).

An. gambiae and *Cx. quinquefasciatus* mosquitoes both display crepuscular peaks of activity whilst *Ae. aegypti* is considered to be a diurnal species (76). Mosquito circadian rhythm activity has been described in detail previously (376, 377), most notably when investigating changes to the circadian patterns of females once they have successfully obtained a blood meal (378). This has been reported to be the result of a downregulation of clock genes once the female has procured a blood meal (375) - indeed there is a strong circadian component with regards to gene expression throughout the mosquito body, particularly in the antenna (379). As noted previously, the antennal fibrillae of male *An. gambiae* become erect during specific periods of the day that tend to be correlated with an increased likelihood of copulatory events (303).

Male and female mosquito activity patterns are clearly distinct, with males tending to show peaks in activity earlier in the day than females – both are more active during nocturnal periods however (380, 381).

Interestingly *Plasmodium* strains of the malaria parasite also exhibit strong circadian activity regulatory mechanisms in order to stay in synchrony with the circadian clock of their host (382, 383). If they become de-synchronised from this rhythm they suffer dramatic decreases in fitness as compared to those that are able to maintain constant time-matching.

Light is generally considered to be the most powerful Zeitgeber for entrainment of the circadian clock, though as mentioned above behavioural events also play a role (384). Entrainment to temperature cycles has also been shown to be possible in mosquito species (385). In *Drosophila melanogaster* ChOs have been proven to be involved in circadian rhythm regulation in terms of both temperature and vibrational entrainment and as such it seems plausible that they could also have a role in the same regulation in mosquito species (33, 386). Given the target of pymetrozine, exposure to the compound could lead to a change in circadian rhythm regulation within the affected mosquito which could then result in sub-optimal feeding or mating behaviour.

2.11. Mathematical modelling

Given the complexity involved in modern disease control programmes, it is essential that as many eventualities as possible are accounted for and the tools involved in these programmes are utilised as effectively as possible. Extensive mathematical modelling of many aspects of mosquito borne diseases has thus been conducted – these range in complexity from the original compartmental Ross model of malaria transmission (and its later development into the Ross-MacDonald model) to Bayesian models of *Ae. aegypti* abundance (188, 387-389).

Given pymetrozine's indirectly fatal nature and the necessity of compound ingestion by an insect for effective exposure rather than cuticular uptake, modelling strategies previously used for fungicides could be appropriate starting points for model development for this toxin (168). Feeding cycle models in general could also be useful as reference points as they provide sufficient complexity to capture the unusual exposure method involved with regards to pymetrozine as well as allowing for investigation into the possible long-term impacts of insecticide spraying (390, 391).

Even these relatively progressive models do not properly account for male actions within the overall population however. Indeed male mosquitoes in general remain unaccounted for and, in the case of pymetrozine in particular, could be significant (392). Spraying of insecticides onto vegetation has not been investigated in depth either, although some modelling with regards to maximising the effectiveness of attractive toxic sugar bait solutions has been attempted (393).

Models of insect mechanosensation have also been reported, with the aforementioned gating spring model of auditory mechanotransduction in *Drosophila* species forming the basis for future investigations described in this thesis into the auditory system of both *Drosophila melanogaster* and several mosquito species (as discussed in sections 6 and 7) (285, 394).

Overall, estimating pymetrozine's potential impact in the field would require a mathematical model that could account for both mosquito sexes whilst including all the potential targets for spraying with the compound. This would necessitate some type of feeding cycle model which allowed for multiple generations to be followed.

3. Project rationale, objectives and hypotheses

3.1. Project rationale

From the literature reports presented in section 2, it is clear that insecticides with different mechanisms of action to the currently existing options are increasingly required for mosquito control programmes. Pymetrozine is a promising candidate for usage based off the compounds' current deployment as a crop spray – its effectiveness against mosquitoes however remains to be tested. This project therefore attempts to ascertain the efficacy of pymetrozine (and by extension the potential of other mechanotoxins) at disrupting copulatory, flight and auditory systems in both *Drosophila* and mosquito species with a view to the inclusion of pymetrozine in future mosquito control measures.

In addition to these experiments, using pymetrozine as a powerful pharmacological tool to ablate mechanosensation will allow for the exploration of insect ChO-dependent mechanosensory systems. Assessing the role of mechanosensation in courtship not only provides concrete support or dismissal of pymetrozine as an pesticide but also enables investigation of what is and what is not necessary for insects to function competitively.

Fundamental aspects of auditory function in mosquitoes are still - to some extent - open for interpretation; pymetrozine can contribute to a greater understanding of the overall system by allowing for comparisons between different states. Whilst the final conclusion of this work will be on the suitability of pymetrozine for mosquito control initiatives, the results demonstrated to support this recommendation should also provide the basis for a deeper comprehension of insect mechanosensation.

Three different mosquito species have been chosen for experimentation – *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus*. All three of these mosquito species are major vectors of disease worldwide and as such are targets of disease control programmes. Each species has also been reported to rely on harmonic convergence for copulation to occur. Significant variation in feeding, oviposition and copulatory behaviour has been observed between these mosquito species – by examining all three therefore it is hoped that the study can be as broad and inclusive as possible.

Careful comparisons of auditory systems can also be made across species, as well as relative assessments of the extent of sexual dimorphism observed; by using three different strains of *An. gambiae* (Kisumu, Ngusso and Tiassale) for example it should be possible to study more closely the auditory systems present within this species.

3.2. Project objectives

The major objectives of this thesis are:

1) To conduct behavioural investigations into the effect of pymetrozine on several aspects of the mechanosensory mediated behaviour of *Drosophila melanogaster*. This includes the potential impact of pymetrozine on fertility, fecundity, life expectancy, flight ability (both with regards to initiation and maintenance), courtship song production and competitive fitness, and is discussed in section 4.

2) To conduct behavioural investigations into the effect of pymetrozine on several aspects of the mechanosensory mediated behaviour of mosquito species; this includes the potential impact of pymetrozine on mosquito larvae as well as fertility and fecundity in adult male and female mosquitoes; this is discussed in section 5.

3) To conduct electrophysiological experiments in conjunction with LDV measurements in order to investigate the mechanical signatures of transducer gating in mosquito species using similar principles as previous reports and pre-existing models developed in *Drosophila melanogaster*; this is discussed in section 7.

4) To conduct electrophysiological experiments to investigate the effect of pymetrozine exposure on these mechanical signatures in both *Drosophila melanogaster* and mosquito species; this includes *Drosophila melanogaster* and mosquito lines that have been reported as expressing insecticidal resistance and is discussed in sections 6 and 8 respectively.

5) To calculate energy gain estimates for male and female mosquitoes following exposure to compounds designed to sever existing efferent feedback loops, as well as following exposure to pymetrozine, with comparisons between the different injection states enabling investigation into the amplification machinery present in both sexes; this is discussed in section 7.

6) To measure the WBF of male and female mosquitoes for all three species, which will then be used to calculate distortion products for comparison with the results of pure tone stimulation experiments designed to identify the peak mechanical sensitivity and nerve response frequencies for all mosquito groups tested; this is discussed in section 7.

7) To calculate displacement gain estimates (using either pure tone or white noise stimulation) for male and female mosquitoes from all species tested in order to investigate sex-specific differences in both the extent of gain observed and the best frequency of mechanical responses; this is discussed in section 7.

3.3. Hypotheses

The major hypotheses of this thesis are as follows:

1) Mechanical signatures of mechanotransducer gating have been identified for *Drosophila melanogaster* and gating spring models of transduction have been published (285). The first hypothesis is thus that the same basic models as used for *Drosophila* species can be used to investigate similar signatures in mosquito species and can allow for quantification of mechanotransducer gating in the JO of mosquitoes; this includes amplification, adaptation and auditory non-linearities.

2) Pymetrozine has been shown to target the Nan-Iav heterodimeric ion channel complex in *Drosophila melanogaster* and acts as an irreversible agonist (27). Given the reported conservation of this channel in mosquito species (318), the second hypothesis is that pymetrozine should have a comparable effect on mosquitoes as it does on *Drosophila melanogaster* in terms of the ablation of ChO mechanosensory function; this includes the effect of pymetrozine on mechanical signatures of mechanotransducer gating.

3) Given the role that the Nan-Iav heterodimeric ion channel complex plays in ChO mechanotransduction (274), the third hypothesis is that exposure to pymetrozine should lead to a disruption in those behaviours (in both *Drosophila melanogaster* and mosquitoes) which are mediated by mechanosensation; this includes flight ability and courtship ritual performance.

4) Efferent synapses have been identified in male *Cx. quinquefasciatus* mosquitoes (314). The fourth hypothesis is that if amplification is transducer based in mosquito species then exposure to pymetrozine should abolish this amplification. In addition to this, if efferent synapses are involved in regulating this amplification then severing these synapses pharmacologically should lead to a change in amplification i.e. synapses act to downregulate/upregulate amplification then severing synaptic function should increase/decrease amplification.

5) Amplification and flagellar frequency tuning are closely correlated with the sexually dimorphic communication behaviour of male and female mosquitoes, a major component of which is harmonic convergence (34, 276). The fifth hypothesis is that the specific frequency tuning of both the flagellum and the maximum nerve response is directly correlated with the tuning of the WBF and the resultant distortion products created by interactions between conspecific male and female mosquitoes.

In total therefore this thesis aims to not only make suggestions as to the efficacy and potential of pymetrozine as an insecticide but by analysing the data collected using pymetrozine as a pharmacological tool also offers future proposals to enhance the general understanding of ChO-mediated mechanosensation in mosquito species.

4. *Drosophila melanogaster* behavioural assays

4.1. Introduction

To date there are no published reports of the effect of pymetrozine (or any other mechanotoxin) on any mosquito species. The little that has been reported on pymetrozine's efficacy and mechanism of action has utilised other insect species, such as *Drosophila melanogaster*, and has so far not fully explored the potential impact of ablating ChO mechanosensory function on the lifecycle of an insect species (22, 23, 27).

Thus by testing various experimental procedures using *Drosophila melanogaster* as a model organism, it seems possible to not only produce data that can be analysed to form the basis of future, more complex experiments in mosquito species but also to investigate the importance of mechanosensation with regards to essential activities in this species; these include flight for example, or courtship behaviour (as detailed in section 2.10).

As discussed in section 2.5, *Drosophila melanogaster* serve as a highly effective model organism for many reasons – among the most important being not only the genetic mutability of the species but also the enormous array of tools and assays which have been reported and published (395-397). This diverse literature enables many specific aspects of various important parts of the *Drosophila melanogaster* lifecycle to be investigated. For example, experiments investigating the fertility and fecundity of various mutant and control flies are possible because of the short generation time of this species (398, 399). Conceptually similar assays can thus be used to investigate whether pymetrozine exposure causes reductions in fertility and/ or fecundity (as had been reported for aphids) (320).

In a similar manner to this flight assays, such as those devised to test either flight initiation or flight maintenance, can also be utilised (400, 401). Whilst flight initiation attempts can be relatively straightforward to quantify, with basic comparisons being drawn between individuals who attempted flights when dropped into containers and those who did not, flight maintenance can be explored in a variety of ways: the multi-camera tracking systems referred to as Flycubes for example, which have been reported to accurately track the flight patterns of *Drosophila melanogaster* in response to visual stimulation, provide an excellent paradigm for such investigations because of the reproducibility of stimulation patterns and the accuracy of the recording equipment (401). Flycubes consist of a free-flight environment containing multiple LCD screens which can present synchronised visual stimuli, alongside high resolution cameras capable of tracking insect flight responses to stimulation (402).

Flycubes take advantage of *Drosophila melanogaster*'s optomotor response to wide-field visual motion by presenting rotating stimuli which cause individual *Drosophila* to rotate in-line with the stimulus (402, 403). By presenting stimuli with different rotational velocities for example, Flycubes are thus able to provide information on the number of flights attempted, how long each flight lasted, the flight pattern produced and the flight response following stimulation (401). This data can then be compared between different *Drosophila* groups to look for significant differences in flight behaviour and maintenance.

Circadian rhythm regulation fulfils a vital role in the control and regulation of numerous behaviours for *Drosophila* and mosquito species (76, 372, 404). *Drosophila melanogaster* has served as an ideal model for circadian rhythm studies in many previous publications and has also previously been reported to require ChO function for proper entrainment to temperature stimuli (369, 386). Given that mosquitoes also entrain to temperature cycles and pymetrozine allows the precise ablation of ChO function, investigating the circadian activity patterns of *Drosophila melanogaster* that have been exposed to pymetrozine (in comparison to controls) will allow for a greater understanding of the role of ChOs in circadian rhythm regulation as well as the formation of hypotheses regarding the potential effect of pymetrozine on the circadian rhythms of mosquitoes (385).

Section 2.10.2 described the courtship routine of *Drosophila melanogaster*, including the production of pulse and sine songs from the male fly. Although the previous literature does not support the idea that auditory feedback is necessary for the production of songs of sufficient quality, the ability of pymetrozine to pharmacologically ablate ChO function (both with regards to audition and proprioception) may result in significant differences being observed (329, 348). The results of such experiments could thus deepen the understanding of the potential role that feedback mechanisms fill with regards to song production.

Finally, the previous experiments described above all investigated the effect of pymetrozine on individuals in non-competitive environments; in the proposed fecundity assay for example male *Drosophila melanogaster* are not provided with competition for females. Providing competition in such environments can enable greater insights into the true extent of pymetrozine's impact which may otherwise remain hidden because of the optimisation of the environment i.e. ample food and no competitors for courtship.

Numerous competitive fitness assays have been described for *Drosophila melanogaster*, which frequently involve two males competing with each other to mate with a single female (405, 406). Thus by exposing one male to pymetrozine and allowing the other to remain unexposed it should be possible to analyse the potential impact that the compound could have on fitness due to the ablation of ChO mechanosensory function.

4.2. Materials and methods

4.2.1. *Drosophila melanogaster* rearing

All *Drosophila melanogaster* stocks, unless otherwise stated, were reared in incubators at 25°C and 60% relative humidity and were provided with food prepared according to the Chippendale recipe. The incubators used a 12 hr: 12hr Light: Dark (LD) cycle which the lines were kept at during all developmental stages.

All wildtype flies used in section 4, with the exceptions of experiments described in sections 4.2.8 and 4.2.11, were the Jörg Albert labs' own wildtype CantonS strain and had no known resistance to any insecticide. Control flies used in experiments described in section 4.2.8 were from the lab of Dr Andrew Straw (Institute of Molecular Pathology, Vienna) and were also from the CantonS line – these flies had no reported insecticidal resistance to any compound. The LH_M–UCL and LH_B–UCL fly lines used in the experiment described in section 4.2.11 were made available by the Dr Max Reuter lab (University College London, London) and had no reported insecticidal resistance (407).

4.2.2. Compound preparation

Pymetrozine: No significant differences had been seen in previous experiments between using DMSO or using milli-Q water as a solvent for pymetrozine, provided the solution was vortexed thoroughly for several minutes (laboratory data, unpublished). Therefore milli-Q water was utilised in all relevant experiments to avoid any possible issues arising in using DMSO as a control substance. A stock solution of concentration 1000 parts per million (ppm) was created by mixing 50mg of pymetrozine with 50ml of milli-Q water. This stock was then shaken and vortexed until the compound had fully dissolved and was diluted to necessary concentrations as required.

4.2.3. Compound exposure – ingestion

Male and female *Drosophila melanogaster* were deprived of food for 24 hours in empty plastic vials containing only damp cotton for humidity control before being transferred to new vials containing 2ml of a mixture composed of 7.5% glucose and 1.5% agar. Either 20 µl of an appropriate concentration of pymetrozine or 20 µl of milli-Q water (for control purposes) had been directly spread on the surface of the food in each individual vial. The flies were kept on the pymetrozine exposed/ unexposed food for a varying length of time depending on the experiment and were then returned to regular stock food. Only 10 *Drosophila melanogaster* were kept in each vial, including control vials, to prevent increases in insect mortality that had been seen previously for pymetrozine exposed *Drosophila melanogaster* if the density of flies per vial was too high.

The length of time during which *Drosophila melanogaster* were allowed to feed from doped food was chosen to minimise the possibility of multiple feedings whilst maximising the number of flies that would feed at all. Pymetrozine exposed *Drosophila melanogaster* have a clear phenotype because of their lack of mechanosensory capabilities – unlike control flies, exposed *Drosophila melanogaster* are slow to move about and reluctant to climb up the walls of a vial (27). The conclusive testing for pymetrozine exposure, which was performed wherever possible, was done using LDV (as described in section 6); this allowed for an unambiguous identification of pymetrozine exposed flies. As this testing was destructive however it could only be done upon the completion of an experiment.

4.2.4. *Drosophila melanogaster* fertility assay

Male and female CantonS virgin *Drosophila melanogaster* were selected upon eclosion using CO_2 sedation and transferred in groups of 10 to vials containing standard fly medium for 24 hours, with male and female flies being kept separately. After this, half of the females were transferred to food doped with pymetrozine at a concentration of1000ppm whilst the remaining half were placed on control food (i.e. food that not been doped with pymetrozine). All males were transferred to control food (with both sexes kept separate).

After two days on this food 40 pairs (each consisting of one male and one female) were placed in individual courtship containers via aspiration. Twenty of these pairs contained a female that had not been exposed to pymetrozine with the other twenty females having been exposed to the compound (no male had been exposed to pymetrozine). Each pair was given 24 hours in which to engage in courtship before the males were isolated and the females transferred to vials containing fresh food. After a further 24 hours on this food, the females were transferred again to another set of vials containing fresh food, where they remained for a final 24 hours before being removed for confirmation of pymetrozine exposure/non-exposure.

All egg counts were done immediately after removing the females from each vial and were then aggregated over each experimental day. In total 40 couples were included in the final analysis; 20 of which contained a female *Drosophila melanogaster* that had been exposed to pymetrozine, whilst the other 20 contained a female that had not been exposed to pymetrozine.

Statistical tests for differences between groups were calculated using Mann-Whitney rank sum tests with a significance level of 0.05 using the statistics software package Sigmaplot (Systat Software Inc., London). Post-hoc calculations of statistical power for the sample sizes and significance level used and effect size observed gave an estimate of 49% power, which is considerably lower than would be usually advised and would therefore lead to an increased likelihood of type II errors.

4.2.5. *Drosophila melanogaster* fecundity assay

Male and female CantonS virgin *Drosophila melanogaster* were selected upon eclosion via CO_2 sedation, after which they were transferred in groups of 10 to vials containing standard fly food medium for 24 hours (with females and males being kept separately). They were then transferred onto either food doped with pymetrozine at a concentration of 1000ppm or control food for two days.

Pairs consisting of one male and one female were then placed together on transparent food that allowed for larval counting. In total there were four different couple phenotypes –both male and female unexposed to pymetrozine (which served as a control), both male and female exposed to pymetrozine and either the male or the female exposed to pymetrozine whilst the other sex remained unexposed. 30 couples from each group were prepared at the beginning of the experiment.

Each couple was allowed to copulate for five days before being transferred to a new vial where they stayed for another five days before being tested for pymetrozine exposure. Larval counting was done immediately upon the couple's removal from the vial. Couples in which either fly died at any point during the experiment were removed from the final analysis – this meant the final analysis included 23 couples from the control group, 22 couples in which only the female was exposed to pymetrozine, 28 couples in which only the male was exposed and 22 couples in which both male and female were exposed.

Statistical tests for differences between groups were calculated using ANOVA on ranks tests with a significance level of 0.05 using Sigmaplot. Post-hoc calculations of statistical power for the sample sizes used and effect sizes observed gave estimates of less than 50% power for each group investigated when compared to the control group, and less than 40% statistical power estimates were calculated for comparisons between groups which contained at least one fly that had been exposed to pymetrozine. This level of statistical power is considerably lower than would be usually advised and would therefore lead to an increased likelihood of type II errors.

4.2.6. *Drosophila melanogaster* life span assay

Male and female CantonS virgin *Drosophila melanogaster* were collected after eclosion via CO_2 sedation and were transferred in groups of 10 to vials containing standard fly food medium for 24 hours (with females and males being kept separately). *Drosophila melanogaster* were then transferred in groups of 10 to either pymetrozine doped food (at a concentration of1000ppm) or control food for two days, with males and females still kept apart. Following this all flies were then transferred to vials containing standard fly food medium, with male and female flies still being maintained separately.

Drosophila melanogaster were either kept alone in individual food vials or in single sex groups of 10. In total there were 48 pymetrozine exposed males and 44 pymetrozine exposed females kept individually, as well as 67 unexposed males and 44 unexposed females; 8 group vials of 10 exposed males and 4 group vials of 10 exposed females were maintained as well, alongside 7 group vials of unexposed males and 4 group vials of unexposed females. All *Drosophila melanogaster*, whether alone or in groups, were transferred into vials containing fresh fly food every 48 hours until all flies involved in the experiment had died. After each transfer fly mortality levels were recorded by counting the number of dead flies in each newly empty vial.

As this study was designed to be purely descriptive and to provide an estimate for fly mortality, no formal calculations for statistical power were made. This means that the results of this study should be treated with caution and should not be considered statistically significant.

4.2.7. Flight initiation assay

Drosophila melanogaster from the CantonS line were sedated using CO_2 upon eclosion and females were inspected under a microscope for apparent wing defects. Those females without apparent wing defects were then transferred into vials containing standard fly food for 24 hours. After this, the flies were transferred in groups of 10 onto fly food doped with pymetrozine at a concentration of either 100ppm, 500ppm or 1000ppm (or a control food that was not doped with pymetrozine). After being kept for two days on their respective food types all flies were transferred onto standard fly food for a further 24 hours.

Paraffin oil was applied to the interior of a 500ml measuring cylinder and a funnel was securely attached to the top of the cylinder (with figure 7 including an outline of the experimental setup). Female flies were then emptied from their vials (each of which contained 10 flies) into the cylinder, whereupon they attempted to initiate flight and adhered to the paraffin oil coating the interior of the cylinder or fell to the bottom. After each vial the position at which each fly landed was noted and the flies were removed from the inside of the cylinder. These results were then aggregated and split into two phenotypes dependant on whether the flies landed in the top ('good fliers') or bottom ('bad fliers') half of the measuring cylinder, and as such were judged to initiate, or not initiate, flight.

The data was then analysed using Fisher's exact test (with an applied Bonferroni correction to account for multiple testing) using Sigmaplot, such that the significance level was set at P = 0.0083. No formal statistical power calculations were made for sample size estimates, with sample sizes being chosen based on a previous report which used a maximum sample size of 84 – all groups investigated had a sample size greater than this except for the controls (with 66 control flies, 89 flies exposed to 100ppm concentration pymetrozine, 149 flies exposed to 500ppm concentration pymetrozine and 159 flies exposed to 1000ppm concentration pymetrozine included in the final analysis) (272).

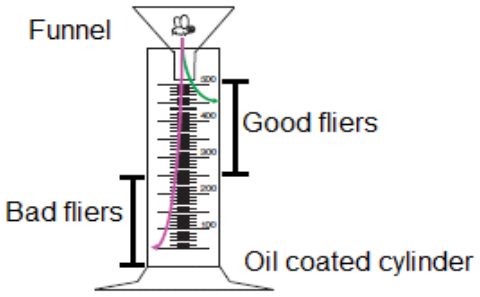


Figure 7. Experimental outline of the flight initiation assay, with the landing areas which denote good or bad fliers being highlighted (adapted from (272)).

4.2.8. *Drosophila melanogaster* Flycube flight assay

The mechanisms behind the Flycube setup have been described in detail previously (401). The Straw lab CantonS line was used for all *Drosophila melanogaster* experiments. 3 day old flies were deprived of food overnight by being placed in a vial containing nothing but water-soaked cotton (for humidity control). The following day the flies were then sedated on ice and females were transferred in groups of 10 to vials containing food doped with pymetrozine at a concentration of either 100, 500 or 1000ppm, or control, unexposed food.

After remaining on the food for 4 hours, the flies were then sedated on ice again and females were individually selected after being checked under a microscope for apparent wing defects. 10 females were placed overnight into each Flycube, which contained soaked cotton wool to provide sufficient humidity but no food source. In total, 6 repeats were made each of the three different concentrations of pymetrozine that were tested, as well as the control group, meaning that 60 flies were tested for each level of exposure and were included in the final analysis.

The Flycube room was kept in constant darkness and at a constant temperature of 25°C in order to counteract any potential electronic heating difficulties. Each Flycube consisted of 5 video screens, as shown in figure 8 part A, which were able to display visual stimuli once an insect had been judged to successfully begin flying (but otherwise remained white) and a glass slide that sealed the top of the cube and prevented escape attempts.

The stimulation type and length was adjustable in accordance with the animal's flight pattern. In this experiment three different types of stimulation were used – a 'figure 8' stimulus which forced the insect to fly in a double loop and two control stimulus patterns (one of which presented stimuli designed to encourage the insect to fly at a constant height whilst the other presented an entirely neutral stimulus) for comparative purposes (401, 402).

As described in section 4.1, *Drosophila melanogaster* turn in accordance with a rotational stimulus in order to negate the effect of the rotational motion. Thus the 'figure 8' stimulus was able to force individual *Drosophila melanogaster* to complete two 180^{O} rotations in the shape of a figure 8 (in a similar style to the trajectories included in figure 8 part B) by providing stimuli with the appropriate rotational velocities; in this case a checkerboard stimulus pattern was used, with control stimuli providing altered versions of the same pattern which were not designed to stimulate rotational movements (401).

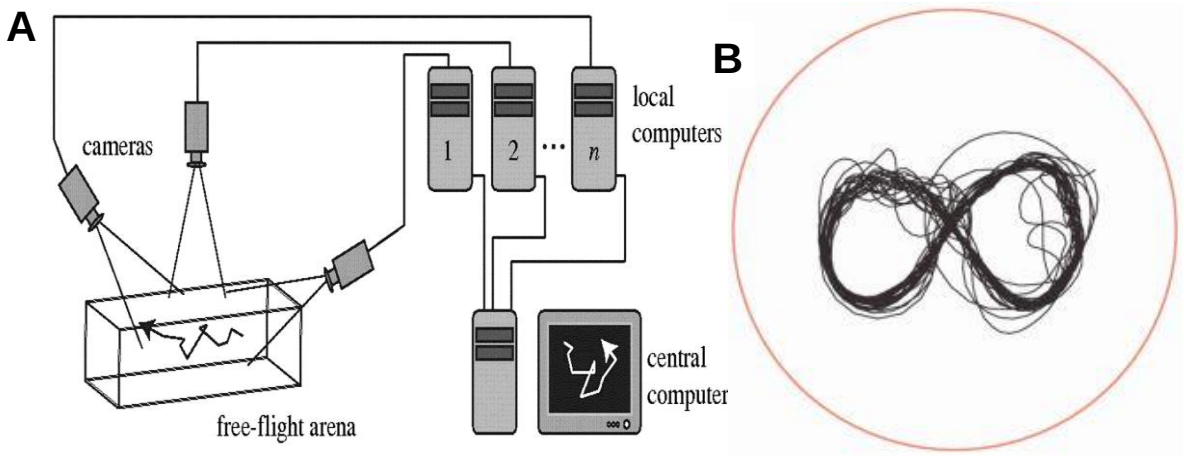


Figure 8. A) The Flycube setup, indicating the positions of several of the cameras as well the computer arrangement required (image taken from (401)).
B) Aggregated flight trajectories from multiple Drosophila melanogaster whilst exposed to the 'figure-8' stimulus paradigm (adapted from (402)).

The Flycube setup involved 5 cameras as depicted in Figure 8 part A, which were able to track flying *Drosophila melanogaster* with high levels of accuracy. Once a fly initiated flight, it was followed until a certain length of time had passed (set here to be 1 second) and the initiation attempt had been judged to be successful. At this point the stimuli were provided in a set pattern – first the standard control stimulus, then the 'figure 8' stimulus and finally a z-control stimulus, with the stimulus pattern being looped if the flight continued for long enough. Only one fly at a time could be monitored by the camera system and so even if multiple flies attempted to follow the stimulation pattern only the flight of the original insect was recorded. Only stimulated flights of at least 1 second in length were saved for analysis.

Once an experiment had been terminated, all data was automatically uploaded to a shared cloud space and basic automated analysis was completed. Flight trajectories recorded during figure 8 stimulus playback were compared to expected flight patterns and the number of complete loops made by individual flies was calculated. This analysis was then aggregated with previous data set analyses to provide a database of the total number of tracked flights per stimulus type, the time length of each flight recorded (which was then aggregated across all flights for each stimulus type to produce an estimate of total flight time per stimulus), 'heat' maps of the most likely fly position during each stimulation type and turning probability in response to 'figure 8' stimulus onset. The turning probability data is of particular interest because it is able to show the median response time of a group of *Drosophila melanogaster* to the 'figure 8' stimulus as well as the likelihood of any response being elicited.

4.2.9. Circadian rhythm temperature entrainment of pymetrozine exposed and unexposed *Drosophila melanogaster* males

CantonS *Drosophila melanogaster* were sedated upon eclosion using CO_2 and virgin males were transferred in groups of 10 to vials containing standard food medium. The next day half of the virgin males were again transferred in groups of 10 to vials containing either food doped with pymetrozine at a concentration of 1000ppm or control food. After spending two days on this food, the males were again sedated using CO_2 and transferred individually to activity tubes containing standard food on one side only. In total 96 exposed males and 96 unexposed males were transferred. The activity tubes were then inserted in groups of 32 into 6 DAM2 monitors (Trikinetics, MA), which were themselves kept in a temperature, humidity and light controlled incubator. An environmental monitor was kept in the incubator in order to ensure that the correct temperature and light programmes were followed consistently.

DAM2 monitors enable an estimation of circadian activity to be calculated by shining a single infrared beam down the centre of each of the 32 vials in the monitor. Each time a fly crossed the centre of a vial (which is designed to reduce as much as possible the fly's vertical movement) the beam was broken and a count was recorded. These counts were them summed together in 15 minutes bins across the length of the experiment.

The following entrainment pattern was used for all flies; first a four day 12 hr: 12hr LD entrainment at a constant temperature of 25°C was used to ensure entrainment to a light stimulus. Then a Dark: Dark (DD) free run, also at 25°C, was completed between 00:00 relative circadian time on day five of the experiment and 16:00 on day eight. After this a four day temperature entrainment cycle was initiated in constant darkness, with temperature shifting between 16 and 25°C every 12 hours. This meant there had been an 8 hour advance in stimulus onset to minimise any potential effects from the first entrainment. Finally, flies entered another DD free run at 25°C which lasted for 3 days.

A collection of scripts called the fly toolbox was used to analyse the summated count data utilising the Matlab software package (MathWorks, Cambridge) (408). Actograms (which display the number of beam breaks recorded for individuals over every 15 minute period in the experiment) of each fly were investigated, with flies that demonstrated no activity in the last 24 hours of the final free run being excluded from any further analysis. In total therefore after applying this selection process 81 pymetrozine exposed males and 88 unexposed males were included in the analysis. Sensitivity analysis calculations using these sample sizes estimated that when using Mann-Whitney rank sum tests at a significance level of 0.05 an effect size of 0.46 could be identified with 90% statistical power.

4.2.10. Effect of pymetrozine on *Drosophila melanogaster* courtship songs

Male and female virgin CantonS *Drosophila melanogaster* were collected upon eclosion using CO_2 sedation and were transferred in groups of 10 to vials containing standard food medium for 24 hours (with both sexes being kept separately). Half of the male and female populations were then transferred to vials containing food doped with pymetrozine at a concentration of1000ppm, with the rest of the males and females being transferred to vials containing control food. After 2 days in these vials all *Drosophila melanogaster* were then transferred to vials containing standard fly food medium, with males and females still being kept apart.

2 days after being transferred to standard fly food medium, males and females were individually sedated on ice and screened for apparent wing defects. Following this one male and one female were chosen from the pymetrozine exposed/ unexposed groups and placed into plastic courtship chambers. These chambers consisted mainly of a small compartment approximately 1cm in diameter and 0.5cm deep. A metal slide in the middle of the compartment allowed for the chamber to be split in order to separate the male and female.

A microphone (Emkay NR 3158 miniature pressure-gradient microphones, Knowles Electronics Inc., Illinois) was positioned directly parallel to the bottom of the chamber for recording purposes. The microphone output was initially run through an amplifier, before being put through a CED 1401 data acquisition board. It was then digitized at a 10 kHz rate using the Spike2 software (Cambridge Electronic Design Ltd., Cambridge). All recordings were made between one and four hours after the incubator lights had turned on (meaning that the flies should have been within the morning peak of activity), with room temperature and relative humidity maintained at 22°C and approximately 55% respectively.

The couple was allowed 10 minutes to recover from the sedation, during which time the slide prevented contact between the sexes. Once the recording started, the slide was removed and the couple was allowed 5 minutes together before the recording was halted. Only songs produced during the 5 minute period immediately following the couple being able to physically interact were analysed. After the recording had finished both male and female *Drosophila melanogaster* were collected so that the effect of pymetrozine exposure/non-exposure could be confirmed using LDV.

Four different couple phenotypes were recorded – both male and female unexposed to pymetrozine (control), both male and female exposed to pymetrozine, only the male exposed to pymetrozine or only the female exposed to pymetrozine. Seven couples from each group were recorded from and included in the final analysis. Pulse and sine songs were identified in the Spike2 recording manually and exported for use in Matlab using criteria based on existing literature reports and laboratory procedures (284, 356, 409).

For a section of the recording to be labelled as a pulse song the following criteria had to be satisfied:

1) Within a time of 0.5s at least 5 pulses must be identifiable

2) The apparent pulse train must have an amplitude of more than double the background noise level; this background level was assessed visually by manually placing horizontal cursors on the upper and lower lowers limits of the noise recorded by the microphones before the slide had been removed

3) If the time window between adjacent pulses was more than twice that of the gap recorded between previous adjacent pulses than a new potential pulse train was judged to have begun

Similarly, for a section of the recording to be labelled as a sine song the following criteria had to be satisfied:

1) The sine train must last for at least 0.5s, during which time the frequency of the train must not change by more than 10%

2) The apparent sine train amplitude must be more than double the background noise level (judged in a similar manner as for the pulse song criteria)

3) A new sine song was determined to have started provided the gap between adjacent songs was longer than 0.1s and the previous criteria were also satisfied

Identified pulse and sine songs were then analysed in Matlab using a bespoke software package (as previously detailed (284)), which provided frequency estimates for each individual sine song and IPI and IPF values for each individual pulse song.

The sine song frequency estimates were calculated by applying fast Fourier transforms to the extracted sine songs after first applying a high-pass filter (>70Hz). Applying the previous criteria for sine song identification allowed for a frequency resolution of less than 3Hz. Peak frequencies could then be identified for individual males.

It was not possible to extract accurate estimates of the IPF by directly applying fast Fourier transforms to the extracted pulse data because the frequency resolution of individual pulses is low whilst applying such transforms to the entire pulse train leads to high levels of noise. To circumvent these issues a Matlab program was used that detected each pulse created within a manually defined region of the extracted recording by comparing the width and power of amplitude peaks above a pre-determined threshold - this detection procedure was then manually screened to ensure that no pulses had been misidentified or missed.

All pulses were centred on their largest amplitude peak, which was then normalised across the train such that all pulses had the same maximum amplitude (with negative pulses being inverted so that their peaks became positive in order to maintain phase coherence). A Hamming window function was applied to this normalised data to minimise noise. The IPI could then be estimated by extracting the time intervals between the maximum amplitude peaks and calculating the median of these intervals.

The individual pulses were then rearranged such that the distance between neighbouring peaks was constant. The distance value was chosen by the program so that the fast Frequency transform of the reconstructed pulse train had a frequency resolution of 1Hz, from which the IPF could be extracted by identification of the peak frequency.

Statistical comparisons between the IPI, IPF, sine song frequency and number of pulse trains produced from males from each of the four groups were then calculated using ANOVA tests in Sigmaplot. Post-hoc calculations of statistical power indicated that whilst calculations for significant differences in the IPI and sine song frequency between groups in which males had not been exposed to pymetrozine and those in which they had been exposed had a high level of power (over 95% in all cases tested) comparisons made between groups in which neither male had been exposed (or both had been exposed) were highly underpowered (<50% in all cases tested).

This was also true for statistical tests involving the IPF and the number of pulse trains produced: none of the statistical tests performed involving these parameters had a power above 50% and as such had a lower statistical power than would normally be advisable. This means that there is an increased likelihood of type II errors and that tests which do not find significant differences between groups should be treated with caution.

4.2.11. *Drosophila melanogaster* competitive reproduction assay

The LH_M–UCL line has a homozygous dominant allele which results in a red eye colour phenotype whilst the LH_B–UCL line has been bred to maintain a homozygous recessive allele, resulting in a brown-eye phenotype. The first generation offspring of a cross between these two lines should therefore solely have a red-eyed phenotype, with only the first generation offspring of two brown-eyed parents having brown eye colour. Adults from these lines, maintained in the Reuter lab (407), were kept for 24 hours in vials containing standard fly food medium (with both lines being kept separately). Both male and female adults were then removed and isolated. Four days after this removal, larvae that emerged from eggs that had been laid were picked and transferred into fresh standard food vials. Each vial contained 50 larvae as a density control measure.

Virgin males from both lines and virgin females from the LH_B–UCL line were selected upon eclosion via ice sedation and were transferred to vials containing standard fly food for two days – male and female flies were kept apart, as were males from each line. Males from both lines were then distributed equally in groups of 10 between vials containing either pymetrozine doped food (1000ppm) or control, unexposed food. All females were placed separately on the control food as well in groups of 10. All flies were kept on this food for 2 days to ensure saturating exposure to the compound.

Following this, pymetrozine exposed and unexposed LH_M–UCL males, pymetrozine exposed and unexposed LH_B–UCL males and LH_B–UCL females were placed individually in separate food vials (following sedation on ice). Pairs of males were then introduced to the vials containing individual females via aspiration. These pairs always consisted of one LH_M–UCL male and one LH_B–UCL male and were of one of three types – both males unexposed to pymetrozine (as a control), only the LH_M–UCL male exposed or only the LH_B–UCL male exposed. The triplets were then left together for 90 minutes before both males were removed from the vial via aspiration and kept separately so that they could be later tested using LDV in order to confirm their pymetrozine exposure phenotype.

Females were then allowed 4 days to lay eggs before also being removed from the vials. The eye colour of the offspring of females which had successfully mated was then checked upon eclosion, with red eyes indicating a LH_M–UCL father and brown eyes indicating a LH_B–UCL father. The final data analysis included 51 control triples, 47 triplets in which the LH_B–UCL male had been exposed to pymetrozine and 34 triplets in which the LH_M–UCL had been exposed (with figure 9 providing a schematic of the experiment).

Previous experiments in the Reuter lab (laboratory data, unpublished) had suggested a small competitive advantage conferred to the LH_M–UCL as compared to the LH_B–UCL line, meaning a slight bias may have been introduced to the control results. A generalised linear model of the results, using eye colour as a variable, was fitted using the statistics software programme R, with the control triplet utilised as a reference (410).

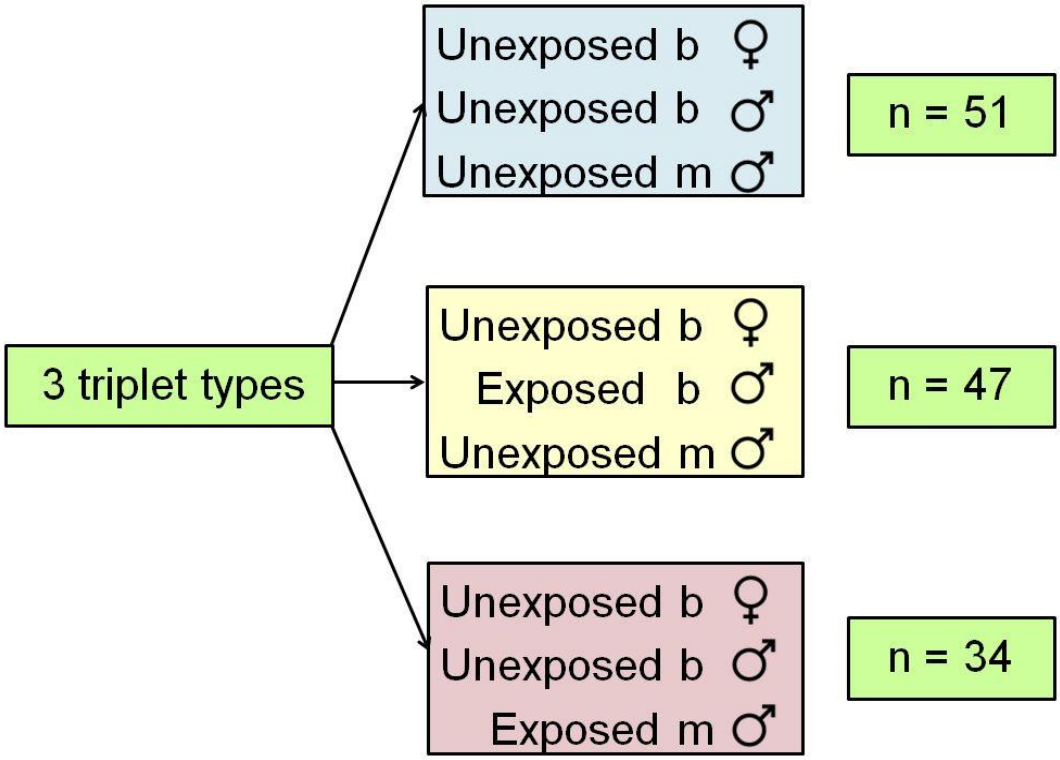


Figure 9. Schematic of triplets used in the competition assay, with b referring to Drosophila melanogaster with brown eyes from the LH_B–UCL line, m referring to Drosophila melanogaster with red eyes from the LH_M–UCL line and exposed/ unexposed referring to whether or not the fly had been exposed to pymetrozine (sample sizes are shown next to each triplet type).

4.3. Results

4.3.1. *Drosophila melanogaster* fertility and fecundity assays

No significant differences were seen in the number of eggs laid by females either exposed to pymetrozine or those that were only exposed to a control (P >0.05). There were also no significant differences seen between any group in the fecundity assay over any five day period or over the entire 15 day length of the experiment (P> 0.05 in all cases tested). Figure 10 contains distributions of egg and larval counts observed for each group tested.

Flies from both experiments had their auditory capabilities tested using LDV (free fluctuations, as defined in section 6.2.6, of representative flies from the fertility assay are shown in appendix A).

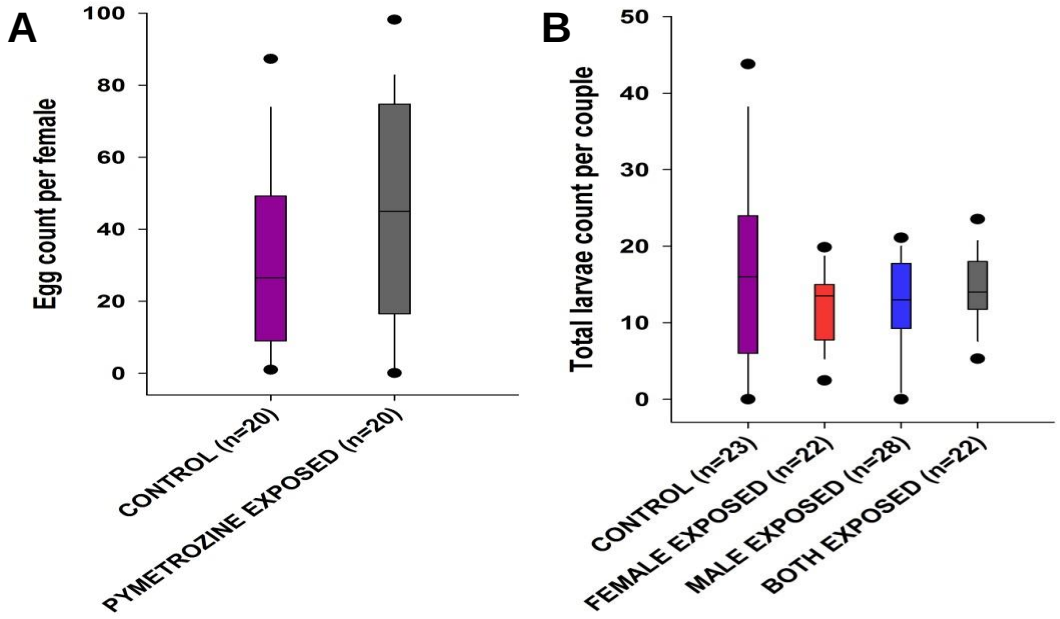


Figure 10. A) Total egg count values per individual female for the two groups involved in the fertility experiment (sample size in brackets, black dots correspond to the 5th and 95th percentiles).

B) Larval count data per couple for all four groups tested for fecundity (sample size in brackets, black dots correspond to the 5th and 95th percentiles).

4.3.2. *Drosophila melanogaster* life span assay

Mortality rates of pymetrozine exposed *Drosophila melanogaster* males and females kept individually were much greater during the first 25 days of the assay, after which a decrease in mortality rate for the exposed flies saw all four groups reach approximately the same percentage of survivors after 90 days. Some exposed flies were capable of living far longer than this however, with ages of over 120 days being reached, as is illustrated in figure 11 part A.

Mortality rates of pymetrozine exposed males and females kept in groups of ten were greater than their unexposed counterparts whilst the median number of flies per vial was greater than 1; once this stopped being the case and vial density was thus reduced then mortality rates stabilised and population numbers became equal throughout the four groups (as seen in figure 11 part B).

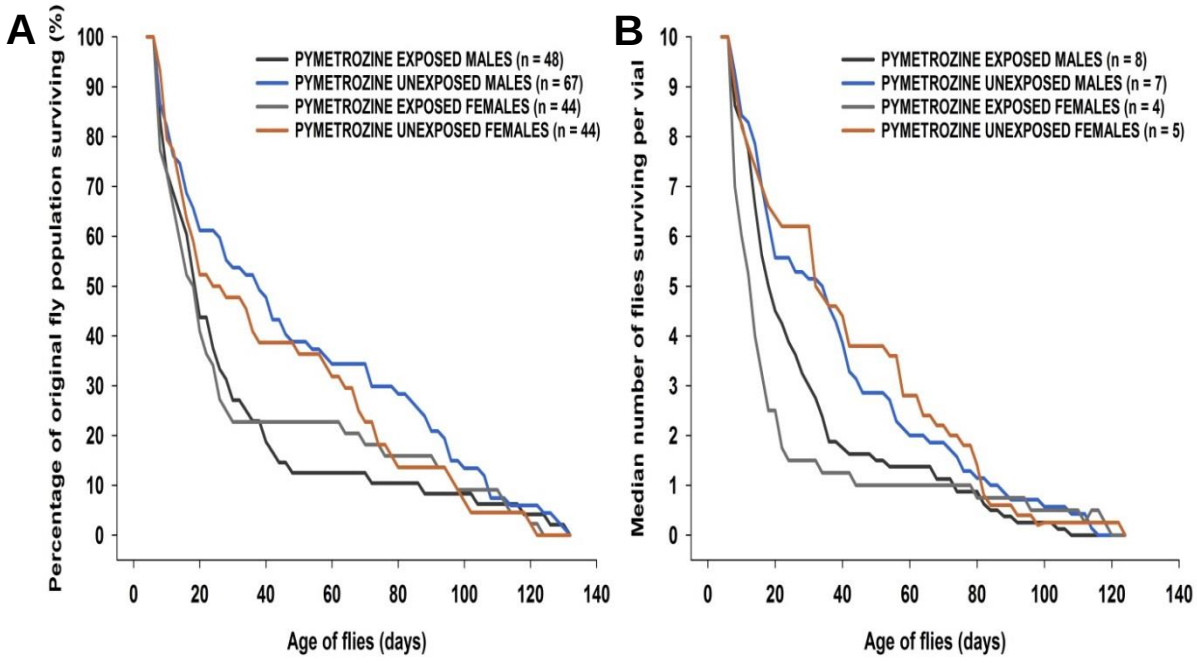


Figure 11. A) Survival rate of each of the four groups over the entire course of the experiment for flies that were kept individually (sample size in brackets).
B) Median number of flies surviving per vial for flies that were kept in groups of ten (sample size given in brackets).

4.3.3. Flight initiation assay

Exposure to pymetrozine at any concentration tested led to a significant decrease in the ability to successfully initiate flight, as can be seen in figure 12 ($P<0.001$ for all comparisons made). The experimental group in which flies which had been exposed to pymetrozine at a concentration of 100ppm had a significantly greater ratio of good to bad fliers than those groups where the exposure concentration of pymetrozine was 500 or 1000ppm ($P<0.005$ for both comparisons made). No statistically significant differences were seen between the group in which exposure level was 500ppm and that in which the level was 1000ppm ($P>0.05$).

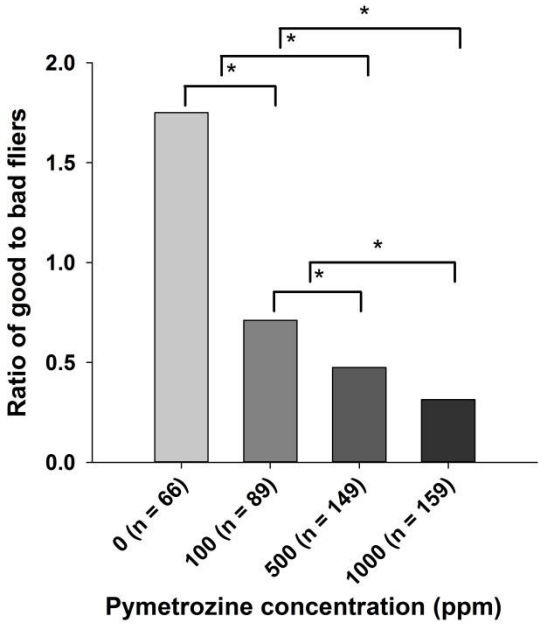


Figure 12. Ratio of good to bad fliers for control flies or flies exposed to pymetrozine at a concentration of either 100, 500 or 1000ppm (sample size in brackets and significant differences between groups are starred).

4.3.4. *Drosophila melanogaster* Flycube flight assay

Control *Drosophila melanogaster* that were not exposed to pymetrozine were able to routinely respond to stimuli in the expected manner. There were 1116 recognised flights during the figure 8 stimulus display resulting in a total flight time of 3266.4s, as compared to 626 and 538 flights for the control and Z-control stimuli which totalled 1061.5s and 932.1s respectively. Average flight times for the three stimuli types were thus approximately 2.93 seconds for the 'figure 8' stimulus, 1.70 seconds for the control stimulus and 1.73 seconds for the Z-control stimulus. The heat maps shown in figure 13 part B demonstrate the ability of the Flycube system to force *Drosophila melanogaster* to fly in the desired 'figure 8' pattern.

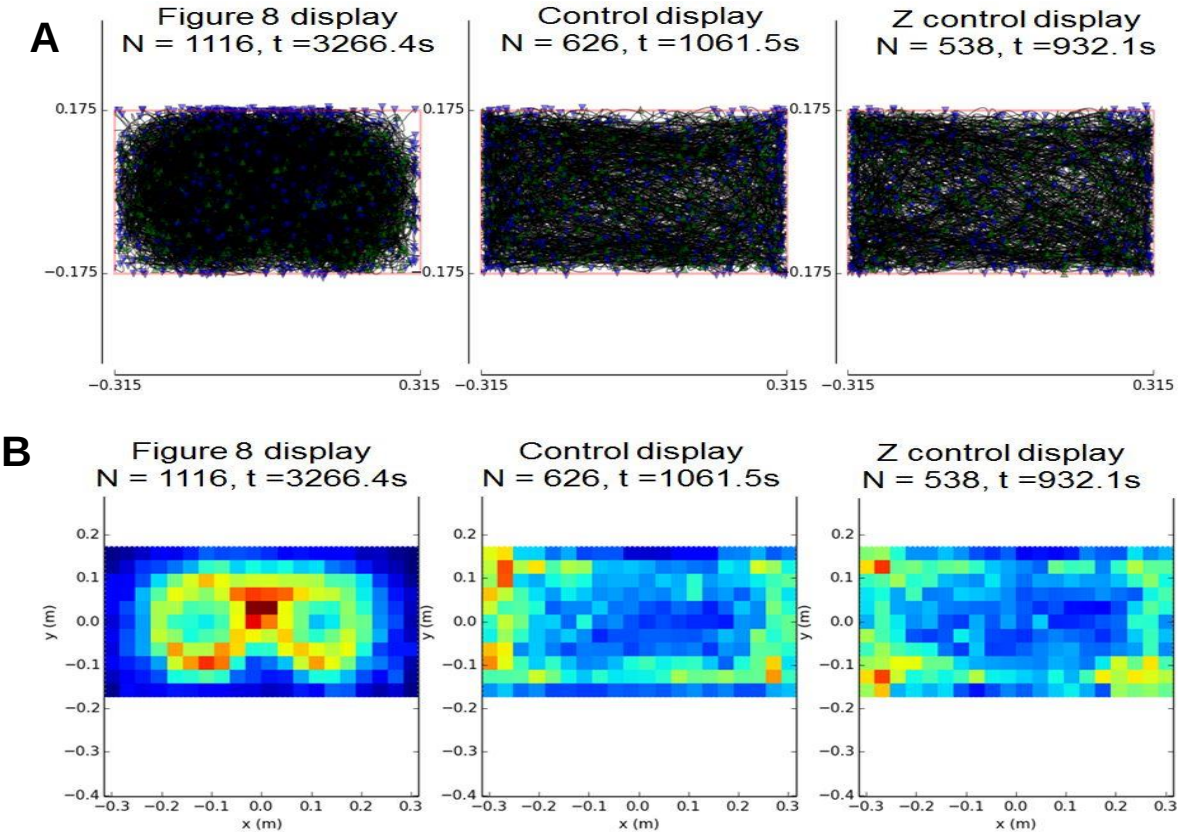


Figure 13. A) Aggregated two dimensional trajectories for each of the three stimuli patterns for control Drosophila melanogaster (n=60), with the number and total time length of trajectories indicated above each stimulus.

B) Two dimensional heat maps indicating the most commonly occupied positions by control Drosophila melanogaster (n=60) being tracked during each stimulus type. The colour gradient goes from blue to red (low to high average occupancy rate).

After pymetrozine exposure (at 1000ppm concentration), the number of *Drosophila melanogaster* flights successfully maintained long enough for the system to begin tracking decreased substantially for every stimulus type. There were only 56 recognised flights during the 'figure 8' stimulus display resulting in a total flight time of 112.1s, as compared to 78 and 90 flights for the control and Z-control stimuli (which totalled 134.0s and 147.3s respectively).

Average flight time for the two control stimuli thus stayed approximately the same as for the control flies (at 1.72 and 1.64 seconds for the control and Z-control stimuli respectively) but was reduced for the 'figure 8' display stimulation to 2.00s. No successful 'figure 8' attempts were recorded by the system, with the heat maps provided in figure 14 part B indicating this clearly, especially when compared to the heat maps of control *Drosophila melanogaster* shown in figure 13 part B.

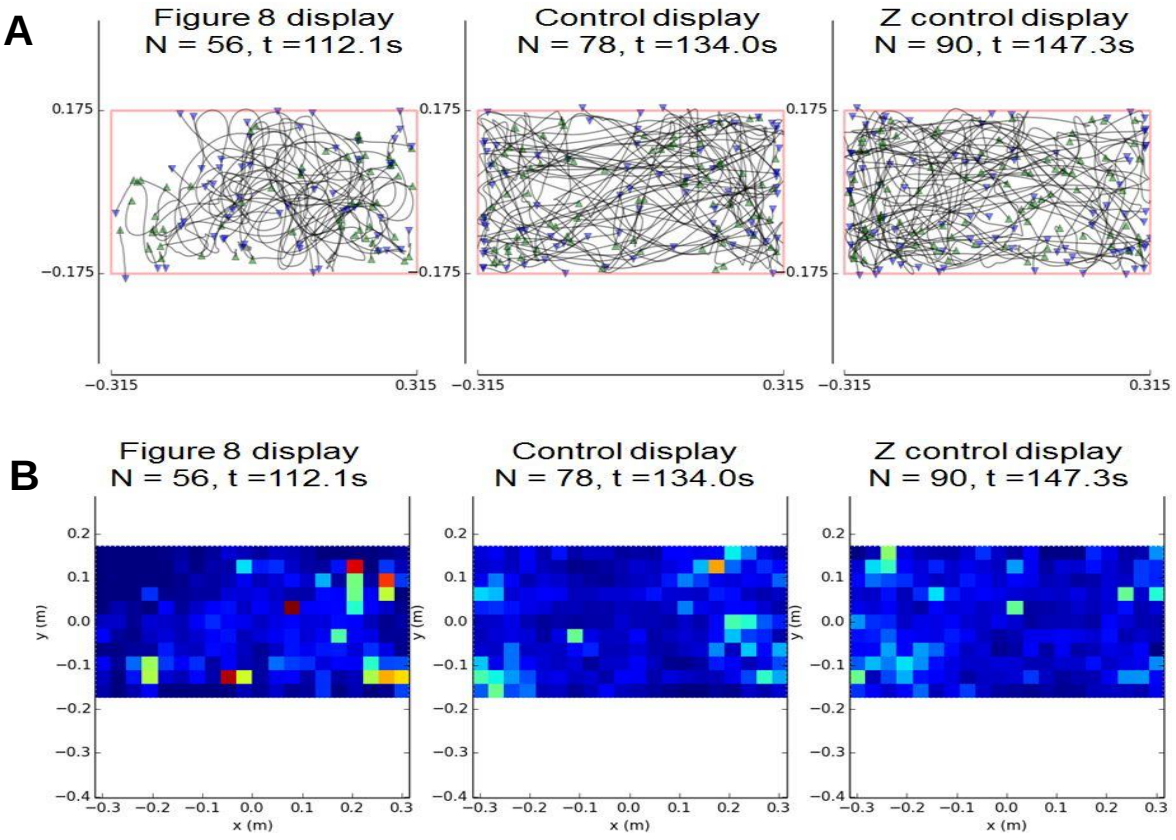


Figure 14. A) Aggregated individual two dimensional trajectories for each of the three stimuli patterns for pymetrozine exposed (1000 ppm) Drosophila melanogaster (n=60), with the number and total time length of trajectories indicated above each stimulus. B) Two dimensional heat maps indicating the most commonly occupied positions by pymetrozine exposed (1000ppm) Drosophila melanogaster (n=60) being tracked during each stimulus type. The colour gradient goes from blue to red (low to high average occupancy rate).

This decrease in successful flight maintenance and the number of trajectories tracked compared to the control is also visible in the results from the other exposure concentrations (to a progressively smaller extent as the concentration level is correspondingly reduced). A dose dependent effect is also visible in the turning probabilities in response to stimulation (demonstrated in figure 15), with increasing concentrations of pymetrozine leading to decreases in turning probability following stimulation onset. This reduction in turning probability is reflected in the elimination of successful 'figure 8' attempts in pymetrozine exposed flies shown in figure 14 part A.

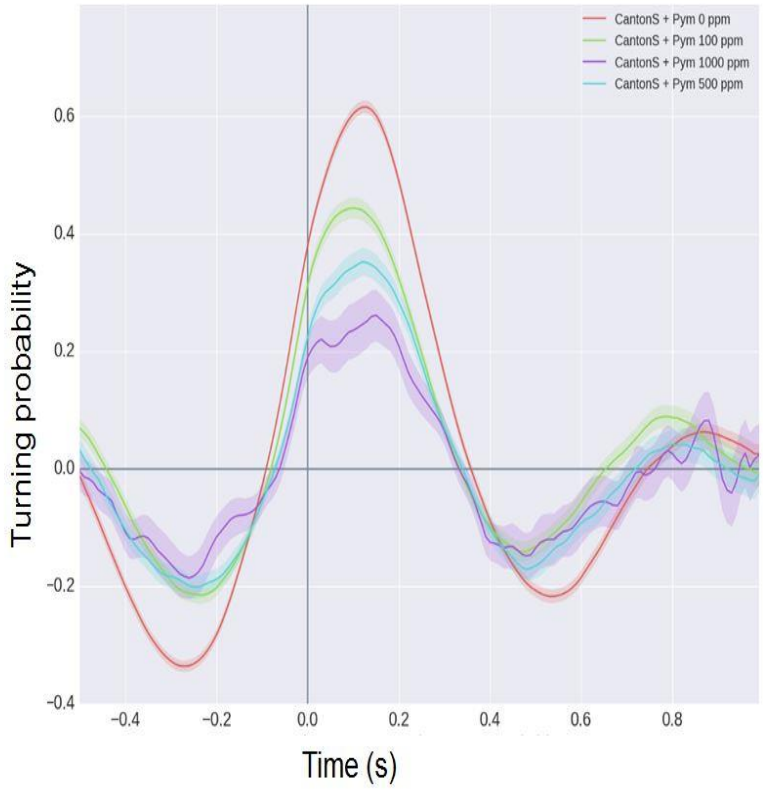


Figure 15. Turning probability after onset of the 'figure 8' stimulus at relative time 0s for each of the pymetrozine exposure levels tested (n=60 for all groups, with dark lines representing median probabilities whilst the shaded areas represent 95% confidence intervals).

4.3.5. Circadian rhythm temperature entrainment of pymetrozine exposed and unexposed *Drosophila melanogaster* males

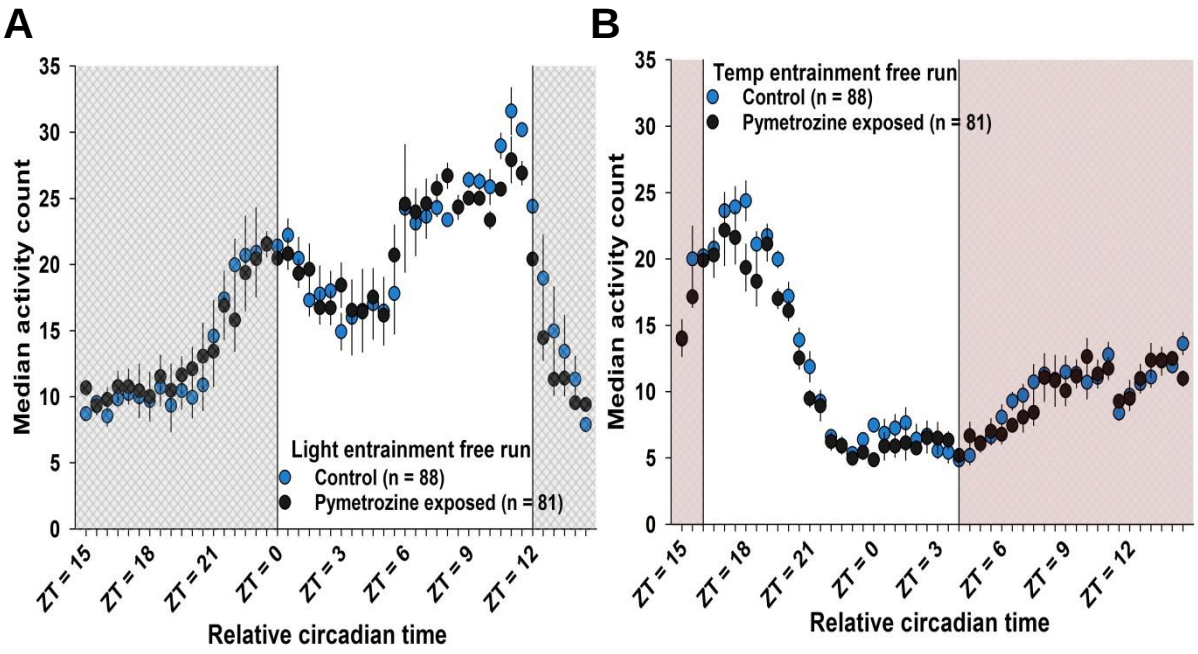


Figure 16. A) Median activity counts during the free run following light entrainment of both pymetrozine exposed and unexposed Drosophila melanogaster, with the white/ dark background segments represent the period during light entrainment in which the incubator lights were turned on/ off respectively (standard errors are represented by vertical lines at each data point).

B) Median activity counts for pymetrozine exposed and unexposed Drosophila melanogaster during the second, post temperature entrainment free run. The white/ red background segments represent the period during temperature entrainment when the temperature was kept at 16/ 25°C respectively (standard errors are represented by vertical lines at each data point).

There were no significant differences in median activity count observed between the pymetrozine exposed and unexposed *Drosophila melanogaster* groups at any time point during either the free run after light entrainment or the free run after temperature entrainment. Two activity peaks are observable for both sets of flies in the free run that followed the light entrainment section of the experiment, whilst only one is observable in the temperature entrainment free run (as displayed in figure 16). Selected representative free fluctuations from flies included in the experiment are included in Appendix B.

4.3.6. Effect of pymetrozine on *Drosophila melanogaster* courtship songs

Significant differences in the IPI were seen between the two groups that contained a male *Drosophila melanogaster* unexposed to pymetrozine and the two groups that featured an exposed male (Control vs only male exposed = P <0.006, Control vs both exposed = P <0.006, only female exposed vs only male exposed = P <0.04, only female exposed vs both exposed = P <0.05). Significant differences were also seen between the same groups for the sine song frequency (Control vs only male exposed = P <0.004, Control vs both exposed = P <0.0003, only female exposed vs only male exposed = P <0.001, only female exposed vs both exposed = P< 0.0001). No other significant differences were calculated for these parameters. Figure 17 below displays these significant/ non-significant differences.

No significant differences were observed between any groups for either the IPF or the number of pulse trains produced (P>0.05 in all cases tested). Table 1 contains median values for all parameters of interest and appendix C contains representative examples of free fluctuations for male and female *Drosophila melanogaster* involved in the experiment.

Table 1. Median IPI, IPF, sine song frequency and number of pulse trains produced for each couple type (standard errors are given in brackets below each median value, with significant differences when compared to the control couples starred).

Couple type	IPI (ms)	IPF (Hz)	Sine frequency (Hz)	Number of pulse trains
Neither exposed (n = 7)	34.20 (0.35)	180.90 (4.08)	135.94 (1.37)	59.0 (9.04)
Only male exposed (n = 7)	*36.28 (0.38)	182.33 (5.30)	*126.17 (2.96)	42.0 (8.67)
Only female exposed (n = 7)	33.12 (0.42)	185.41 (5.71)	132.24 (1.73)	36.0 (6.39)
Both exposed (n = 7)	*36.61 (0.28)	174.80 (6.65)	*127.49 (1.78)	36.0 (6.22)

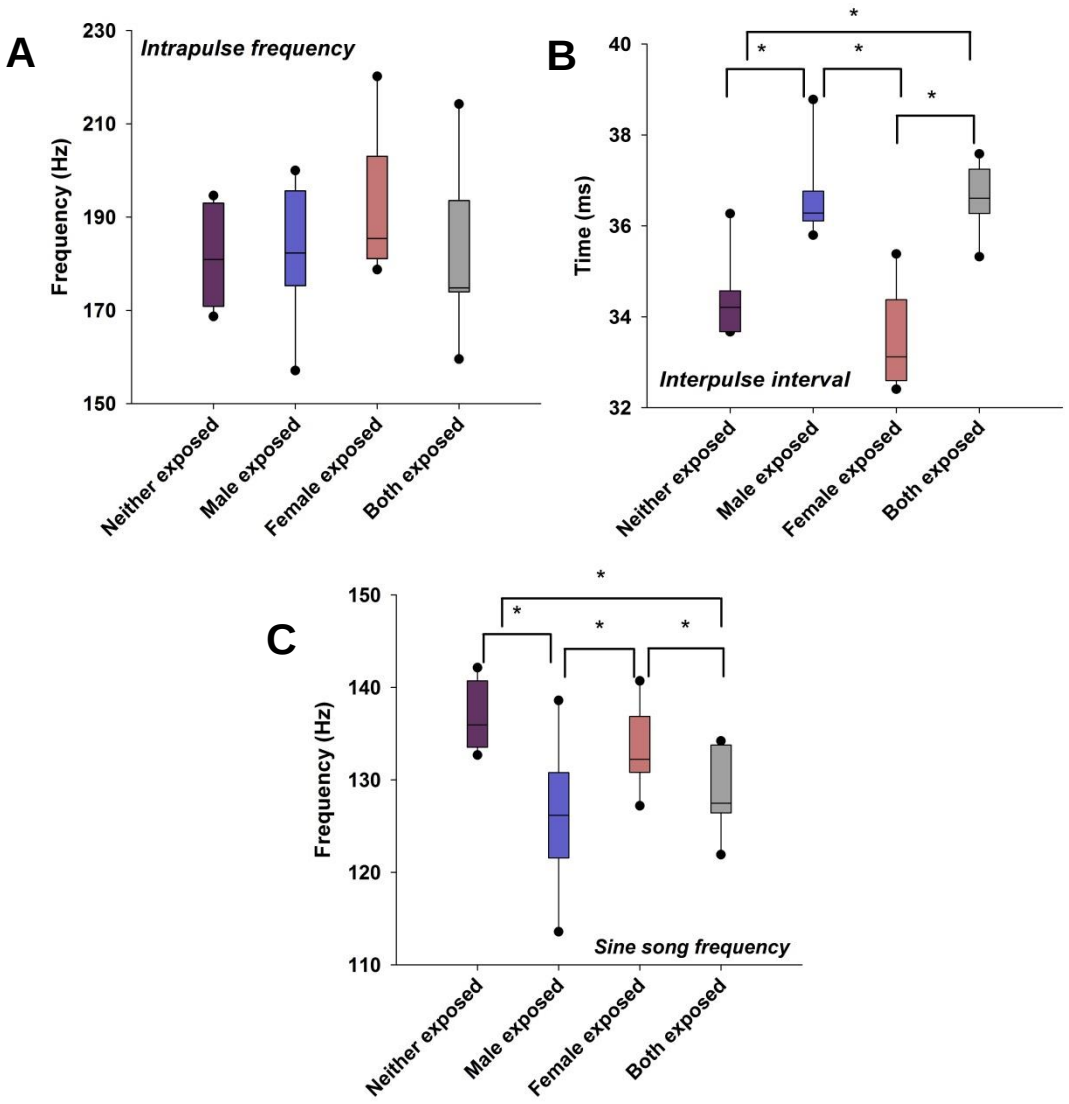


Figure 17. A) Intrapulse frequency values for each of the four groups (black dots correspond to the 5th and 95th percentiles).

B) Interpulse interval values for each of the four groups (significant differences are starred, black dots correspond to the 5th and 95th percentiles).

C) Sine song frequency values for each of the four groups (significant differences are starred, black dots correspond to the 5th and 95th percentiles).

4.3.7. *Drosophila melanogaster* competitive reproduction assay

As shown in table 2, exposure to pymetrozine significantly decreased the likelihood of a male *Drosophila melanogaster* from either line successfully out-competing an unexposed rival male and copulating with a female, with exposure to pymetrozine leading to a significant reduction in the percentage of offspring having the eye colour of the male that was exposed. Appendix D contains examples of free fluctuations for males involved in the experiment

Table 2. Percentage of females from all triplet types whose offspring had brown eyes and the associated significance values (-/+ refers to pymetrozine unexposed and exposed males respectively, starred values represent significant differences when compared to the reference couple).

Triplet type (males)	Number of triplets	Percentage of triplets with brown eyed offspring	Z value (Pr(>z))
Lh_B-UCL - Lh_M-UCL -	51	43.1%	Ref (-)
Lh_B-UCL - Lh_M-UCL +	34	85.3%	*3.628 (0.000286)
Lh_B-UCL + Lh_M-UCL -	47	23.4%	*-2.04 (0.041306)

4.4. Discussion

4.4.1. The effect of pymetrozine on *Drosophila melanogaster* fecundity and fertility

There was no evidence found in the data presented in section 4.3.1 to support the idea that reductions in insect fecundity previously reported for aphids after pymetrozine exposure could extend to *Drosophila melanogaster*, with no significant differences being calculated between control and pymetrozine exposed females with respect to either egg laying or production of larvae (meaning that fertilised, viable eggs had been produced) (320).

Females which had copulated with pymetrozine exposed males also produced viable offspring in statistically similar numbers as those who had mated with control males, suggesting that pymetrozine does not affect male *Drosophila melanogaster* fertility.

These results agree with previous reports that female mechanosensory mutants (for example *hemingway* mutants, which have abolished auditory mechanical amplification) have wildtype levels of fertility – the equivalent male mutants, as well as male *tilB* mutants, are sterile because of the roles played by these genes with regards to ciliary motility which pymetrozine is unlikely to replicate given it's mechanism of action (411, 412). Although these findings do not necessarily by themselves rule out possible effects on mosquito fertility or fecundity, they do reduce the probability that such an effect exists.

Both tests were underpowered however and as such the lack of a significant difference between the control groups and groups exposed to pymetrozine may be the result of a type II error – previous studies investigating differences in the number of offspring produced by female flies required comparison groups of at least 50 females in order to cope with the high degree of variability observed in offspring counts between individuals (413, 414). As such it would require experiments including much larger sample populations to reach concrete conclusions on the possible effect of pymetrozine on fertility and fecundity in *Drosophila melanogaster*.

4.4.2. The effect of pymetrozine on *Drosophila melanogaster* lifespan

Taken together, the two experiments within the lifespan assay shown in section 4.3.2 provide some support for the idea that density plays a significant role in survival probability after pymetrozine exposure, with significantly higher mortality rates seen for vials containing multiple pymetrozine exposed flies (both male and female) than for control vials, with these rates reducing once fly density had been reduced. This concurs both with anecdotal evidence that *tilB* mutants demonstrate similar mortality rates when kept in comparatively dense vials (laboratory data, unpublished) and with published reports that increased adult density leads to decreases in *Drosophila* life span (415, 416).

Mutations to olfactory, gustatory and other sensory peripheral neurons have been reported to affect *Drosophila melanogaster* lifespan and mortality rates, and as such mechanosensory deficits have the potential to induce similar reductions in survival probabilities in relatively challenging environments (417, 418). Retaining mechanosensory capability in crowded environments could therefore provide a competitive advantage.

However the results of the test where all flies were housed individually, in which both male and female flies exposed to pymetrozine had greater mortality rates than unexposed individuals, suggest that fly density may not be the most significant factor determining fly lifespan. Indeed, the results of this assay seem to imply that pymetrozine itself may have an impact on short-term mortality, with those exposed flies that died early potentially having consumed more of the compound than those that survived for longer.

There could also be compensatory mechanisms involved, or some degree of potential reversibility (although no such indications have so far been identified in laboratory tests), such that after a certain length of time the fly is able to adapt to pymetrozine exposure and is fully capable of obtaining lifespans on the scale as unexposed *Drosophila melanogaster*. Residual levels of geotaxis have been identified in *Nan* and *Iav* null mutants which are entirely abolished from pymetrozine exposed flies, suggestive of compensatory mechanisms which attempt to fill the role that should be occupied by ChOs (27).

These lifespan assays were designed to be exploratory and test the upper potential limits of the lifespan of pymetrozine exposed *Drosophila melanogaster,* and as such do not carry substantial statistical weight. Potential significant differences between groups (or the lack thereof) do not carry statistical significance and further, larger assays using group sizes in line with the published reports would be necessary to fully explore the impact that pymetrozine could have on *Drosophila melanogaster* mortality rates (419, 420).

4.4.3. The effect of pymetrozine on *Drosophila melanogaster* flight initiation and maintenance

Given that *Drosophila melanogaster* use at least three different sensory-motor reflexes to maintain only the vertical aspects of flight, ablation of ChO-mediated sensation via pymetrozine could be expected to significantly reduce flight ability – this was indeed the major conclusion of sections 4.3.3 and 4.3.4, with pymetrozine exposure leading to reductions in both flight initiation and maintenance (421).

Beyond this, there also appears to be a dose-dependent effect of the compound with further significant differences calculated between different exposure concentrations as concentration increased. This may be the result of inefficient exposure to the compound at lower concentrations, with toxin levels within the insect being insufficient to affect every ChO present. The retention of some level of ChO function could thus be enough to maintain some (reduced) flight capability. As the exposure concentration is increased so is the likelihood of complete ablation of all ChOs in the individual fly and therefore further deterioration of flight ability. Such dose dependent effects on flight (and climbing) have been identified for CO_2 exposure for example (422).

Whilst there was no statistically significant difference found between *Drosophila melanogaster* exposed to pymetrozine at either 500 or 1000ppm in terms of flight initiation capability, there was a further reduction in flight turning probability at the higher exposure level – this could be the result of different internal mechanisms which control flight initiation and turning in *Drosophila* species (421, 423, 424). This suggests that, based upon these results, the highest concentration of pymetrozine possible should be tested for use in future trials, though this should be adjusted according to safety and tolerability concerns.

Whilst the Flycube system is highly efficient at tracking the flight patterns of *Drosophila melanogaster* and can provide a wealth of information about the resulting flight behaviour, it is unable to provide data regarding groundspeed variability and cannot force individual flies to initiate flight behaviour (which can lead to relatively low numbers of flight attempts) (29, 328). Further tests which utilise tethered flying *Drosophila melanogaster* to investigate changes in groundspeed in conjunction with JO neuron activity could provide further detail on the role of ChO neurons in flight maintenance, and the resulting effect of pymetrozine exposure on these processes (325).

4.4.4. The effect of pymetrozine on *Drosophila melanogaster* circadian rhythms

The ability of pymetrozine exposed *Drosophila melanogaster* to entrain to temperature changes, as evidenced by the lack of significant differences observable in section 4.3.5 between the control and pymetrozine exposed groups in terms of their second free run activity pattern, was surprising given the prior literature on the requirement of functioning ChOs for *Drosophila melanogaster* to entrain to temperature (386). The impact of pymetrozine was assessed after the experiment using LDV (with examples shown in appendix B) and every fly that had been exposed to pymetrozine showed a clear and expected phenotype, suggesting that the lack of a significant difference between the two groups is not the result of non-exposure to pymetrozine in the test group.

Previous studies investigating the importance of ChOs for temperature entrainment used mutants that completely lacked all ChO function (386). It could therefore be the case that pymetrozine exposed *Drosophila melanogaster* are still able to entrain to a temperature Zeitgeber because there are potentially multiple sensory input pathways to ChOs and only one of these is affected by the compound. For example, *Drosophila* ionotropic receptor 25a has been shown to form part of a pathway to the *Drosophila melanogaster* circadian clock that is able to detect changes in temperature in the absence of all currently reported temperature sensors located in the antenna (373). The ability of pymetrozine exposed *Drosophila melanogaster* to entrain to vibrational stimulation could provide greater insight into the various potential sensory input pathways to ChOs and the circadian clock (33).

As a result of the *Drosophila melanogaster* experiment, it seems unlikely that pymetrozine would have an effect on temperature entrainment of mosquito circadian rhythms and so may not affect the clock-related activity patterns observed for both males and females, particularly with regards to biting frequency and habits (379, 425).

4.4.5. The effect of pymetrozine on *Drosophila melanogaster* courtship

Male *Drosophila melanogaster* are the producers of both pulse and sine songs within a couple. That the differences in IPI and sine song frequency described in section 4.3.6 are seen between both groups which contain pymetrozine unexposed males and both those that contain exposed males, in addition to the sizable extent of the differences seen, suggests pymetrozine has a significant effect on both pulse and sine song production in males.

Both the median IPI and IPF values for each group lay within the ranges previously reported for *Drosophila melanogaster* (approximately 35ms and between 150 to 250Hz respectively); however there was an observed reduction in median sine song frequency when compared to the expected value of 150Hz in all groups tested. This could be the result of the documented changes in major song component characteristics in response to temperature shifts (426, 427).

Given that previous reports did not find auditory feedback to be required for successful production of sufficient quality songs (as discussed in section 2.10.2), and that unpublished laboratory data suggested that ablating single antennal function led to increases in IPF but not IPI, it is possible that the pharmacological effect of pymetrozine in some way led to the demonstrated increase in IPI and decrease in sine song frequency. The statistical power of tests for differences in IPF between different groups was too low however to conclusively determine that the lack of significant differences is real and not the result of a type II error.

This pharmacological effect for example is not limited to just ChOs within the JO and should extend to all ChOs throughout the fly body. This could then lead to wing beat control problems, which would lend further credence to the previously presented evidence in sections 4.3.3 and 4.3.4 of a reduction in flight capability following pymetrozine exposure (as well as the aforementioned literature described in section 2.10.1 reporting the necessity of a functional JO for proper flight control) (29, 325).

There is also the possibility that pymetrozine has a secondary target (as although a very high concentration of the compound has been reported to be necessary to affect other ion channel complexes, only a few such channels have been tested (27)) and it is the impact of the toxin on this target that results in an altered song phenotype – for example *Drosophila* species with mutations in the *slowpoke* locus have recently been found to have relatively lower frequency sine songs than controls (428).

These significant changes to important pulse and song characteristics are likely to be related to the decrease in competitive fitness detailed in section 4.3.7 for pymetrozine exposed male *Drosophila melanogaster*. Given sufficient time and a low fly density, male *Drosophila melanogaster* were still able to copulate with females after pymetrozine exposure and as such this change in song structure does not complete eliminate reproductive viability. It may however severely decrease the likelihood of outcompeting rivals during copulation attempts (especially when taken in tandem with the other effects that pymetrozine has on insect physiology), which is not only interesting from a theoretical stand-point but also from the point of view of control programme design.

As the IPI has been reported to be an essential characteristic of a pulse song (which is itself deemed crucial to successful courtship events) whilst the sine song is considered to some extent unnecessary in *Drosophila melanogaster*, it could be that this parameter specifically is responsible for the reduction in fitness observed for males following pymetrozine exposure (343, 344).

The possibility that it is specifically a reduction in comparative song quality that led to the apparent reduction in fitness demonstrated in section 4.3.7 could be further investigated by repeating the experiment with the same male phenotypes but this time using only females that had been exposed to pymetrozine; if pymetrozine unexposed males no longer hold a competitive advantage this would suggest that song quality is the major determining factor in mate selection (346). If pymetrozine exposure still results in a reduction in male fitness then this could instead be due to other effects of pymetrozine on the males, with compound exposure leading to a loss of proprioception and thus a reduction in physical fitness for example (429). It seems reasonable to hypothesise however that it is combination of all of the effects of pymetrozine exposure (loss of proprioception, change to song characteristics etc.) that results in the calculated decrease in competitive fitness.

Regardless of the exact cause, using a directly competitive environment shows the extent of the difficulties facing pymetrozine exposed *Drosophila melanogaster* males during courtship. If pymetrozine is able to reduce competitive fitness in male mosquitoes in addition to impacting courtship behaviour in both sexes then the compound could become an attractive proposition for mosquito control.

5. Mosquito behavioural assays and physiological measurements

5.1. Introduction

The results described in section 4 showed the impact that pymetrozine exposure can have on *Drosophila melanogaster*, with significant reductions in flight ability and competitive fitness noted. Whilst *Drosophila melanogaster* may be able to serve as a basic model organism, in order to investigate the potential effects that pymetrozine may have on mosquito species investigations utilising mosquitoes must be performed.

Modern mosquito control programmes employ multiple intervention methods in order to maximise the impact on mosquito populations (as discussed throughout section 2.3) (430, 431). This includes larval source management, with identification of larval sources proving vital to control programmes targeting *Aedes* and *Anopheles* species for example (62, 144).

Whilst a variety of different larvicides are currently used (with many more proposed for use), the unique mechanism of action demonstrated by pymetrozine would make any potential larvicidal effect of particular interest for future use as an intervention (432-434). Testing the potential impact of pymetrozine exposure on *An. gambiae* larvae would therefore provide information regarding the compound's efficacy with regards to larval stages of the mosquito lifecycle.

Although pymetrozine's potential as a larvicide should not be neglected, given the compound's effect on auditory function in *Drosophila melanogaster* and the results of the competition assay presented in section 4.3.7, it also seems applicable to investigate the potential impact on mosquito fertility and fecundity (27). Previous reports have assessed the impact of interventions or mutations on fertility and fecundity, and thus have provided the basis for investigations using pymetrozine (435, 436).

Repeating such tests using *An. gambiae* mosquitoes (which are highly relevant given the role of this species as a major vector of disease) would therefore be able to provide evidence as to whether pymetrozine exposed male mosquitoes are able to mate with unexposed females or, alternatively, whether pymetrozine exposed female mosquitoes are able to copulate with unexposed males.

Experimental results shown in section 4 suggested that whilst pymetrozine led to a significant reduction in the competitive fitness of male *Drosophila melanogaster*, in the absence of competition exposed males were still able to copulate with females and no significant differences were found in the fertility and fecundity of pymetrozine exposed female *Drosophila melanogaster* (though those studies were found to be underpowered). Given the differences in courtship routines however between mosquito and *Drosophila* species it may be the case that pymetrozine has a more potent effect on mosquito courtship; investigations into this topic using *An. gambiae* mosquitoes will help to determine the pymetrozine's potential for future use for targeting mosquito species.

The results of *Drosophila melanogaster* flight initiation and maintenance assays which compared different levels of pymetrozine exposure (as described in sections 4.3.3 and 4.3.4 respectively) seemed to show not only an effect of pymetrozine on flight ability but also a dose-dependent relationship between increases in compound concentration and decreases in flight ability. Given the importance of flight to mosquito species, it would be therefore of interest to investigate whether pymetrozine has a similar effect on mosquitoes as it does on *Drosophila melanogaster* (56, 76).

However, mosquitoes have a significantly different flying style than *Drosophila* species because of differences in wing placement on the body as well as body mass increases (76, 437). The visual system of mosquitoes is also less well-understood and analysed than the corresponding *Drosophila melanogaster* system (438-440). Thus before the experiments conducted in section 4.3.4 can be repeated for mosquitoes, an exploratory study is necessary in order to optimise parameter settings in the Flycube and ensure that mosquitoes not exposed to pymetrozine are able to respond to stimuli in a regulated and controllable manner.

The majority of the data so far published using LDV to investigate insect auditory function used displacement data that was non-angular i.e. did not provide the angular movement of the antenna but instead gave the direct displacement (285, 394). Providing angular displacement data however removes systematic biases associated with measuring force deflections at different lengths along the flagellum (288).

Calculation of the antennal length for both male and female mosquitoes from each species that will be investigated in section 7 will thus enable the future conversion of flagellar displacement data into angular flagellar deflections if necessary and will also enable future comparisons between energy and displacement gain estimates calculated for different species and sexes (as calculated in section 7) to be viewed in the context of the distance of the laser focus point from the pedicel.

Given that this thesis will focus on the three species detailed in section 2.1 (*Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus*) because of their relevance as major disease vectors, these measurements will be taken for male and female mosquitoes from these species.

The tuning of male and female mosquito antennal systems (in terms of their mechanical sensitivity and JO tuning) has been reported to depended on the WBF of males and females in that species, particularly with regards to distortion products formed by the interaction of male and female flight tones (34, 276, 358). Although WBF estimates have been reported for all three species investigated in this thesis (as described in section 2), the WBF can change according to environmental factors such as temperature (34, 357).

As such it is vital that WBF estimates are calculated in the exact environmental conditions present during mosquito experiments performed throughout this thesis so that accurate estimates of both the fundamental flight tones and the resulting distortion products can be calculated. This will allow the results of future investigations into the frequency tuning of the mosquito flagellum detailed in section 7 to be compared and contrasted with this data.

5.2. Materials and methods

5.2.1. Mosquito rearing

All, *Ae. aegypti* and *An. gambiae* (Kisumu strain) and *Cx. quinquefasciatus* (Muheza strain) mosquitoes used for experiments were provided by Shahida Begum from the London School of Hygiene and Tropical Medicine. All mosquitoes were reared using a 12 hr: 12hr LD cycle at 26°C and 75% relative humidity and were fed using a 10% glucose mixture. Horse blood feeding, where appropriate, was completed by a trained research assistant using the Hemotek system (Discovery Workshops, Accrington).

5.2.2 Compound preparation

Pymetrozine: Preparation of the compound was identical to that described in section 4.2.2 for feeding experiments in *Drosophila melanogaster.*

5.2.3. Compound exposure – ingestion

Male and female experimental mosquitoes were deprived of food overnight before being given a mixture of 1000ppm pymetrozine stock solution and a 10% glucose solution which was created in such a manner as to give the required pymetrozine concentration – control mosquitoes were provided with a solution containing only 10% glucose. The mosquitoes were exposed to pymetrozine exposed/ unexposed food for 6 hours before it was replaced with non-doped food.

The length of time during which the mosquitoes were allowed to feed from doped food was chosen to minimise the possibility of multiple feedings whilst maximising the number that would feed at all. Confirmation of effective pymetrozine exposure was made using LDV wherever possible, though this could only be done after the conclusion of the experiment as this form of testing is destructive.

5.2.4. Larvicidal assay

Following World Health Organisation (WHO) guidelines (441), groups of 30 third instar *An. gambiae* larvae were added to test cups that already contained 100ml of water. All larvae were screened under a microscope after being transferred to confirm their general health. 10 ml of a solution containing the relevant concentration of pymetrozine was added to the appropriate cup to produce a final dilution of the compound – the final concentrations tested were 1, 10, 100 and 1000ppm. A control solution was also tested which was composed only of the solvent used to dissolve pymetrozine (in this case milli-Q water), and as such did not contain any pymetrozine.

Each concentration tested (as well as the control) included a replicate, meaning that 60 larvae in total was analysed for each dilution group included in the series. Sufficient food was added to each cup to prevent larval death from starvation.

The cups were kept between 25 to 27°C with constant 70% humidity and had a 12hr: 12hr LD photoperiod. Mortality was measured at 24, 48 and 72 hours after the beginning of the experiment. Statistical comparisons were made between the control group and groups that had been exposed to some concentration of pymetrozine using Fisher's exact test with the Bonferroni correction to account for multiple testing using Sigmaplot, such that the significance level was set at $P = 0.0125$.

WHO guidelines for larvicidal assays recommend that at least five replicates are made for each compound concentration tested in order to reach an accepted level of statistical power, meaning that this assay as conducted should be considered statistically underpowered and therefore has an increased likelihood of type II errors.

5.2.5. Impact of pymetrozine on reproductive success in males and females

An *An. gambiae* colony was blood fed in the laboratory of Dr Gareth Lycett at the Liverpool School of Tropical Medicine, and then allowed to lay eggs, which were reared until the pupal stage. The pupae were then sexed under a microscope with males and females being transferred to separate vials at a constant density of 10 pupae per container. After emergence the mosquitoes were given one meal of 10% glucose food before being deprived of food overnight.

All mosquitoes were then given 6 hours to feed from a glucose solution containing pymetrozine (at a concentration of either 50, 100 or 1000ppm) or from a control solution that did not contain pymetrozine. There were in total seven different experimental groups; apart from the control group in which neither sex had been exposed to pymetrozine, all groups contained one sex exposed to the compound (with a concentration of either 50, 100 or 1000ppm depending on the group) and the other entirely unexposed.

Between 50 and 55 female mosquitoes and 45 and 50 male mosquitoes were aspirated into different courtship containers, where they were provided with a constant source of glucose. The containers were stored at a constant humidity and temperature of 75% and 26°C respectively in a room that was kept on a 12hr: 12hr LD cycle with dawn and dusk photoperiods included. Blood meals were provided on both the second and third day that the mosquitoes were kept in the containers and blood feeding was confirmed visually for the majority of females.

After five continual days of containment female mosquitoes were aspirated into individual chambers containing damp cotton to allow them to lay eggs. The females were then monitored over the following 72 hours and any mosquito that laid any number of eggs was recorded. These eggs were then monitored to confirm whether hatching occurred, which would therefore indicate prior insemination of the female mosquito.

The total number of females included in the final analysis is included in table 4 below i.e. 39 females from the control group, 45 females exposed to pymetrozine at a concentration of 50ppm, 50 females exposed to pymetrozine at a concentration of 100ppm, 28 females exposed to pymetrozine at a concentration of 1000ppm, 12 pymetrozine unexposed females coupled with males exposed to pymetrozine at a concentration of 50ppm and 33 unexposed females coupled with males exposed to pymetrozine at a concentration of 1000ppm were included in the final analysis.

Statistical testing was conducted using Fisher's exact test with the Bonferroni correction to account for multiple testing using Sigmaplot. Calculation of post-hoc tests of statistical power indicated that statistical power throughout all comparisons was <50%, which is much lower than would usually be advised and as such statistical test results should be treated cautiously because the study is highly statistically underpowered.

5.2.6. Mosquito Flycube flight assay

The experimental set up was similar to that described previously for *Drosophila melanogaster* in section 4.2.9 with 5 to 7 day old female *Cx. quinquefasciatus* mosquitoes being sedated on ice, checked for apparent wing defects and then placed overnight in the Flycubes in groups of three in order to judge the ability of the Flycube system to track mosquito flight patterns as well the mosquito response to stimulation. The stimulus types presented were identical to those presented to *Drosophila melanogaster* in section 4.2.9 and the experimental length, environment and analysis procedure were also duplicated.

In total 12 different mosquitoes were included in the final analysis, none of which had been exposed to pymetrozine. As this was an exploratory study, no formal statistical power calculations were made.

5.2.7. Mosquito flagellum length measurements

Male and female *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* mosquitoes between 3 and 6 days old were first sedated on ice and then held by the thorax in a pair of inverted forceps. Using a pair of sharpened forceps under a microscope, the flagellum of each mosquito was removed from the pedicel and placed into a buffer solution. The flagella were then transferred to a microscope slide and a LSM 800 Zeiss confocal microscope (Zeiss, Cambridge) was used to examine each individual antenna. Images were taken of antennae which were judged to be intact. These images were then analysed using the LSM image browser software which enables measurement of each flagellar section.

Radial symmetry of the mosquito flagellum was assumed, meaning that it was assumed that the maximum length (as well as the width) of each flagellomere was identical in every 2-dimensional plane. The length and width of each section was recorded and then used to calculate the surface area of each flagella segment. The total flagellar surface area was then determined from these individual computations.

In total 8 *Ae. aegypti* female flagella, 10 *Ae. aegypti* male flagella, 9 *An. gambiae* female flagella, 22 *An. gambiae* male flagella, 23 *Cx. quinquefasciatus* female flagella and 16 *Cx. quinquefasciatus* male flagella were included in the analysis.

Statistical testing was done using Wilcoxon signed rank tests in Sigmaplot. Post-hoc statistical power tests found that whilst comparisons between the surface areas of male and female *Ae. aegypti* and *Cx. quinquefasciatus* had power estimates of over 95% in both cases, the same comparison made between male and female *An. gambiae* was significantly underpowered (<20%), and as such there was an increased likelihood of a type II error. All statistical comparisons of the antennal length were found to have a statistical power of over 90% in post-hoc tests.

5.2.8. Mosquito wing beat frequency measurements

3 to 6 day old male and female *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* mosquitoes were sedated using ice and then attached to a wire filament using blue-light cured dental glue. The filament was glued to the dorsal side of the insects' thorax in such a way that the mosquito was still able to freely move its wings. The wire was then attached to a micromanipulator atop a vibration isolation table and the individual mosquito was given 5 minutes to recover from sedation.

The same microphone as used in the *Drosophila melanogaster* courtship song experiments described in section 4.2.11 (Emkay NR 3158 miniature pressure-gradient microphones) was placed perpendicular at a set distance of 1cm from the mosquito being measured, along with the same amplifier and spike2 hardware and software setup as previously used. The wing beat of the mosquito as it attempted to escape from its' tether was then captured using the microphone. Each recording lasted for 5 minutes before the mosquito was removed from the setup.

Individual spectrograms of 5s segments of wing beats were analysed in spike2, with a Hanning window being applied to the data. Both the first and second harmonics were then identified using a 4096 point FFT. In total 8 *Ae. aegypti* females, 10 *Ae. aegypti* males, 10 *An. gambiae* females, 11 *An. gambiae* males, 11 *Cx. quinquefasciatus* females and 11 *Cx. quinquefasciatus* males were included in the analysis.

Statistical analysis was conducted using ANOVA on ranks tests in Sigmaplot. Post-hoc power calculations estimated that whilst the statistical power of comparisons between mosquitoes of the same species but a different sex were always above 95%, the statistical power of comparisons between same sex mosquitoes of different species was less than 40% in all cases tested (apart from the comparison made between male *Ae. aegypti* and *An. gambiae* mosquitoes, for which a statistical power of greater than 80% was calculated). This indicates that whilst comparisons between conspecific male and females were unlikely to have a high probability of type II errors occurring, the likelihood of these errors occurring for comparisons between species was much greater than would be usually advised.

5.3. Results of mosquito behavioural assays and physiological measurements

5.3.1. Larvicidal assay

Significant differences were calculated for comparisons between the control group and the *An. gambiae* larval group exposed to pymetrozine at concentrations of 1000ppm ($P<0.01$). For all other statistical comparisons no significant differences were identified between compared groups, in particular when comparing the control group with the groups exposed to pymetrozine at concentrations of 1 and 10ppm (as is evident in table 3).

Table 3. Number of surviving An. gambiae larvae 24, 48 and 72 hours after pymetrozine exposure for each of the five concentrations of pymetrozine tested (numbers in brackets are the percentage of the starting population remaining at that time point, starred values are significantly different from the equivalent control group values).

Pymetrozine concentration (ppm)	Larvae surviving at 24 hours	Larvae surviving at 48 hours	Larvae surviving at 72 hours
0 (control)	60 (100%)	60 (100%)	57 (95%)
1	60 (100%)	59 (98.3%)	58 (96.7%)
10	60 (100%)	56 (93.3%)	54 (90%)
100	52 (86.7%)	50 (83.3%)	49 (81.7%)
1000	25* (41.7%)	15* (25%)	0* (0%)

5.3.2. Impact of pymetrozine on reproductive success in male and female mosquitoes

There was no data recorded for the group containing unexposed females and males exposed to a pymetrozine concentration of 100ppm due to damage sustained to the courtship container during the experiment. The group of unexposed females and males exposed to 50ppm concentration pymetrozine also suffered losses, but some females remained for transfer to individual vials (table 4 includes a full list of the number of females transferred and the percentage of those that were transferred that laid eggs, with figure 18 representing this data graphically).

Whilst it does appear that either male or female exposure to high concentrations (i.e. 100ppm or greater) of pymetrozine reduced the number of fertile females found to almost zero, with no fertile females found in the group that contained females exposed to pymetrozine at a concentration of 1000ppm, no statistically significant differences were identified between any of the groups.

Table 4. The number of egg laying and fertile females for each experimental group (values in brackets are percentages of the total population).

Treatment group (Females/ Males)	Number of females	Number that laid eggs	Number of fertile females
0ppm/ 0ppm (control)	39	7 (17.9%)	5 (11%)
50ppm/ 0ppm	45	16 (35.6%)	11 (24.4%)
100ppm/ 0ppm	50	4 (8%)	1 (2%)
1000ppm/ 0ppm	28	0 (0%)	0 (0%)
0ppm/ 50ppm	12	1 (8.3%)	1 (8.3%)
0ppm/ 1000ppm	33	3 (9.1%)	2 (6.1%)

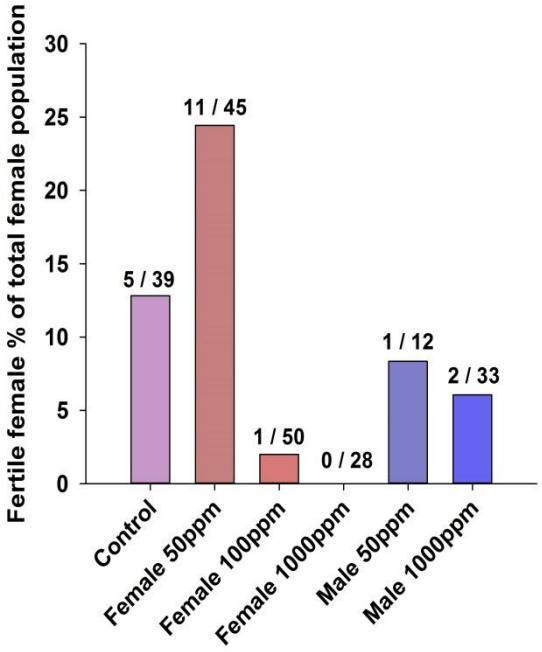


Figure 18. Number of fertile females for each experimental group, with the numbers above each bar representing the relevant absolute number of fertile females as well as the total female population above each bar.

5.3.3. Mosquito Flycube flight assay

245 flights were recorded for mosquitoes whilst the 'figure 8' stimulus was being displayed, with a total flight time of 694.3s – this is in comparison to 332 and 698 flights for the control display and z-control display respectively (which resulted in total flight times of 963.9s and 1896.5s for each stimulus type). Flight durations for each of the stimulus types thus averaged 2.83 (8figure 8' stimulus), 2.90 (control stimulus) and 2.72 (Z-control stimulus) seconds, with the control flight times being much greater than previously seen for *Drosophila melanogaster* and so suggesting that the mosquito flight patterns were not significantly more affected by the test stimulation than by the control. The heat maps shown in figure 19 part B indicate that the flight response to the 'figure 8' and control stimuli was similar (though no statistical test was completed to confirm the potential statistical validity of this statement).

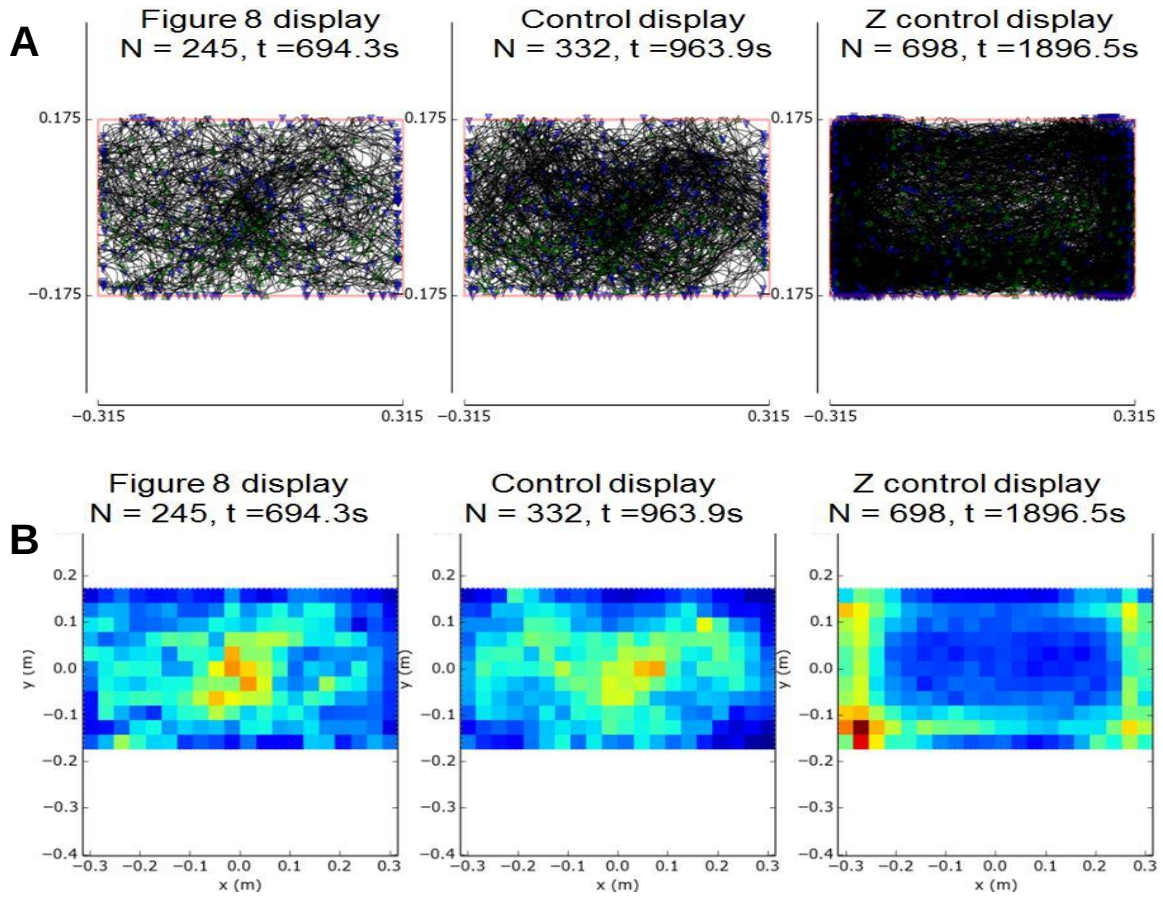


Figure 19. A) Aggregated individual two dimensional trajectories for each of the three stimuli patterns for Cx. quinquefasciatus female mosquitoes (n=12), with the number and time length of trajectories indicated above each stimulus.

B) Two dimensional heat maps indicating the most commonly occupied positions by female Cx. quinquefasciatus mosquitoes (n=12) being tracked during each stimulus. The colour gradient goes from blue to red (low to high average occupancy rate).

5.3.4. Mosquito flagellum length measurements

There is a statistically significant reduction in overall length and surface area of the flagellum for *Ae. aegypti* and *Cx. quinquefasciatus* male mosquitoes compared to females from the same two species ($P < 0.001$ for all comparisons). Male *An. gambiae* have a significantly longer flagellum than female mosquitoes from the same species but there was no significant difference identified between the two sexes from this species in the terms of the total flagellar surface area (as seen in table 5). Appendix E contains a complete table which includes median values for each flagellomere individually.

Table 5. Median values for total length of the flagellum from the point of laser focus to the pedicel, the total length of the entire flagellum and the total surface area of the entire antenna (standard errors are given in brackets below the median values, significant differences between conspecific female and male mosquitoes are starred).

Species/ sex	Length from focus point to base (µm)	Total length (µm)		Total surface area (μm^2)
Ae. aegypti females (n= 8)	1282.30 (18.20)	*1795.83 (20.09)		*154550.5 (2897.9)
Ae. aegypti males (n= 10)	1244.11 (17.74)	1630.86 (21.52)		118863.5 (2266.7)
An. gambiae females (n= 9)	1119.26 (11.72)	*1548.03 (12.08)		140408.8 (3250.7)
An. gambiae males (n= 22)	1551.68 (18.06)	1736.68 (10.32)		139051.0 (1288.1)
Cx. quinquefasciatus females (n= 23)	1447.17 (14.03)	*1863.21 (24.24)		*159103.1 (5515.7)
Cx. quinquefasciatus males (n= 16)	1066.57 (7.96)	1400.99 (9.65)		99313.3 (1224.0)

5.3.5. Mosquito wing beat frequency measurements

Whilst significant differences were identified between the different sexes of each species ($P < 0.001$ in all cases), no statistically significant differences were seen between females of different species ($P > 0.05$ in all cases). *Ae. aegypti* males had a significantly higher WBF than *An. gambiae* males ($P < 0.05$), but no other male comparison was found to be statistically significant. The ratio (shown in table 6) of male to female WBF of about 1.5 in all three species suggests that frequency matching during copulation attempts occurs at the second male, and third female, harmonic (in line with published reports).

Table 6. Median first and second harmonic estimates for all species and sexes (number in brackets indicates standard error values) alongside ratios of male to female first harmonics, as well as previously reported first harmonic estimates: Ae. aegypti published data is from (352), An. gambiae published data is sourced from (353, 442), whilst Cx. quinquefasciatus published data is from (354).

Species/ sex	Median first harmonic/ Hz	Median second harmonic/ Hz	Ratio of male to female first harmonic	Previously reported first harmonic/ Hz
Ae. aegypti females (n= 8)	405.0 (22.0)	810.0 (44.9)		430.6 (10.8)
Ae. aegypti males (n= 10)	624.5 (21.6)	1249.0 (43.8)	1.54	636.7 (15.1)
An. gambiae females (n= 10)	384.0 (16.7)	768.0 (42.2)		492.6 (2.4)
An. gambiae males (n= 11)	558.0 (10.2)	1116.0 (20.4)	1.45	769.0 (3.9)
Cx. quinquefasciatus females (n= 11)	390.0 (10.5)	780.0 (20.8)		428.3 (9.6)
Cx. quinquefasciatus males (n= 11)	583.0 (15.8)	1166.0 (32.0)	1.49	542.4 (18.2)

5.4. Discussion

5.4.1. Effect of pymetrozine on *An. gambiae* larvae

Very high concentrations of pymetrozine were required in order to lead to the significant levels of direct larval mortality reported for other compounds, as can be seen in section 5.3.1. This suggests that pymetrozine may not be a suitable for use as an larvicide given that the major challenges associated with larvicidal interventions tend to be associated more with regards to the most effective methods to locate and destroy larvae rather than which compounds should be used to do this, especially as efficacious larvicides already exist (62, 443, 444).

However, the aforementioned unpublished laboratory evidence that *Drosophila melanogaster* larvae exposed to pymetrozine doped food before eclosion which are then transferred to food not exposed to pymetrozine demonstrate severe reductions in auditory function (similar to the level observed for adults who are exposed to pymetrozine) when compared to controls may indicate that direct larval mortality may not be necessary for pymetrozine to be effective. If this effect holds for mosquito species then adult mosquitoes which emerge from the pupal stage with ablated ChO function may be sufficiently competitively disadvantaged that they are unable to contribute to disease transmission. Targeting larval stages is an essential part of mosquito control strategies for certain species (such as *Aedes* species) and as such new methods which increase the range of options available could still be useful (62).

This hypothesis would require testing however by exposing mosquito larvae to pymetrozine, allowing adults to emerge in the absence of pymetrozine and then using LDV techniques (described in detail in section 6 and discussed with regards to mosquitoes in sections 7 and 8) to measure the antennal state of the adult mosquito. This would immediately provide information as to whether pymetrozine exposure in the larval stage can result in altered adult auditory function.

Using WHO recommendations as a guideline, insufficient replicates were completed for each concentration of pymetrozine tested (441). This means that the results of the statistical tests should be treated with caution as there is an increased likelihood of statistical errors. A greater number of replicates would be necessary before conclusions can be drawn as to whether pymetrozine exposure directly leads to significant increases in larval mortality at low concentration levels.

5.4.2. Effect of pymetrozine on *An. gambiae* female fertility and fecundity

It has been reported that *An. gambiae* colonies tend to reproduce poorly in cages, with a small proportion of the females performing most of the egg-laying (445, 446).This theory is supported by the low number of fertile females present in the control group (seen in section 5.3.2) and therefore it is difficult to draw clear conclusions from comparisons made between different groups.

As such, whilst the results presented do not necessarily contradict the theory that pymetrozine can be used to not only target female mosquitoes in order to prevent biting attempts and reduce fertility but could also be used against males with the aim of decreasing mating success – unfortunately they also do not provide strong evidence that pymetrozine can have a large impact on mosquito fertility because of the low sample sizes.

The low sample sizes for each group, with few females from any group laying eggs, make it difficult to draw conclusions from statistical comparisons between groups because the experiment was statistically underpowered. In order to determine with any sense of accuracy the effect of pymetrozine on male and female mosquito fertility and fecundity many more replicates are necessary in order to account for the small proportion of females in each colony who lay eggs. Alternatively, a different mosquito species could be used in which a greater proportion of the females lay eggs (such as an *Aedes* species) (447).

5.4.3. The potential of Flycubes for tracking mosquito flight patterns

Whilst the results presented in section 5.3.3 show the Flycube system can accurately track mosquitoes during flight, they also unfortunately demonstrate that the stimuli provided was not able to recreate the earlier results seen for *Drosophila melanogaster*.

Although there are currently existing systems able to track mosquito flight patterns in their natural settings, the ability of the recording set-up to recognise and record mosquito flight patterns as well as the vast array of stimulus types that are available as part of the Flycube setup means that future tuning of the system to mosquitoes could enable precise tracking of individuals in response to different sensory cues – for example auditory stimuli which match the WBF of the relevant mosquito species (448). More experiments based upon previous publications (both with regards to the Flycubes and the basic underlying mechanisms of flight maintenance and sensory control) are ultimately necessary to more accurately understand the necessary stimulation types for the mosquito species before experiments utilising pymetrozine exposed mosquitoes are conducted (437, 449, 450).

5.4.4. Mosquito flagellar length and surface area measurements

The median length and surface area measurements of the flagellum for each mosquito species and sex (as estimated in section 5.3.4) are in reasonable agreement to prior reports available in the literature. A previous paper estimated the flagella surface area of *Anopheles gambiae s.s.* females to be in the region of 127000 μm^2, as compared to the value given in section 5.3.4 of approximately 140000 μm^2 for this species; the estimated flagellar length however was calculated to be 1136 µm, which is far smaller than the estimate calculated in this thesis (451). In addition to this a separate report estimated the length of a male mosquito flagellum to be approximately 1300 µm (452).

For *Ae. aegypti*, the length of the female flagellum has been reported as 2mm (±0.1mm), whilst the equivalent value for males was estimated as 1.6mm (±0.1mm) (363). Whilst the value calculated for female *Ae. aegypti* in section 5.3.4 is smaller than this reported value (at approximately 1.8mm), the estimate for male mosquitoes from this species is in agreement with the published report.

Whilst the distance from the pedicel to the focus point of the laser is statistically indistinguishable for male and female *Ae. aegypti*, there are significant differences between the same values for male and female *An. gambiae* and *Cx. quinquefasciatus* (with male *An. gambiae* having significantly longer flagella than females whilst male *Cx. quinquefasciatus* have smaller flagella than females from the same species). These differences in length are also seen between separate species.

The reduction in overall surface area for *Ae. aegypti* and *Cx. quinquefasciatus* male mosquitoes compared to females from the same two species is most likely the results of the measurement process itself, with causes the destruction of the fibrillae which form a visually distinct part of the male auditory system and are far less evident for females (76, 363). The absence of fibrillae drastically reduces the total available surface area of the male flagellum whilst the females are left comparatively unaffected. This is also true for *An. gambiae* males, but the greatly increased length of the male flagellum in this species compared to the females potentially resulted in no significant differences in surface area being calculated between the two sexes.

This antennal length data can now serve as a reference for future data conversions into flagellar angular displacements and as such could help to improve the comparability between different mosquito species of force-displacement data (as discussed in section 7).

5.4.5. Mosquito wing beat frequency measurements

The WBF estimates for *Cx. quinquefasciatus* and, in particular, *Ae. aegypti* mosquitoes from both sexes (as described in section 5.3.5 and included in table 6) match closely with previous reports, with the ratio of male and female fundamental WBFs in both species being approximately 1.5 (1.49 and 1.54 for *Cx. quinquefasciatus* and *Ae. aegypti* respectively) (352, 354).

Both male and female *Anopheles gambiae* mosquitoes however had far lower WBF values than the values published in the literature (353). This could be the result of many possible environmental and genetic variables, including changes in the rearing room temperature, differences in the strain background or the number of generations spent as a laboratory colony (306, 355, 442). Regardless of the cause, this decrease in WBF was found in both males and females and so the ratio of fundamental WBFs between the sexes was kept reasonably constant (1.45 compared to the 1.56 ratio previously calculated) (353).

This difference in estimated WBF could also indicate deeper issues with regards to the design of WBF measurement experiment; applying glue to the thorax of a mosquito may change the mechanics and resonance properties of the mosquito's thoracic box, with an increased stiffening of the system leading to potentially greater than expected WBF estimations (453). The gluing process could also potentially change the flight system in some other manner that would then lead inaccurate estimations of the natural WBF of the mosquitoes as compared to measurements made whilst the mosquito was free-flying – future estimates could therefore be made using a flight arena in which mosquitoes which are not tethered can be recorded from (358).

Whilst the differences in WBF between male and female mosquitoes were sufficiently large enough that significant differences were found (with every species having a difference between male and female median values larger than 150Hz), the sample sizes used for each group did not provide sufficient statistical power to allow for reasonably powered statistical comparison tests to be calculated for within-sex judgments across different species to be made. In order to produce more precise measurements of mosquito WBFs, which are essential for accurate estimation of relevant distortion products (as discussed in sections 2.10.2 and 7.3.6), both the inclusion of larger sample sizes and the utilisation of free-flying mosquitoes are required.

6. *Drosophila melanogaster* pymetrozine vibrometry and electrophysiology

6.1. Introduction

Experiments utilising LDV have allowed for intricate examinations of the sensory systems of many different types of insects, including mosquitoes, *Drosophila* species, bees and crickets (284, 289, 454, 455). Experiments utilising *Drosophila melanogaster* are particularly relevant for this thesis, with extensive investigation of signatures associated with mechanotransduction in the fly's antennal ear having been conducted and reported (274, 282). *Drosophila melanogaster* serves as an ideal tool for investigation into these signatures because of the accessibility of the arista and the genetic malleability associated with this model organism (276).

These signatures (as described for *Drosophila melanogaster* and discussed also in section 2.7) form the basic definitions for the active hearing process and include:

1) Power gain by motile mechanosensory cells i.e. active amplification of the antennal ear's mechanical stimulation (283)

2) self-sustained otoacoustic emissions that are the result of uncontrolled amplification (282)

3) increased antennal sensitivity in response to decreases in stimulus intensity as a result of a compressive nonlinearity associated with transducer gating (456)

4) increased frequency selectivity due to continuous cycle-by-cycle amplification (282, 290)

Given that auditory function in *Drosophila melanogaster* is reliant on ChOs, and pymetrozine has been demonstrated to ablate mechanosensory functionality in this organ, it is of interest to investigate the impact that the compound could have on these signatures not only with a view to inclusion in control programmes but also with regards to the fundamental machinery behind these processes (27).

There are two possible exposure methods that could be utilised in order to investigate the potential effect of pymetrozine on *Drosophila melanogaster* auditory function; oral pymetrozine ingestion or direct injection of the compound.

Ingestion experiments have the clear benefit that, compared to injection protocols, they more accurately mimic the method of exposure to pymetrozine that insects would face in the wild (26). Injection experiments are also limited in that the individual fly can become damaged as a result of the experimental procedure.

On the other hand, injection experiments also have some clear advantages – they allow for clear comparison between pre- and post-pymetrozine states within a single insect, removing the possibility that changes to the auditory system were due to a cause other than pymetrozine, and the full impact of the compound on the auditory system can be clearly observed as the entire body is soaked in pymetrozine. It also removes the possibility that the absence of a nerve response to stimulation is the result of incorrect placement of a recording electrode.

In addition to this the only previously published work regarding pymetrozine's effect on *Drosophila melanogaster* auditory functions utilised compound injection as the preferred exposure method (27). Thus throughout section 6 injection experiments were preferred for all detailed electrophysiological analyses to ensure that any changes in auditory function can be ascribed to pymetrozine exposure.

As described in sections 2.5 and 4.1, one of the major benefits of utilising *Drosophila melanogaster* as a model organism is the extensive library of genetic tools available; this has enabled the creation and maintenance of numerous *Drosophila melanogaster* lines which demonstrate resistance to insecticides used in mosquito control programmes through a variety of mechanisms (including direct expression of mosquito resistance genes) (257, 457, 458). The existence of *Drosophila melanogaster* lines which demonstrate resistance to insecticides from each of the major pesticide classes allows for experiments to investigate the potential presence of cross-resistance mechanisms between pymetrozine and pyrethroids for example. It also enables investigations into the potential impact of resistance expression on auditory function.

Comparisons between before and after pymetrozine injection in terms of both the free fluctuations of the individual fly (as shown before in appendices A – D in terms of feeding experiments) will allow for any changes in antennal function as measured by laser vibrometry to be identified using previously published methods of mathematical analysis (described in full in section 6.2.6) (283). Analysis of force step stimulation electrophysiology, in line with previous reported methodologies, will also enable estimation of the compound effect on not only the CAP response to stimulation but also key parameters which form an essential part of gating spring models of mechanotransduction in ChO (as described in sections 2.7 and 6.2.8) (285, 394).

6.2. Materials and methods

6.2.1. *Drosophila melanogaster* rearing

All *Drosophila melanogaster* stocks, unless otherwise stated, were reared identically to those investigated in section 4; all lines were kept in incubators at 25°C and 60% relative humidity and were provided with food prepared according to the Chippendale recipe. The incubators used a 12 hr: 12hr LD cycle which all lines were kept at during all developmental stages.

6.2.2. Compound preparation

Ringer: A saline solution was produced for control injection purposes following a previously published external saline solution guideline (459); this consisted of 5mM KCL, 135mM NaCl, 0.5mM $CaCl_2$, 2Mg Cl_2, 5mM N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) and 36mM sucrose, with an overall solution pH of 7.4.

Pymetrozine: Preparation of the compound was identical to that described in section 4.2.2 for feeding experiments in *Drosophila melanogaster.*

6.2.3. Insecticide resistant *Drosophila melanogaster* crosses

Pyrethroid resistance: yw/Y; UAS-AaegCYP9J28 7:1 males were crossed with w^{1118};; HR-GAL4 females to produce w^{1118}/Y; UAS-AaegCYP9J28 7:1;HR-GAL4 male offspring (hereon referred to as J28 7:1xGAL4), which were used for testing. Similarly, w^{1118}/Y; UAS-AaegCYP9J28 8:1;HR-GAL4 male offspring (hereon referred to as J28 8:1xGAL4) were produced by crossing yw/Y; UAS-AaegCYP9J28 8:1 males with w^{1118};; HR-GAL4 females. Finally, yw/Y males were crossed with w^{1118};; HR-GAL4 females, which resulted in w^{1118}/Y; HR-GAL4 (hereon referred to as w^{1118}xGAL4) males. These males were used as a control for both previously mentioned lines. Insecticidal resistance to pyrethroids is mediated in these lines via production of a specific enzyme capable of rapidly metabolising the insecticide (457).

Cyclodiene resistance: Bloomington line number 1675 (hereon referred to as BL1675) has the genotype $Rdl^{MD\text{-}RR}$, meaning that all males tested should demonstrate resistance to all cyclodiene-based compounds. This resistance is the result of a single amino acid mutation within the second membrane-spanning domain of the *Drosophila melanogaster* GABA receptor that renders the receptor insensitive to cyclodiene-based insecticides (458).

Carbamate resistance: Bloomington line number 1283 (hereon referred to as BL1283) has the genotype T(3;4)86D, kar^{1} ry^{41} Ace^{1}/MRS, with the balancer being required to avoid lethality. All males should be resistant to carbamate-based compounds due to a single nucleotide mutation in the *Drosophila* acetylcholinesterase gene which results in the production of a resistance-conferring enzyme (460).

DDT resistance: Resistant and susceptible lines (hereon referred to as 91-R and 91-S respectively) came pre-selected and were maintained in the lab. The 91-S line was used as a control for not only the 91-R line but also for both the BL1283 and BL 1675 lines. All resistant males demonstrate DDT resistance because of the way that the lines were originally selected; this resistance is considered to be polygenic in nature and influenced by multiple chromosomes (461, 462).

6.2.4. Compound exposure method - injection

Drosophila melanogaster injection experiments followed an identical protocol regardless of the specific line under investigation: first micro-capillaries (Harvard Apparatus, Cambridge) were pulled in a micropipette puller (Sutter Instruments, CA). These sharpened capillaries were then filled with either a control ringer solution (consisting of deionised water that had been diluted in the ringer solution described in section 6.2.3. to the desired concentration) or a ringer solution containing pymetrozine at a concentration of 1000ppm. When required, the relevant solution was injected into the thorax of the insect in such a way as to flood the body and so increase the likelihood that the compound was able to circulate to the head and as such reach the JO.

All experiments were divided into three stages – before injection, after ringer and after pymetrozine. This allowed for investigation of potential significant differences between pre- and post-ringer injection states. Differences between these states are discussed in greater detail in appendix F - there was a small but significant decrease in CAP amplitude of between 15 and 25% for all *Drosophila melanogaster* and mosquito species tested following ringer injection

No individuals from any *Drosophila melanogaster* line examined appeared to show any significant mechanical differences when comparing before and after ringer injection states and the best frequency of the antenna did not change significantly for any species experimented on. For the purposes of the major body of this thesis, comparisons drawn during injection experiments were done by contrasting the post-ringer state only with the post-compound of interest state in order to simplify procedures.

6.2.5. Vibrometry preparation and procedure – free fluctuations

All *Drosophila melanogaster* antennal free fluctuation recordings and electrophysiology experiments were conducted in conjunction with a PSV-400 Laser Doppler Vibrometer with an OFV-70 close-up unit (70 mm focal length) and a DD-500 displacement decoder, for which the mounting procedure has been described in detail previously (284). Male and female *Drosophila melanogaster* were first glued, ventrum-down, using blue-light cured dental glue to the top of a Teflon rod. Glue was then applied sparingly to the entirety of the fly (except for the antennae and wherever possible the spiracles, particularly those placed at the thorax-leg transition points) in order to minimise disturbances caused by movement of the insect. After this the fly's left arista was completely glued to the head whilst the second antennal segment of the right antenna was stuck down to prevent unwanted movements – as such only the third antennal segment and arista of the fly's right antenna should be able to move freely. The mounted fly was then placed in a micro-manipulator atop a vibration isolation table. The fly's right arista was arranged such that it was perpendicular to the Laser Doppler Vibrometer system.

Using the aristal tip as a reference/ focus point for the laser, aristal displacements were measured using the Laser Doppler Vibrometer system (as shown in figure 20). All measurements were taken without the arista being stimulated, meaning that the antennal movement should be compromised solely of a passive motion due to thermal bombardment and an active component because of mechanical feedback from mechanosensory neurons. A CED 1401 A/D converter was utilised to ascertain displacement output at a sampling rate of 100 kHz in the Spike 2 software programme. All experiments involved 3-6 day-old flies, with room temperature maintained between 20 and 22°C.

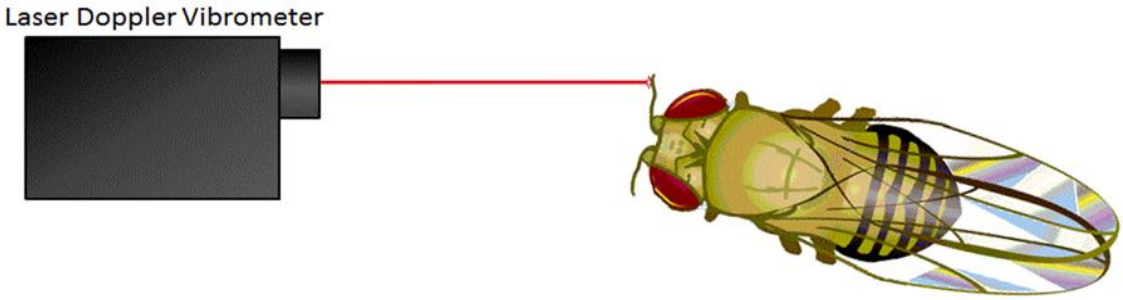


Figure 20. The Laser Doppler Vibrometer experimental setup for measuring unstimulated, free fluctuations of the antennal ear, with the laser point focussed on the tip of the Drosophila melanogaster's arista (figure provided by Nicholas Boyd-Gibbins, Jörg Albert lab).

6.2.6. Free fluctuation fitting procedure

After unstimulated, free fluctuations of the *Drosophila melanogaster* antennal receiver were recorded a fast Fourier transform of the antennal velocity amplitudes was calculated for frequencies between 1 and 3200 Hz for all *Drosophila melanogaster* lines investigated. Recording measurements within the frequency range below 51Hz contained a significant level of noise and were excluded from these analyses.

These velocity amplitudes were then fitted using a modified function based upon a previously described forced damped oscillator function (283) – whilst the original function was used for squared displacement amplitudes ($X^{2}{}_{(\omega)}$) this modified version was instead used for velocity amplitudes ($\dot{X}_{(\omega)}$) by converting between the function domains:

$$X^2(\omega) = \frac{(F_O / m)^2}{({\omega_0}^2 - \omega^2)^2 + (\omega.\frac{\omega_0}{Q})^2}; X^2(\omega) = (\dot{X}(\omega).\omega)^2$$

where F_O is the external force strength, m is the antennal apparent mass, ω is the angular frequency, ω_0 is the natural angular frequency and Q is the quality factor (which is defined as $Q = m\omega_0/\gamma$, where γ is the damping constant).

By taking advantage of these two equivalence relationships the following velocity amplitude fit function can be created:

$$\dot{X}(\omega) = \frac{F_O / m}{\sqrt{\omega^2.(({\omega_0}^2 - \omega^2)^2 + (\omega.\frac{\omega_0}{Q})^2)}}$$

This function provides estimates for F_O/m, ω_0 and Q, which could then be further utilised to calculate other parameters; of particular relevance were the best frequency and the overall stiffness of the antennal receiver. This data was then aggregated across individuals from groups of interest, which allowed for overall population estimates to be made. The velocity fit function well described the antennal velocity data obtained during free fluctuation recordings, with typical R^2 values of over 0.98 (with figure 21 containing an example of such a fit).

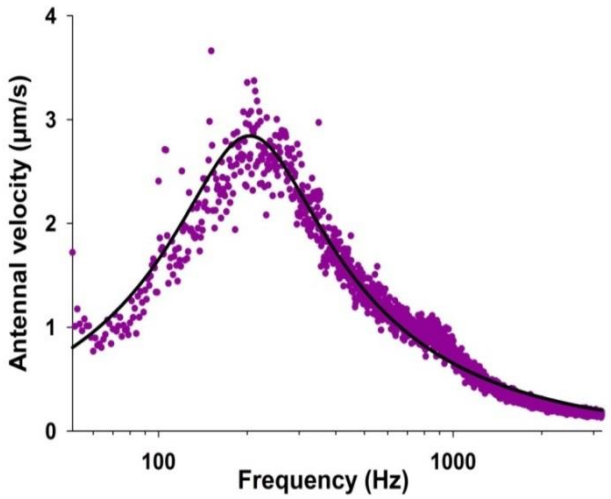


Figure 21. Free, unstimulated fluctuation data for an individual Drosophila melanogaster from the 91-S line, with a frequency range of between 51 – 3200Hz and individual data points (in purple) representing Fourier-transformed velocity amplitudes at each frequency contained within the transform range. The solid black line shows the velocity amplitude function fits for the recording.

The effective stiffness of the antenna was calculated by utilising the assumption that the system could be modelled as a forced-damped oscillator in order to take advantage of the Equipartition theorem:

$$\frac{1}{2}K\left\langle x^2\right\rangle = \frac{1}{2}K_B T$$

where *K* is the effective stiffness of the oscillator, $\left\langle x^2\right\rangle$ is the sum of the squared Fourier displacement amplitudes, K_B is the Boltzmann constant (1.38 × 10^{-23} J/K) and T is the absolute temperature (estimated at approximately 295 K). Rearranging this equation gives:

$$K = \frac{K_B T}{\left\langle x^2\right\rangle} = \frac{K_B T}{\int X^2(\omega)d\omega}$$

In total, 7 *Drosophila melanogaster* males from each of the 91-S, 91-R, BL1283 and BL 1675 lines were analysed, as well as 8 males from the J28 7:1 xGAL4 line and 10 males from each of the w^{1118}xGAL4and J28 8:1 xGAL4 lines. Statistical comparisons of the antennal best frequency and stiffness were made between before and after pymetrozine states using paired before and after t-tests (with a significance level of 0.05) using Sigmaplot. Post-hoc calculations of statistical power consistently showed statistical power estimates of over 95% for all comparisons made.

6.2.7. Stimulated recordings – force steps

All *Drosophila melanogaster* investigated during stimulated antennal recordings were first mounted in the same manner as described for free fluctuation recordings in section 6.2.5. A charging electrode was then inserted into the insect in order to raise its electrostatic potential, compared to ground, to -20V. Two electrostatic actuators were brought into position roughly symmetrically around the arista. These actuators allowed for electrostatic stimulation of the arista once the insect's electrostatic potential reached the aforementioned level (394).

A recording electrode was inserted into the base of the right antenna in order to measure mechanically-evoked compound action potentials (CAPs) - the charging electrode provided a reference point for these CAP recordings. Figure 22 provides a diagrammatic layout of the experimental set-up used during stimulated recordings. The type of stimulus provided to the arista changed depending on the particular experiment being conducted and is described in more detail in the following sections.

Unstimulated fluctuation measurements of the arista were recorded at the start and end of each injection stage of the experiment in order to judge the retention of auditory function throughout the experiment; flies whose free fluctuations showed a shift in best frequency of more than 10% prior to pymetrozine injection were not included in the final analysis (this percentage shift was adopted as a control measure based on the results of previous experiments and mainly served as an estimate of declining auditory function as a result of experimentation).

All force step recordings were conducted in line with previous reports (282, 285). This required the production of a symmetric step stimulus using the electrostatic actuators which lasted for 50ms and was calibrated to push the antenna to a maximum displacement of ± 8000nm. This force step was then exponentially attenuated such that the final step stimulus resulted in an antennal displacement of ±40-50nm. At each step the antennal displacement and CAP response were recorded, as well as the stimulus itself – figure 23 part A contains examples of each of these response types. This data set allowed for investigation of numerous aspects of the auditory system, such as changes in antennal dynamical stiffness in response to changes in stimulus intensity (as discussed in section 6.2.8).

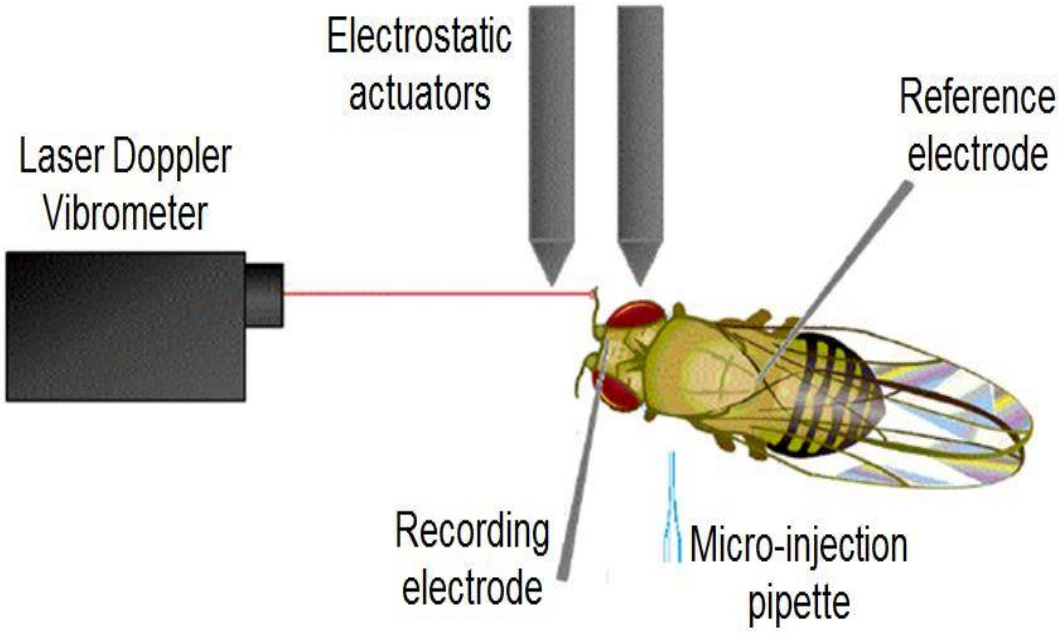


Figure 22. The Laser Doppler Vibrometer experimental setup for measuring stimulated fluctuations of the antennal ear as well as compound action potentials which propagate as a result of stimulation, with the laser point is focussed on the tip of the Drosophila melanogaster's arista (figure provided by Nicholas Boyd-Gibbins, Jörg Albert lab).

6.2.8. Stimulated recordings – force step analysis procedure

After the onset of a force step stimulus, the *Drosophila melanogaster* antennal receiver displays an initial displacement overshoot in the direction of the stimulus before rapidly recoiling before reaching a steady state displacement. These three steps (overshoot, recoil and then excursion) are reflective of the direct mechanical gating and adaptation of transducer channels in both vertebrate hair cells and the transducer machinery for hearing in *Drosophila* species (285, 463, 464). CAP responses are produced with a sub-millisecond delay following the aristal displacement as a result of the stimulation. This process is illustrated in figure 23 for both control and pymetrozine exposed *Drosophila melanogaster*.

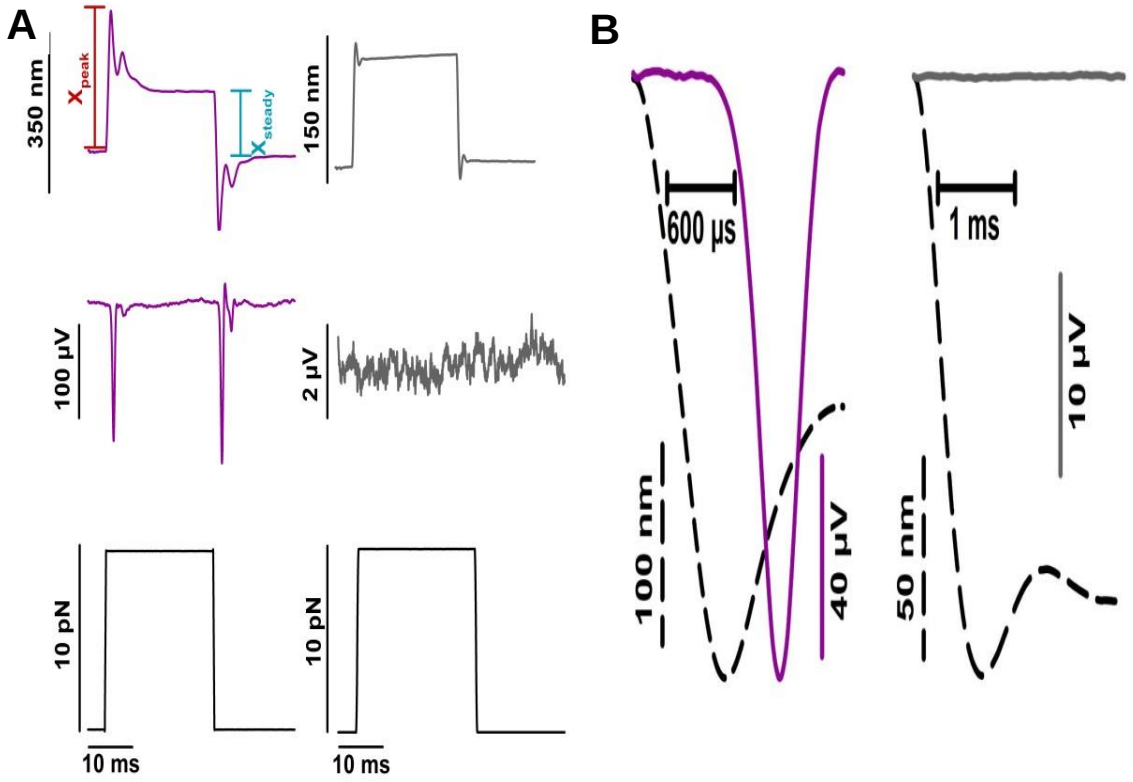


Figure 23. A) Flagellar displacements (top) and CAP amplitudes (middle) in response to the corresponding force steps (bottom) for Drosophila melanogaster before pymetrozine exposure (in purple) or after exposure (in grey) – X_{PEAK} and X_{STEADY} are highlighted as part of the flagellar displacement plot.
B) Flagellar displacements (dashed line) and CAP amplitudes (solid line) for pymetrozine unexposed (left, purple) or exposed (right, grey) Drosophila melanogaster showing the time delay between flagellar and nerve responses (stimulus onset is at start of flagellar displacement and force is constant throughout).

Following exposure to pymetrozine, *Drosophila melanogaster* auditory systems no longer produce CAP responses to stimuli and the previously mentioned signatures of mechanotransduction are also greatly reduced. Instead the aristal displacement to equivalent force step sizes is more than halved, with the majority of this decrease coming as the result of a reduction in the size of the initial overshoot. This initial overshoot displacement (hereon referred to as X_{PEAK}) is highlighted in red in figure 23 part A, with the steady state displacement (hereon referred to as X_{STEADY}) being shown in blue.

Whilst it has been reported that X_{STEADY} largely scales linearly with increasing force (as demonstrated in figure 24 below), X_{PEAK} shows a nonlinear force-displacement relationship in the auditory systems of fully functional *Drosophila* species (285). The corresponding dynamic stiffness equations, assuming that *m* is the apparent antennal mass, *F* is the external force and $\ddot{X}_{PEAK}$ is the acceleration calculated at the initial displacement peak, are:

$$K_{PEAK} = \frac{\delta(F - m\ddot{X}_{PEAK})}{\delta X_{PEAK}}; \quad K_{STEADY} = \frac{\delta F}{\delta X_{STEADY}}$$

This nonlinear behaviour can be modelled by using a two-state gating spring transducer model (285) and is no longer present after pymetrozine exposure. The decreases in K_{PEAK} for small force amplitudes are thus greatly reduced, though they not abolished.

Assuming that X_{STEADY} is linearly proportional to force, K_{STEADY} should remain constant across all force step displacement magnitudes – this assumption does not seem to hold completely true however, as is discussed in detail in section 7.3.3.4. In any case, K_{STEADY} provides important information on the steady state elasticity of the antennal joint. Taken in conjunction with $K_{INFINITY}$, which is defined as the asymptotic stiffness of the antennal receiver in response to large force stimuli, the gating spring stiffness (K_{GS}) can be calculated. K_{GS} is defined as the difference between $K_{INFINITY}$ and K_{STEADY} (i.e. $K_{INFINITY} - K_{STEADY}$) and provides an estimate of transducer mechanical integrity (394).

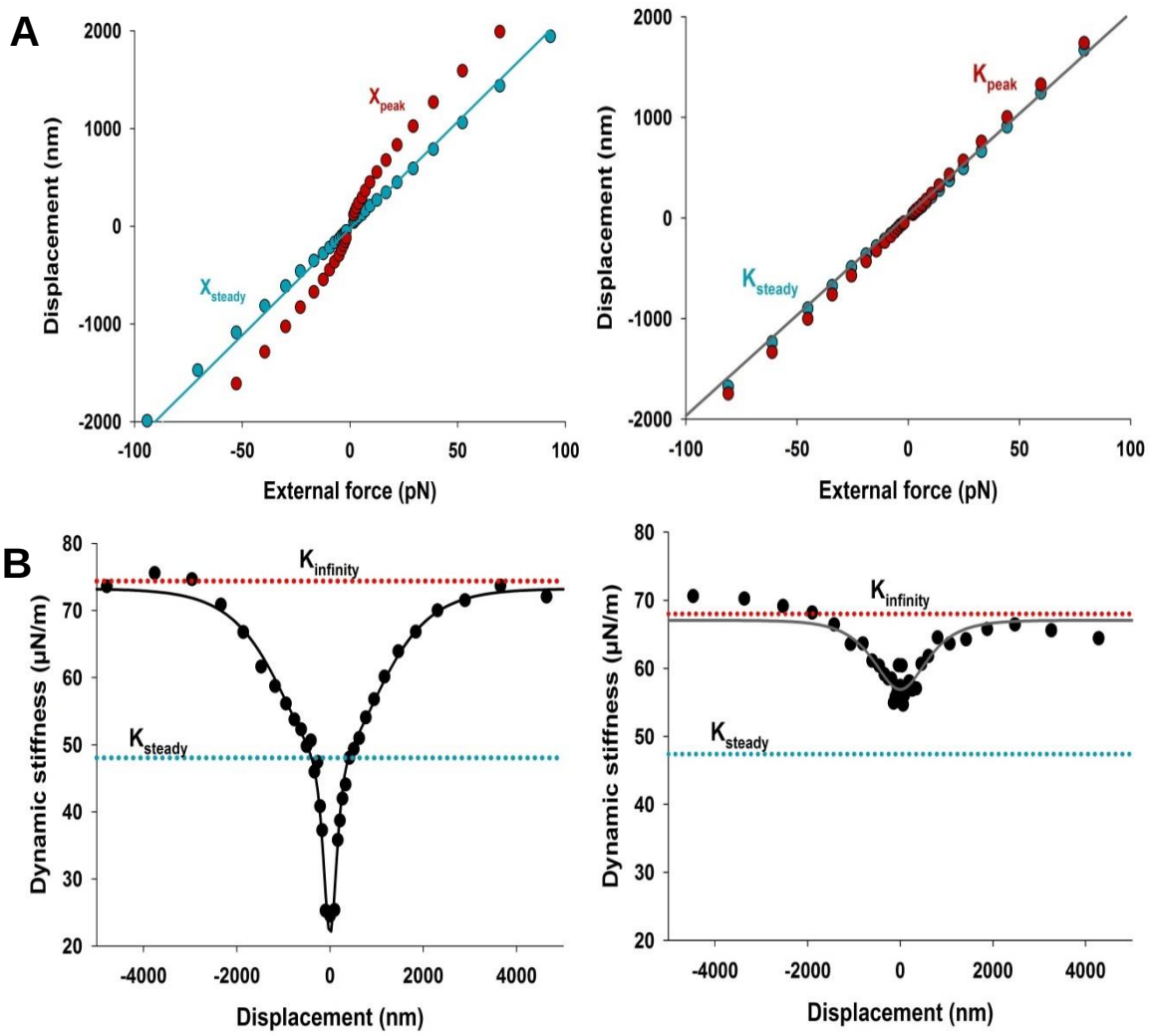


Figure 24. A) X_{PEAK} and X_{STEADY} calculations for pymetrozine unexposed (left) and exposed (right) flies – solid lines represent the best fit to the X_{STEADY} data.
B) Dynamic stiffness calculations for $K_{INFINITY}$ (red dashed line), K_{STEADY} (blue dashed line) and K_{PEAK} for pymetrozine unexposed flies (left) and exposed (right) flies (solid black lines represent two state model fits of two independent transducer populations).

As mentioned above, a two state gating spring model of a single transducer population has previously been reported to describe the ion channel populations of the *Drosophila melanogaster* auditory system in JO (285), with force being defined as:

$$F = K_{INFINITY} X - p_O(X) N z + F_0 - m \ddot{X}_{PEAK}$$

where F is the external force, X is the antennal displacement, $p_O(X)$ is the ion channel open probability at displacement X, N is the number of ion channels, z is the single channel gating force and F_0 is a constant offset term, with the other terms as defined above.

Electrophysiology experiments utilising force steps allow for fitting of this model to force-displacement data for the antennal receiver, which then provides estimates of N, z, $K_{INFINITY}$ and X_0 (the displacement at which the ion channel open probability is equal to 0.5).

The ion channel open probability for a single transducer population is defined as:

$$p_O(X) = \frac{1}{1 + e^{-\frac{z(X - X_0)}{K_B T}}}$$

where $p_O(X)$ is the ion channel open probability at displacement X, K_B is the Boltzmann constant and T is the absolute temperature, with the other terms as defined above. For all *Drosophila melanogaster* analyses X_0 was determined to be 0nm i.e. the resting position.

A model of two independent transducer populations was further developed from this starting point as a result of the identification of different functional roles for the different populations of scolopidia contained in the JO (327). This adapted model follows the same fundamental principles as the single transducer population model but allows for two separate ion channel populations to exist – these are denoted as the sensitive and insensitive populations to reflect their sensitivity, or lack of sensitivity, to auditory stimulation (as discussed in section 2.7) (394). These developments first require a reformulation of the force equation given above so that the displacement-dependent antennal stiffness can be calculated:

$$K(X) = K_{INFINITY} - \left(\frac{Nz^2}{K_B T}\right) p_O(X)(1 - p_O(X))$$

Introducing a second, independent transducer population requires supplementing this stiffness equation with another term to include the effects of this additional ion channel population:

$$K(X) = K_{INFINITY} - \left(\frac{N_S z_S^2}{K_B T}\right) p_{OS}(X)(1 - p_{OS}(X)) - \left(\frac{N_i z_i^2}{K_B T}\right) p_{Oi}(X)(1 - p_{Oi}(X))$$

where N_S, z_S and $p_{OS}(X)$ are the number of ion channels, the single channel gating force and the ion channel open probability at displacement X for the sensitive population (with N_i, z_i and $p_{Oi}(X)$ being the equivalent parameters for the insensitive population).

The ion channel open probability equation also requires modification in order to account for the newly introduced transducer population:

$$\frac{p_{OS}(X)+p_{Oi}(X)}{2}=\frac{1}{2}\left(\frac{1}{1+e^{-\frac{z_S(X-X_0)}{K_BT}}}+\frac{1}{1+e^{-\frac{z_i(X-X_0)}{K_BT}}}\right)$$

where all terms are as defined above.

For all *Drosophila melanogaster* gating spring model analyses included throughout this thesis, this two state model of two independent transducer populations was utilised for displacements between ±5000nm. This provided information on the two apparent types of ion channel populations in the system, including the estimated population sizes and single channel gating forces, as well as $K_{INFINITY}$. The previously reported standard apparent antennal mass of 5ng was used for all *Drosophila melanogaster* analysis (394).

In total, 7 male flies from each *Drosophila melanogaster* line investigated (i.e. 91-S, 91-R, BL1283, BL 1675, w^{1118}xGAL4, J28 7:1xGAL4 and J28 8:1xGAL4) were included in the final force-step analysis. Statistical comparisons of $K_{INFINITY}$, K_{STEADY} and K_{GS} were made between before and after pymetrozine states using paired before and after t-tests (with a significance level of 0.05) in Sigmaplot. Post-hoc calculations of statistical power consistently showed statistical power estimates of over 90% for all comparisons made, except for the statistical comparison made between before and after pymetrozine states for K_{GS} for *Drosophila melanogaster* from the 91 – S line. The statistical power in this case was calculated to be less than 30%, which therefore indicates a higher likelihood of a type II error than would usually be recommended.

6.3. Results of *Drosophila melanogaster* pymetrozine injections with respect to vibrometry and electrophysiology

6.3.1. Effective stiffness and best frequency changes

Table 7 contains the statistically significant increases in best frequency seen for all lines tested, with the minimum increase observed being 200 Hz (with a maximum of approximately 400Hz). The free fluctuation plots for male *Drosophila melanogaster* shown in figure 25 are demonstrative of changes seen after pymetrozine exposure throughout all experiments – an increase in best frequency from between 100-300Hz to around 500Hz in association with a decrease in maximum antennal velocity amplitude in line with a statistically significant increase in effective stiffness (as shown in table 8 and figure 26).

Table 7. Median best frequency values for all Drosophila melanogaster lines tested comparing post-ringer and post-pymetrozine states (standard errors given in brackets) as well as P-values for statistical comparisons between the two states.

Drosophila melanogaster line	Best frequency median after ringer (Hz)	Best frequency median after pymetrozine (Hz)	P-value
91-S (n = 7)	210.48 (10.4)	603.94 (31.0)	P = 0.00002
91-R (n = 7)	249.20 (12.3)	443.25 (26.5)	P = 0.000435
BL1283 (n = 7)	281.88 (18.3)	494.17 (15.3)	P = 0.000006
BL 1675 (n = 7)	239.33 (9.28)	544.45 (28.5)	P = 0.00001
w^{1118}xGAL4 (n = 10)	144.08 (4.46)	389.59 (19.7)	P = 0.000001
J28 7:1 xGAL4 (n = 8)	178.09 (5.94)	385.27 (12.75)	P = 0.000009
J28 8:1 xGAL4 (n = 10)	164.42 (9.75)	370.62 (27.8)	P = 0.000034

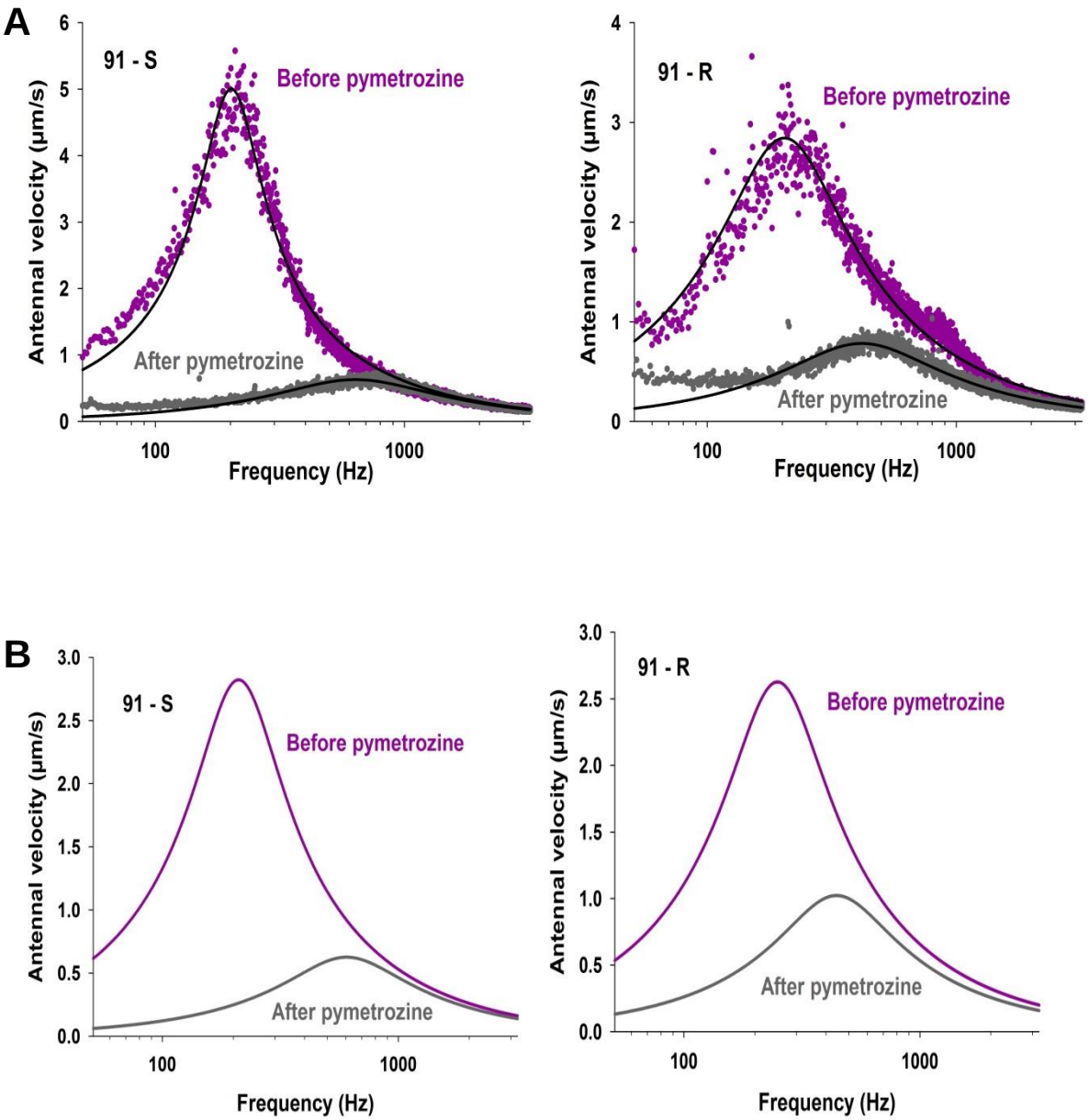


Figure 25. A) Free, unstimulated fluctuation data for a 91-S fly (left) before and after pymetrozine, with the equivalent states in a 91-R fly shown to the right. The frequency range for both flies is 51 – 3200Hz, with individual data points (in purple and grey for before and after pymetrozine injection states respectively) representing Fourier-transformed velocity amplitudes at each frequency contained within the transform range. Solid black lines show the velocity amplitude function fits for each recording. B) Velocity amplitude function fits produced using median population values for each of the fit parameters both before (in purple) and after (in grey) pymetrozine for 91-S (left) and 91-R (right) lines.

Significant increases in the calculated effective stiffness were also seen for all *Drosophila melanogaster* lines (as is demonstrated in figure 26) – table 8 contains all effective stiffness estimates in both post ringer and post pymetrozine states.

Table 8. Median values of effective stiffness for all Drosophila melanogaster lines investigated comparing post-ringer and post-pymetrozine states (numbers in brackets are standard errors) as well as P-values for statistical comparisons between the two states

Drosophila melanogaster line	Effective stiffness median after ringer (µN/m)	Effective stiffness median after pymetrozine (µN/m)	P-value
91-S (n = 7)	1.9 (0.32)	58.9 (6.56)	P = 0.000064
91-R (n = 7)	1.4 (0.13)	19.6 (6.87)	P = 0.016
BL1283 (n = 7)	2.5 (0.40)	63.1 (5.84)	P = 0.000042
BL 1675 (n = 7)	3.5 (0.83)	48.5 (3.57)	P = 0.000035
w^{1118}xGAL4 (n = 10)	0.3 (0.03)	20.1 (16.22)	P = 0.002
J28 7:1 xGAL4 (n = 8)	0.7 (0.07)	15.5 (3.19)	P = 0.008
J28 8:1 xGAL4 (n = 10)	0.4 (0.09)	18.3 (3.63)	P = 0.016

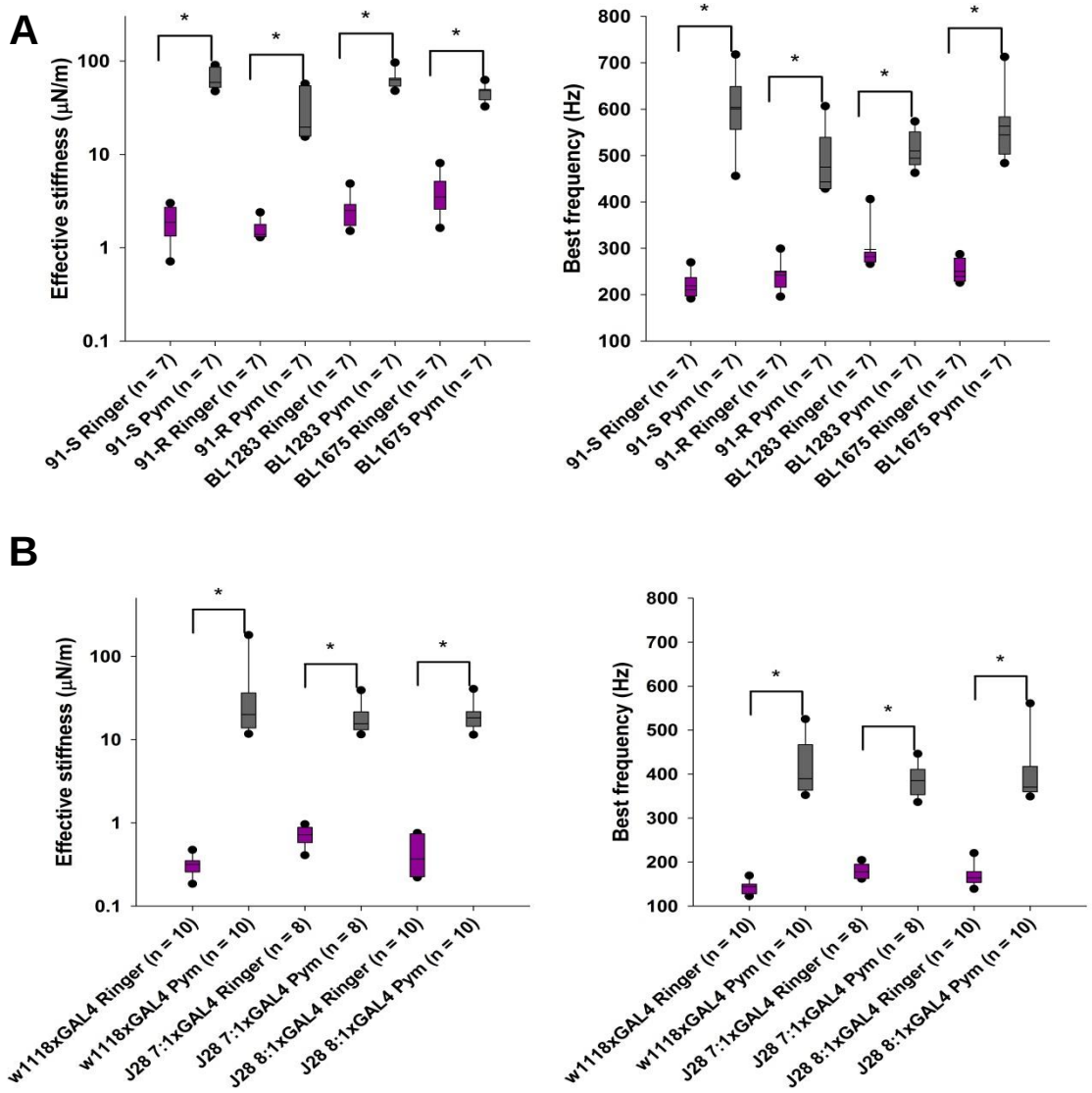


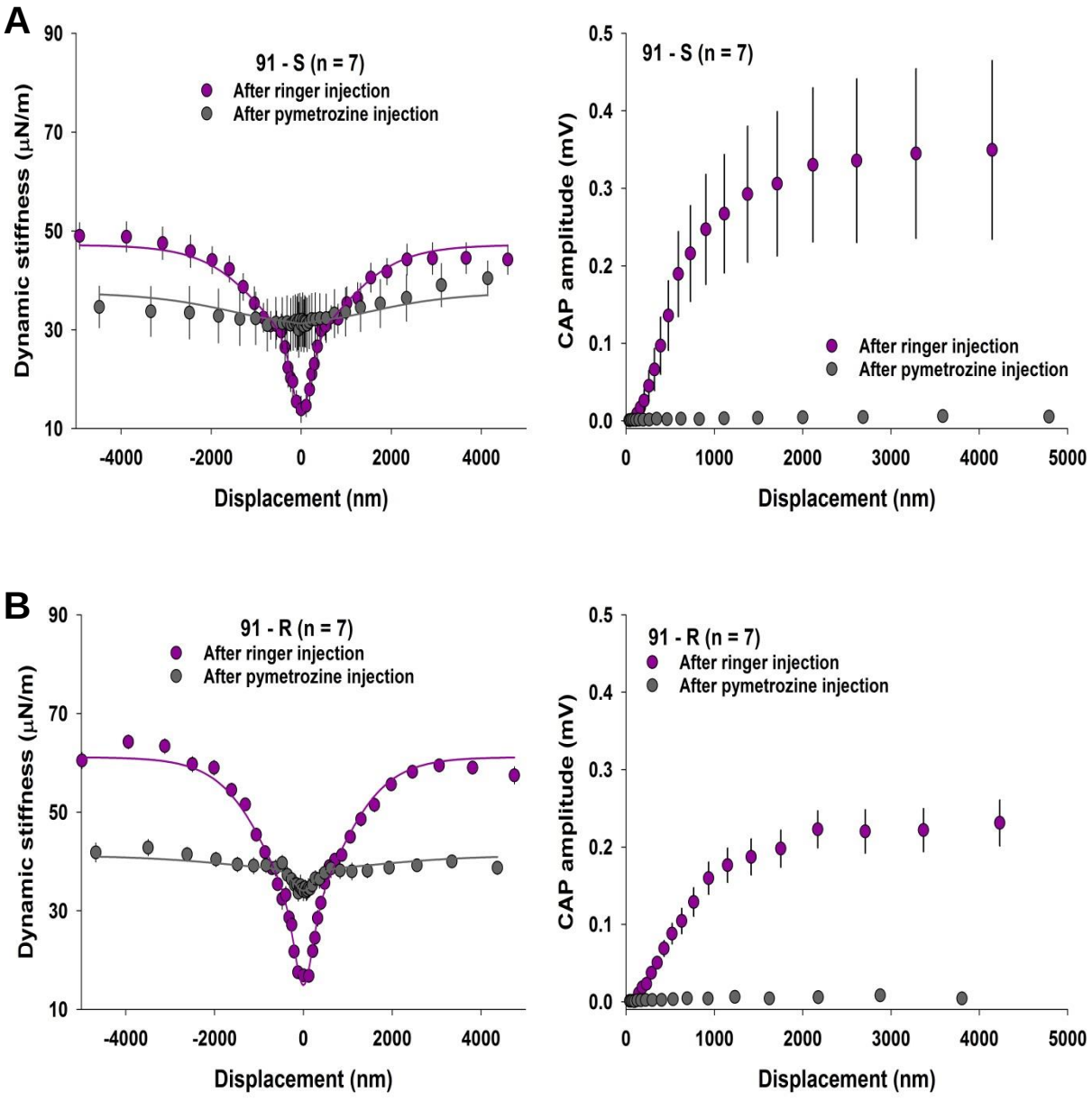
Figure 26. A) Effective stiffness values for all Drosophila melanogaster lines investigated comparing post-ringer and post-pymetrozine injection.

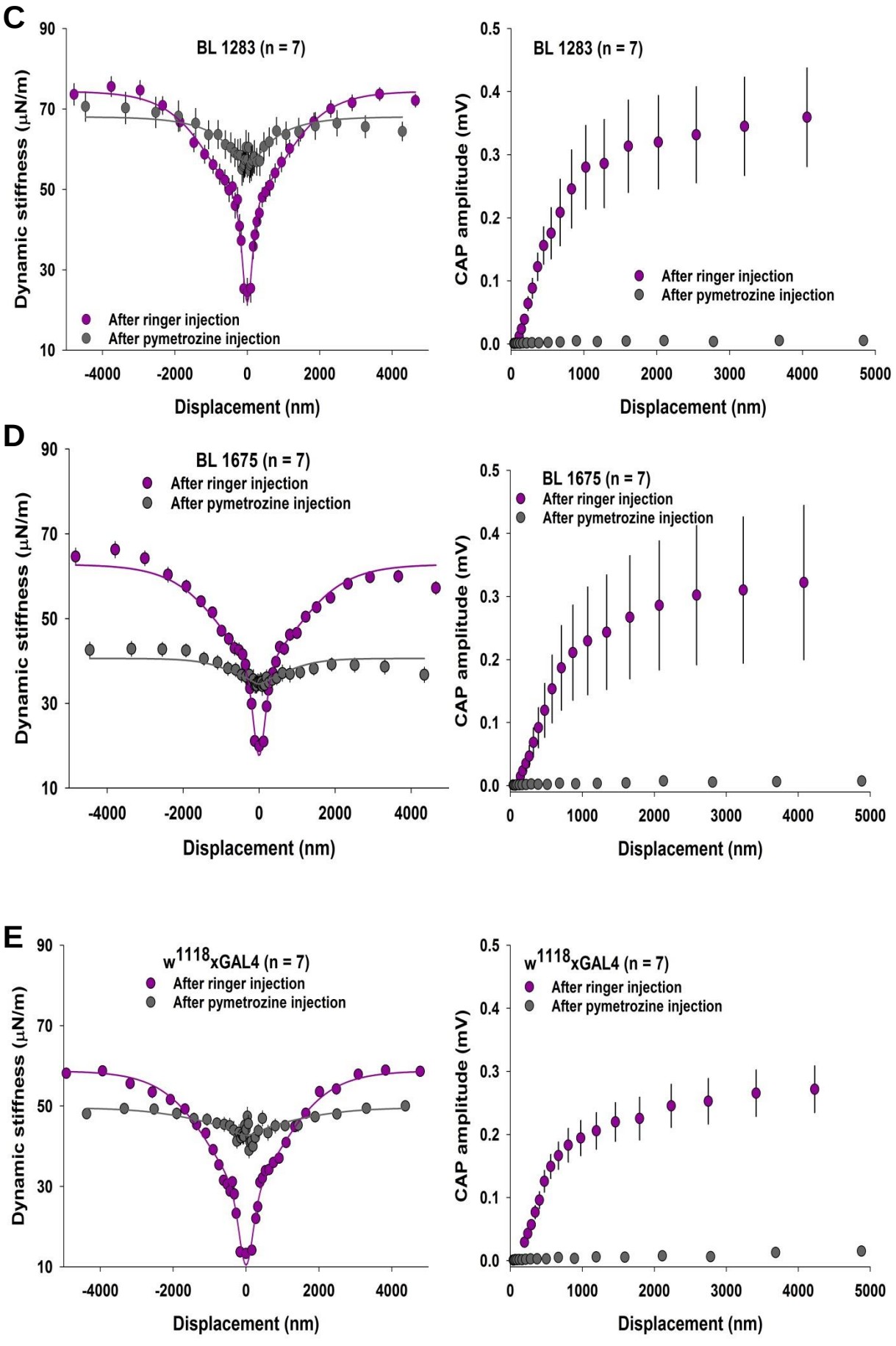
B) Best frequency values for different Drosophila melanogaster lines comparing post-ringer and post-pymetrozine injection.

For both A) and B), from left (also labelled in graph): 91-S, 91-R, BL1283, BL 1675, w^{1118}xGAL4, J28 7:1xGAL4 and J28 8:1xGAL4. Starred values indicated significant differences between groups (with resistance types being documented in section 6.2.3) and black dots correspond to the 5^{th} and 95^{th} percentiles.

6.3.2. Force step electrophysiology recordings

Full lists of median parameters obtained by fitting the two state model of two independent transducer populations to the force displacement data obtained after ringer injection (but prior to pymetrozine injection) are available in tables 29 and 30 in appendix G. Following pymetrozine injection, there was a severe ablation of auditory transduction in all *Drosophila melanogaster* lines tested (as is evident in figure 27). CAP responses to stimuli were no longer produced and changes in dynamic stiffness around the resting position became less pronounced, although not entirely eliminated for some lines.




C
BL 1283 (n = 7)
Dynamic stiffness (μN/m)
Displacement (nm)
After ringer injection
After pymetrozine injection
CAP amplitude (mV)
D
BL 1675 (n = 7)
E
w1118xGAL4 (n = 7)

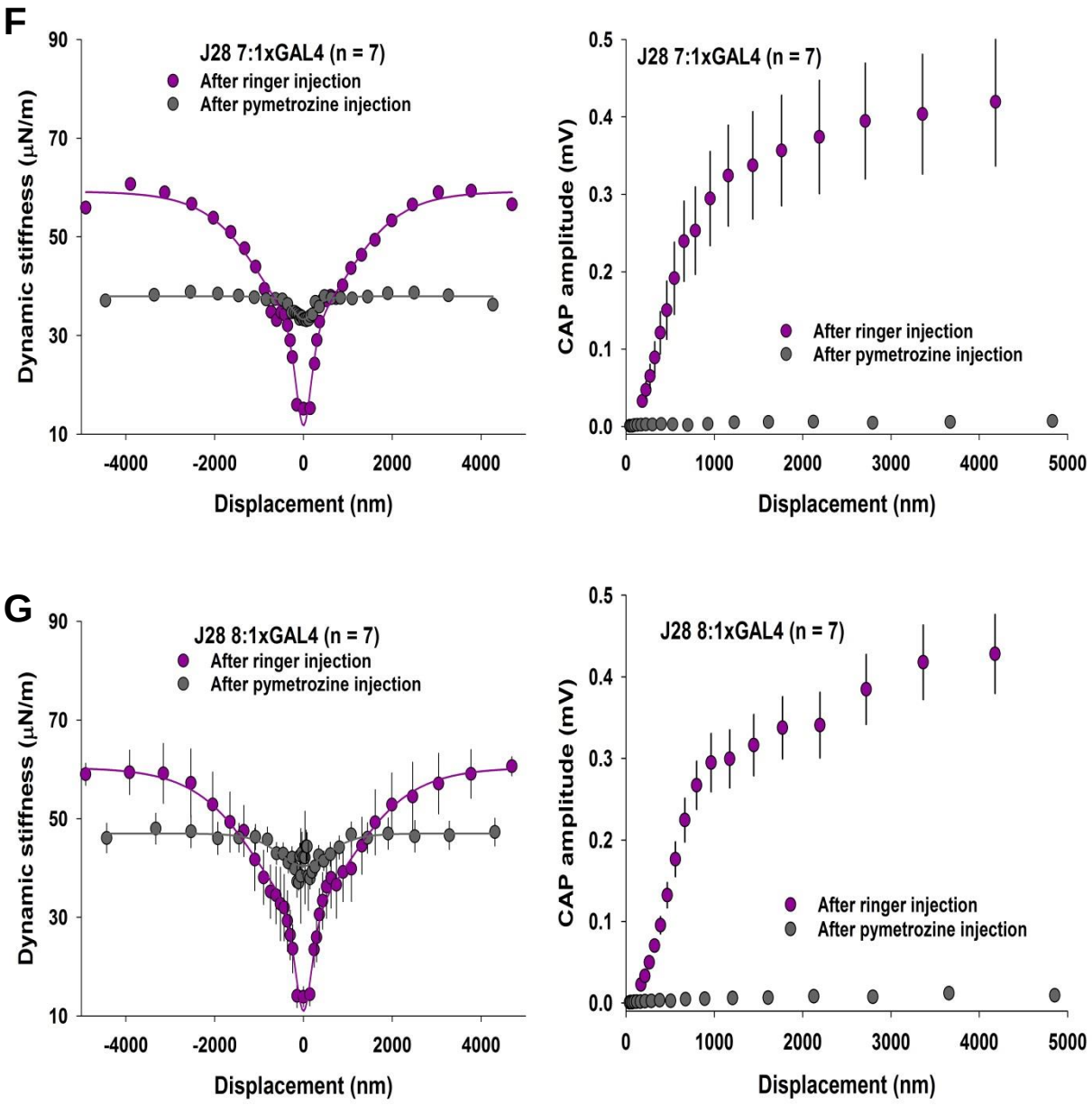


Figure 27. Changes in dynamical stiffness and CAP amplitude in response to changes in antennal displacement to a maximum displacement of ±5000nm comparing post-ringer (in purple) and post-pymetrozine (in grey) states for each of the control and resistant Drosophila melanogaster lines investigated: A) 91-S, B) 91-R, C) BL1283, D) BL 1675, E), w^{1118}xGAL4, F) J28 7:1xGAL4 and G) J28 8:1xGAL4. Individual data points represent median values calculated at that displacement, with vertical black bars representing standard errors.

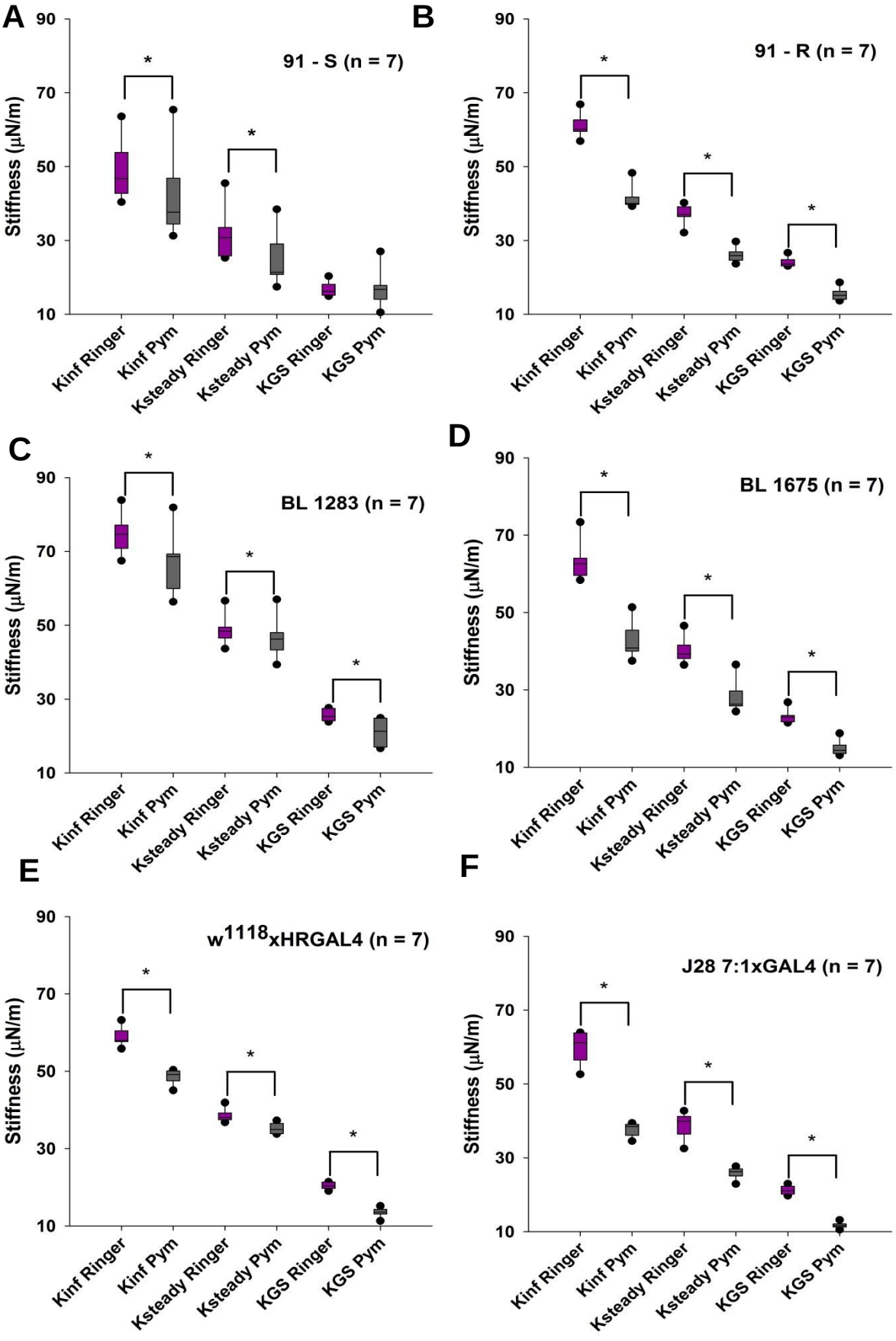
Significant changes to $K_{INFINITY}$ and K_{STEADY}, and thus by extension K_{GS}, were also identified, with significant decreases being calculated for all three of these parameters across all *Drosophila melanogaster* lines investigated (as shown in tables 9 and 10 as well as figure 28) – with the exception of the 91–S line, for which K_{GS} was not found to be statistically significantly different when comparing between the post ringer and post pymetrozine states.

Table 9. Median values for dynamic stiffness parameters after ringer injection and after pymetrozine injection for the following Drosophila melanogaster lines (standard errors given in brackets): 91-S, 91-R, BL 1283 and BL 1675. P-values for all statistical comparisons are also included.

	91 – S (n = 7)	91 – R (n = 7)	BL 1283 (n = 7)	BL 1675 (n = 7)
$K_{INFINITY}$ after ringer (µN/m)	47 (3.0)	60 (1.2)	75 (2.0)	63 (1.8)
$K_{INFINITY}$ after pymetrozine (µN/m)	38 (4.4)	40 (1.2)	69 (3.1)	41 (1.8)
Significance value	P = 0.013	P = 0.00006	P = 0.002	P = 0.0001
K_{STEADY} after ringer (µN/m)	31 (2.6)	37 (1.0)	48 (1.5)	39 (1.2)
K_{STEADY} after pymetrozine (µN/m)	21 (2.7)	26 (0.7)	46 (2.1)	26 (1.5)
Significance value	P = 0.001	P = 0.016	P = 0.013	P = 0.016
K_{GS} after ringer (µN/m)	16 (0.7)	24 (0.5)	25 (0.6)	23 (0.7)
K_{GS} after pymetrozine (µN/m)	17 (1.9)	15 (0.6)	21 (1.3)	14 (0.7)
Significance value	P = 0.992	P = 0.0002	P = 0.004	P = 0.0001

Table 10. Median values for dynamic stiffness parameters after ringer injection and after pymetrozine injection for the following Drosophila melanogaster lines (standard errors given in brackets): w^{1118}xGAL4, J28 7:1xGAL4 and J28 8:1xGAL4. P-values for all statistical comparisons are also included.

	w^{1118}xGAL4 (n = 7)	J28 7:1xGAL4 (n = 7)	J28 8:1xGAL4 (n = 7)
$K_{INFINITY}$ after ringer (µN/m)	58 (0.9)	61 (1.5)	59 (1.6)
$K_{INFINITY}$ after pymetrozine (µN/m)	49 (0.7)	39 (0.6)	47 (3.1)
Significance value	P = 0.0001	P = 0.002	P = 0.000458
K_{STEADY} after ringer (µN/m)	38 (0.6)	40 (1.2)	40 (0.7)
K_{STEADY} after pymetrozine (µN/m)	35 (0.5)	26 (0.5)	34 (2.4)
Significance value	P = 0.0001	P = 0.002	P = 0.016
K_{GS} after ringer (µN/m)	20 (0.3)	21 (0.4)	21 (1.0)
K_{GS} after pymetrozine (µN/m)	14 (0.5)	12 (0.3)	12 (1.8)
Significance value	P = 0.00016	P = 0.002	P = 0.008


A
91 - S (n = 7)
B
91 - R (n = 7)
C
BL 1283 (n = 7)
D
BL 1675 (n = 7)
E
w1118xHRGAL4 (n = 7)
F
J28 7:1xGAL4 (n = 7)
Stiffness (µN/m)
90
70
50
30
10
*
Kinf Ringer
Kinf Pym
Ksteady Ringer
Ksteady Pym
KGS Ringer
KGS Pym

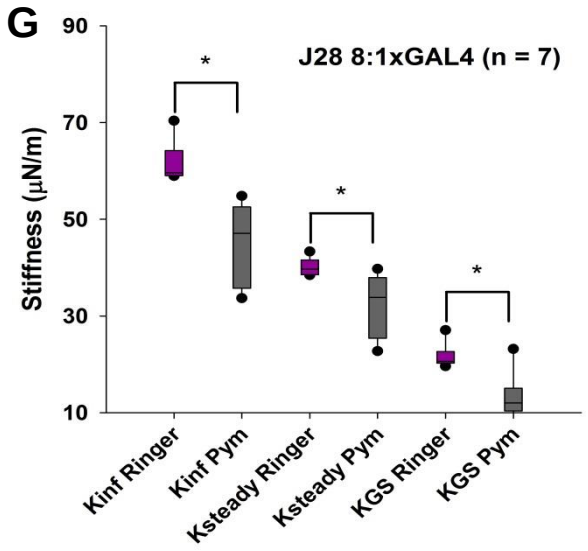


Figure 28. Estimated stiffness parameter values (including $K_{INFINITY}$, K_{STEADY} and K_{GS}) in both post-ringer and post-pymetrozine states for all Drosophila lines investigated (significant differences between states are starred, black dots correspond to the 5th and 95th percentiles): A) 91-S, B) 91-R, C) BL1283, D) BL 1675, E), w^{1118}xGAL4, F) J28 7:1xGAL4 and G) J28 8:1xGAL4.

6.4. Discussion

6.4.1. The effect of pymetrozine on *Drosophila melanogaster* auditory systems as measured by free fluctuations

Best frequency and effective stiffness estimates calculated from the free fluctuation fits for all *Drosophila melanogaster* lines tested in section 6 prior to pymetrozine exposure were in broad agreement with previous published values (274, 284, 310). In addition to this, all *Drosophila melanogaster* lines tested in section 6.3.1 showed significant increases in both best frequency and effective stiffness after pymetrozine injection compared to after ringer injection. The frequency values reached after pymetrozine injection were similar to those previously found in section 4 for susceptible *Drosophila melanogaster* from the wildtype CantonS line exposed to pymetrozine via oral ingestion (as shown in the free fluctuations provided as evidence of pymetrozine's effect on auditory function in appendices A - D); in particular, these CantonS results were similar to estimates of the 91-S and w^{1118}xGAL4 lines, which lacked insecticidal resistance and were thus used as controls for other lines which demonstrated some form of resistance..

The changes in best frequency and effective stiffness in pymetrozine injected insects are comparable to the differences previously reported when comparing control flies with auditory mutants (283); for example, $nompA^{2}$ and btv^{5PI} and mutants had reported median best frequencies of approximately 406 and 528Hz, and median effective stiffnesses of approximately 35 and 60 μN/m, respectively. As such, pymetrozine injected *Drosophila melanogaster* from the 91-S, 91-R, BL1283 and BL1675 lines measured in this section match closely with btv^{5PI} mutants, whilst the pymetrozine injected three UAS-based lines were to some extent similar to $nompA^{2}$ mutants.

Another ChO mutant, $tilb^{2}$, was reported to have a much greater best frequency and effective stiffness (approximately 750 Hz and 120 μN/m respectively) than any of the *Drosophila melanogaster* lines tested here, and as such had an antennal receiver much more similar to a dead fly than an alive, pymetrozine exposed fly (283).

The three UAS-based lines (including the line used as a control) all had lower best frequency and effective stiffness values than the other insecticidal resistant lines tested before pymetrozine injection (though still with the expected range of values for *Drosophila melanogaster* (283, 284)), suggesting that this is the result of the UAS construct rather than any insecticidal resistance mechanism; after pymetrozine injection the three lines still demonstrated significant increases in both best frequency and effective stiffness.

6.4.2. The effect of pymetrozine on *Drosophila melanogaster* auditory systems as measured by force step stimulation electrophysiology

For all *Drosophila melanogaster* lines tested in section 6.3.2, regardless of insecticidal resistance status, pymetrozine exposure caused an elimination of CAP production and a reduction in the dynamical stiffness decreases seen in response to relatively small stimuli that were present after ringer injection (with the lack of CAP response to stimulation in agreement with that previously reported for pymetrozine exposed *Drosophila melanogaster* (27) as well as for ChO mutants such as *tilB* (412)).

The decrease in dynamical stiffness around the resting position seen in *Drosophila melanogaster* with fully functional ChO and mechanotransduction machinery appears to be somewhat maintained in several lines after pymetrozine injection. In all cases however this decrease is greatly reduced, indicating that the auditory nonlinearity (a hallmark of the active hearing process) has been mostly abolished by pymetrozine (282). It could also be possible that the remaining nonlinearity is not an auditory nonlinearity but is instead related to other processes, with non-auditory nonlinearities being previously reported for *NompC* null *Drosophila melanogaster* mutants for example (394).

The partial retention of some level of compliance is particularly interesting when comparing the 91–R DDT resistant line to its' control line, 91-S. Whilst susceptible 91–S flies appear to lose all compliance increases after exposure to pymetrozine, 91–R flies still show an apparent decrease in dynamical stiffness around the resting position (in figure 27). This could potentially indicate some level of cross-resistance to pymetrozine, but the significant reduction in dynamic stiffness changes (in addition to the complete ablation of CAP responses to stimulation) suggest that if any resistance is present in this line it is not sufficient to maintain wildtype levels of auditory function after pymetrozine exposure.

For all lines tested there were clear and significant reductions in K_{STEADY} and $K_{INFINITY}$ which led to a significant decrease in K_{GS} in all lines except the 91-S line (as demonstrated in table 9). Similar reductions in K_{STEADY} and $K_{INFINITY}$ have been reported for NompC null *Drosophila melanogaster* mutants as well as *Drosophila melanogaster* whose auditory neurons were ablated (394). As this decrease in K_{GS} was also significant for the 91–R resistant line (for which the 91–S line acts as a control), this could potentially indicate that there exists some difference in the molecular substrates of these two lines. The 91–S line does appear however to be completely susceptible to pymetrozine as there is a complete elimination of CAP response and compliance changes around the resting position following pymetrozine injection.

Whilst there was a clear and complete abolition of ChO mechanosensory function (as measured by LDV) in all *Drosophila melanogaster* lines tested using injection protocols, no lines were tested using oral ingestion exposure methods. Given that ingestion is the regular method of exposure, and that direct compound injection could be more efficacious than ingestion exposure procedures, future experiments investigating the impact of pymetrozine could utilise ingestion procedures for comparative purposes (26).

Changes in dynamic stiffness around the resting position for several *Drosophila melanogaster* lines (for example 91-S flies) were also to some extent noisy compared to other lines. Whilst the sample sizes for each group were sufficient to identify significant differences between stiffness parameters of interest, increasing the sample sizes of future investigations could help to reduce this noise level. Other insecticidal resistant *Drosophila melanogaster* lines are available for testing (including lines resistant to spinosad for example), and increasing the number of resistant mechanisms studied would not only provide information on potential occurrences of cross-resistance with pymetrozine but also offer potential insights into how insecticide resistance could affect auditory systems (465).

7. Mosquito vibrometry and electrophysiology

7.1. Introduction

For male mosquitoes (particularly those from each of the three species described in section 2.1, which are the focus of this thesis) to increase the probability of successfully identifying and locating conspecific females their auditory systems must remain tuned within specific frequency ranges (34). This process is made more complicated by the high level of background noise produced by mosquito swarm (76, 290). Female mosquitoes are also poor sound radiators and produce relatively faint emissions during flight (305).

As such the male mosquito antennal receiver must not only be selective in distinguishing what could potentially be a female of the same species that is available for courtship but must also be sensitive enough to differentiate the female in the first place. This requires an auditory system that is able to both inject large amounts of energy (should that be required) and maintain a constant frequency tuning (290).

Though these auditory requirements may be more stringent for male mosquitoes, a large burden is also placed on females from some species as well because of the nature of mosquito courtship rituals (363). In species where harmonic convergence forms an essential part of the courtship ritual (for example, *Ae. aegypti, An. gambiae* and *Cx. quinquefasciatus*), both male and female mosquitoes require significant levels of auditory function in order to match their WBFs (34, 352).

These three mosquito species are particularly relevant for disease control programs because of their status as major vectors of multiple diseases (as discussed in sections 2.1 and 2.3) and will be the focus of all experiments conducted in section 7.

The mosquito antennal system is thus the result of trade-offs between these multiple requirements. Previous studies have used laser vibrometry to investigate the frequency selectivity and tuning that underlies these systems, particularly with regards to copulatory activities (289, 354, 358). These reports have not however necessarily examined potential mechanical signatures that arise from the direct gating of mechanotransducers, with mechanotransduction in mosquito auditory systems not fully explored (as discussed in section 2.8, although some aspects of these signatures, such as auditory nonlinearities, have been studied) (276, 287).

Such signatures of transducer gating (as described in section 6.1) have been extensively examined for *Drosophila melanogaster* however, with the application of gating spring models (as discussed in section 6.2.8) having been reported to be sufficient to describe this simple, one dimensional system (285).

These models have enabled the estimation of transducer populations and gating forces, alongside antennal stiffness projections (282). Applying these models therefore to mosquito species could therefore provide a wealth of information about mosquito auditory transduction processes, though the inherent additional complexity of the mosquito antennal system (for both males and females) means that this application needs to be done carefully and any conclusions that are made require caution.

Previous reports have also estimated energy gain in *Drosophila melanogaster* lines, which allowed for comparisons of energy injection between controls and ChO mutants (283). Applying these principles to male and female mosquitoes from different species (by comparing the free fluctuations of the flagellum of individual mosquitoes in active and passive states) would enable estimation of the extent of auditory sexual dimorphisms in these species.

Displacement gain calculations could provide insight into these sexual dimorphisms as well, with previous calculations being made in mosquito and *Drosophila* species using either white noise or pure tone stimulation (34, 272, 284, 289). Pure tone stimulation at specific frequencies tends to be used for displacement gain calculations because pure tones are potentially more biologically relevant than white noise stimulation (which covers a far larger range of frequencies but at a reduced intensity), with pure tones having the potential to model mosquito wing beats.

White noise stimulation however ensures as broad a stimulus as possible however such that a large range of frequencies can be investigated. Therefore given the nature of the two stimuli, comparing the results of pure tone stimulation with white noise stimulation could help to explore the extent of frequency selectivity present in mosquito auditory systems.

Whilst these investigations would provide quantifiable information regarding the differences between the auditory systems of male and female mosquitoes, using the already established vibrometry and electrophysiology protocols (detailed in section 6.2.8) could also allow for hypotheses to be made about the systems of frequency selectivity within the mosquito antenna.

For example, one possible method mosquitoes could use to maintain a constant frequency tuning is to minimise dynamic stiffness reductions that result from direct mechanotransducer gating - this would then decrease the extent of nonlinearity within the auditory system and reduce changes to the flagellar best frequency associated with transducer gating (466). This can be investigated by comparing the changes in the K_{PEAK} and K_{STEADY} (as defined in section 6.2.8) in response to decreasing antennal displacements and estimating the dynamic stiffness of the system in the absences of the steady state stiffness.

In addition to this, utilising extensions to the gating spring model and the ion channel open probability function (also as described in section 6.2.8) would allow for exploration of the conceptual existence of multiple transducer populations (which have been identified for *Drosophila melanogaster* (394)) in mosquito species.

Finally, spontaneous oscillations of the flagellum remain unique to males in the absence of compound injection - these oscillations, which can have displacement amplitudes on the order of hundreds of micrometres, may therefore play a male-specific role during mosquito courtship rituals but the mechanisms which control these oscillations are not fully understood (289, 308). The recent identification of efferent feedback loops within the auditory system of male *Cx. quinquefasciatus* mosquitoes could suggest the potential involvement of such loops in regulating these oscillations (314).

Injecting compounds which have been reported to sever such efferent feedback networks in other organisms into mosquitoes should therefore reveal the underlying mosquito auditory system (310, 467). This would enable the potential presence of such systems in both male and female mosquitoes from each of the different species investigated to be examined, as well as exploring the necessary requirements for spontaneous oscillations to occur. Sexual dimorphisms between the responses of male and female mosquitoes can also be analysed so that differences between the sexes in terms of auditory function can be described.

7.2. Materials and methods

7.2.1. Mosquito rearing

Unless otherwise noted, all mosquito species were reared in an identical manner as described in section 5: all *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* (Muheza) mosquitoes used for experiments were provided by Shahida Begum from the London School of Hygiene and Tropical Medicine. All mosquitoes were reared using a 12 hr: 12hr LD cycle at 26°C and 75% relative humidity and were fed using a 10% glucose mixture.

7.2.2 Compound preparation

Ringer: Preparation of the ringer solution was identical to that described in section 6.2.2 for injection experiments in *Drosophila melanogaster*.

Pymetrozine: Preparation of pymetrozine was identical to that described in section 4.2.2 for feeding experiments in *Drosophila melanogaster.*

TTX: A 100µM stock TTX solution was obtained from Professor Jonathan Ashmore (University College London, London). This was diluted to 5µM in the ringer solution previously described, with this concentration being used in all injection experiments.

TeNT: 166µl of the aforementioned ringer solution was injected into 25µg of a powdered version of the compound to obtain a 1 µM solution that could be used as a stock. 20 µl of this solution was then further mixed with 480 µl of ringer solution to create a 20nM solution that was used for all injection experiments.

7.2.3. Compound exposure method – injection

Injection experiments in mosquitoes entailed a similar protocol as described in section 6.2.3 for *Drosophila melanogaster* injection experiments: first micro-capillaries (Harvard Apparatus, Cambridge) were pulled in a micropipette puller (Sutter Instruments, CA). These sharpened capillaries were then filled with either a control ringer solution (consisting of deionised water that had been diluted in ringer to the desired concentration to match the compound of interest) or a ringer solution containing the compound of interest (pymetrozine, TTX or TeNT). Upon injection of either control or compound, the solution was injected into the mosquito in such a way as to flood the body and so increase the likelihood that the compound was able to circulate to the head and as such reach the JO.

All experiments were again divided into three stages – before injection, after ringer and after compound of interest. This allowed for investigation of potential significant differences between both pre- and post-ringer injection states as well as post-ringer and post-compound of interest injection states. Similar to *Drosophila melanogaster*, reductions in maximum CAP responses were observed after ringer solution injection but in general the mechanics of the system did not significantly change (appendix F contains representative comparisons for *Ae. aegypti* females and more discussion on this topic). As such, all comparisons made for injection experiments compared post-ringer and post-compound of interest states to simplify procedures.

7.2.4. Mosquito vibrometry preparation

Whilst the major principles of mounting were the same for both mosquitoes and *Drosophila melanogaster*, the increased size (with regards to both the entire body and more specifically the antennal system) of individual mosquitoes from each of the mosquito species tested resulted in minor modifications being made to the mounting procedure to ensure that whilst the flagellum remained free to move the rest of the mosquito body was firmly held in place. This meant that mosquitoes were glued dorsal-side down and as such glue was applied to each leg individually to prevent interference with the measurements.

Once the mosquito was mounted, the experimental setup was identical to that described in section 6.2.7 for *Drosophila melanogaster*, with the exception that the laser point was no longer focused on the antennal tip – instead the bottom of the third flagellomere from the tip was used as the focal point for female mosquitoes from all three species, whilst the corresponding point for males was the bottom of their second flagellomere from the tip.

This change in laser focus point was introduced to minimise disturbances in free fluctuations noted when focussing on the antennal tip, whilst also attempting to maximise stimulated displacements and providing as stable a focal point as possible. The frequency range of fluctuation measurements was also increased to between 1 and 10000Hz (with a 1.5625Hz resolution), in order to investigate the possibility of high frequency harmonics becoming a factor in any recording.

Male mosquitoes that were considered to be spontaneously oscillating at the beginning of an experiment were not included in any of the analyses – this was judged on the basis of the maximum flagellar displacements identified during free fluctuation measurements, with males demonstrating displacements greater than 1µm being considered to be spontaneously oscillating. No female mosquitoes were judged to demonstrate spontaneous oscillations in the absence of solution injection.

Electrophysiological experiments utilising force step stimuli were completed in the same manner as for *Drosophila melanogaster* and as such are as described in section 6.2.8, up to and including the type and duration of stimuli.

7.2.5. CO_2 sedation experiments – free fluctuation measurements

For CO_2 sedation experiments the mounting procedure was identical to regular experiments but the mounted mosquito was instead put inside a rectangular steel chamber (6 x 6 x 2.5cm), which was held in place opposite the vibrometer by a micromanipulator. A small glass window in the one of the sides of the chamber was positioned perpendicular to the vibrometer and the mosquito was placed inside the chamber so as to still allow for free fluctuation measurements to be taken.

A cavity under the chamber allowed for CO_2 to enter the chamber and sedate the mosquito, which was kept as close to the cavity as possible. A flow regulator (Flowbuddy, Flystuff) enabled a constant flow rate of 3 l/min to be maintained. A plastic case (3.5 x 2.5 x 2.5cm) was placed surrounding the mosquito in order to prevent rapid losses of CO_2 once the gas flow had been halted.

After an initial unstimulated fluctuation recording had been taken to assess the health of the antennal system pre-sedation, CO_2 was allowed to flow into the chamber for one minute, with fluctuation measurements being taken in a constant loop to judge the state of the auditory system. At that point, the flow was stopped and a free fluctuation of the passive state of the flagellum was recorded. After 5 minutes of recovery time had elapsed, another free fluctuation was recorded so that the level of recovery could be analysed.

Mosquitoes which did not exhibit signs of recovery of auditory function were excluded from the final analysis; this recovery was judged by assessing the maximum antennal velocity amplitude reached after cessation of CO_2 flow as well as the best frequency of the flagellum, with shifts of more than 10% in either of these values compared to the pre-sedation state resulting in the exclusion of the mosquito from the data set.

7.2.6. CO_2 sedation experiments – electrophysiological recordings

Force step electrophysiological experiments (as described in section 6.2.8) conducted whilst a mosquito was sedated by CO_2 required the insertion of charging and recording electrodes into the mosquito and thus prevented the use of the plastic case. They also required a constant flow of CO_2 within the chamber to ensure that the mosquito did not awaken whilst the recordings were being taken. As the flow could cause disruptions to laser measurements of the smallest stimuli this required a significantly greater than normal amount of force steps to be recorded for averaging purposes, with a balance being maintained between a completely sedated state and allowing the mosquito time to recover from the effects of sedation before the auditory system became permanently damaged.

In practice this entailed regularly halting the gas flow within the chamber to allow the mosquito to recover, confirming the apparent health of the auditory system by taking a free fluctuation recording so that comparisons could be made between the current state and the original baseline and confirming the CAP amplitude response to stimulation in the active state before sedating the mosquito again and proceeding with the step recordings. A complete set of force steps was recorded in the active state prior to any sedation attempts to provide a control for each mosquito recorded from.

7.2.7. Free fluctuation fitting procedure

Unstimulated, free fluctuations of the mosquito flagellum were analysed using a similar procedure to that described for *Drosophila melanogaster* in section 6.2.6: flagellar free fluctuations were recorded and a fast Fourier transform of the antennal velocity amplitudes was calculated for frequencies between 1 to 2000Hz for all mosquito species. Recording measurements within the frequency range below 101Hz contain a significant level of noise and were excluded from these analyses. These velocity amplitudes were then fitted using the same velocity amplitude fit function as described for *Drosophila melanogaster*, from which estimates of the parameters F_O/m, ω_0 and Q for mosquito species were obtained.

52 *Ae. aegypti* females, 39 *Ae. aegypti* males, 42 *An. gambiae* females, 35 *An. gambiae* males, 37 *Cx. quinquefasciatus* females and 43 *Cx. quinquefasciatus* males were included in the analysis of non-sedated mosquitoes; 35 *Ae. aegypti* females, 29 *Ae. aegypti* males, 33 *An. gambiae* females, 24 *An. gambiae* males, 28 *Cx. quinquefasciatus* females and 31 *Cx. quinquefasciatus* males were included in the analysis of sedated mosquitoes.

7.2.8. Energy gain calculations

In order to estimate the level of energy gain in the auditory system, ratios of the total fluctuation power in the active and passive auditory systems of mosquitoes were calculated. The procedure builds upon a previously published approach (282, 283) and thus follows the following rationale: the passive energy of the system can be assumed to be equal to the sum of the squared Fourier displacement amplitudes in the passive state, $\left\langle x_p{}^2 \right\rangle$, multiplied by the spring constant k_p as well as a proportionality constant α:

$$E_p = \alpha k_p \left\langle x_p{}^2 \right\rangle$$

The passive spring constant itself is equal to the product of the antennal apparent mass, m, and the square of the natural frequency of the system, $\omega_p{}^2$:

$$k_p = m\omega_p{}^2,$$

Therefore it can be assumed that:

$$E_p = \alpha k_p \left\langle x_p{}^2 \right\rangle = \alpha m \omega_p{}^2 \left\langle x_p{}^2 \right\rangle$$

Applying the same procedure to the active state provides an equivalent equation for that state:

$$E_a = \alpha k_a \left\langle x_a{}^2 \right\rangle = \alpha m \omega_a{}^2 \left\langle x_a{}^2 \right\rangle$$

Energy gain is defined here as

$$Energy\,gain = \frac{E_a - E_p}{E_p}$$

Thus:

$$Energy\,gain = \frac{\alpha m \omega_a{}^2 \left\langle x_a{}^2 \right\rangle - \alpha m \omega_p{}^2 \left\langle x_p{}^2 \right\rangle}{\alpha m \omega_p{}^2 \left\langle x_p{}^2 \right\rangle}.$$

Assuming that the proportionality constant α is the same in both active and passive states, this can be finally simplified to:

$$Energy\,gain = \frac{\omega_a{}^2 \left\langle x_a{}^2 \right\rangle}{\omega_p{}^2 \left\langle x_p{}^2 \right\rangle} - 1$$

In order to calculate energy gain therefore it is necessary to obtain the best frequency estimates, as well as the sum of the squared Fourier displacement amplitudes, in both the active and passive states - passive states of the auditory system are estimated by sedating the insect using CO_2. The best frequency estimates are obtained by fitting the velocity amplitude fit function described in section 6.2.6 to the fast Fourier transforms of the antennal velocity amplitudes in the active and passive states whilst the sum of the squared Fourier displacement amplitudes is estimated by integrating the displacement squared fit function also described in section 6.2.6 over all angular frequency values, i.e.

$$\left\langle x_i{}^2 \right\rangle = \int_0^\infty x_i{}^2(\omega) d\omega$$

In total, 35 *Ae. aegypti* females, 29 *Ae. aegypti* males, 33 *An. gambiae* females, 24 *An. gambiae* males, 28 *Cx. quinquefasciatus* females and 31 *Cx. quinquefasciatus* males were included in the final analysis (complete lists of sample sizes used in every energy gain calculation made for all mosquito species tested in section 7 are given in the tables included in appendix H).

Tests for significant differences between male and female mosquitoes were made using Wilcoxon signed rank tests with a significance level of 0.05 in Sigmaplot. Post-hoc statistical power calculations gave estimates of below 50% power for comparisons made between male and female mosquitoes for both *Ae. aegypti* and *An. gambiae* species. This indicates that the comparisons were underpowered, with an increased likelihood therefore of a type II error occurring.

7.2.9. Mosquito apparent antennal mass estimations

All *Drosophila melanogaster* step recordings analysed in section 6 used an apparent antennal mass estimated from CO_2 sedation experiment results (283). No such apparent antennal mass has previously been reported for mosquito species however and as such it was necessary to calculate new values, using the same method as for *Drosophila melanogaster*. Thus individual mosquitoes were sedated, with free, unstimulated fluctuations of the mosquito antenna being recorded whilst the mosquito was sedated. A harmonic oscillator model was then fitted to the resulting curve, which enabled extraction of the apparent antennal mass.

This follows from the procedure previously reported in the literature (283): assuming that the mosquito auditory system is in a passive state (i.e. there is no active injection of energy into the auditory system) then the fluctuations of the mosquito flagellum should obey the Equipartition theorem (as in section 6.2.6):

$$\frac{1}{2}K\left\langle x^2\right\rangle = \frac{1}{2}K_B T$$

where K is the effective stiffness of the oscillator, $\left\langle x^2\right\rangle$ is the sum of the squared Fourier displacement amplitudes, K_B is the Boltzmann constant (1.38×10^{-23} J/K) and T is the absolute temperature (estimated at approximately 295 K).

Assuming that K is equal to the spring constant of the oscillator system, K_S (i.e. $K = K_S$), whilst the mosquito is sedated then the relationship between the spring constant, apparent antennal mass m and the natural frequency of the system ω_0 can be modified accordingly:

$$K_S = m\omega_0{}^2$$

$$\Rightarrow m = \frac{K_B T}{\omega_0{}^2\left\langle x^2\right\rangle}$$

Therefore, in order to obtain apparent mosquito antennal mass estimates the natural frequency of the oscillator system and the sum of the squared Fourier displacement amplitudes are required.

Both of these values can be estimated using methods described in section 7.2.8 which detailed the energy gain calculation procedure, with the natural frequency of the system being approximated from the velocity amplitude fit function and $\langle x^2 \rangle$ following from

$$\langle x_i^{\,2} \rangle = \int_0^\infty x_i^{\,2}(\omega) d\omega$$

It was assumed that the apparent antennal mass remained constant regardless of whether the mosquito auditory system was in an active or a passive state (an assumption reported to be reasonable for *Drosophila melanogaster* (283)).

For many mosquitoes two passive state fluctuations were taken; one before and one after pymetrozine. No significant differences in the apparent antennal mass estimates were found between the two states using paired before and after t-tests with a significance level of 0.05 in Sigmaplot, and tests for normality found the data to be normally distributed. In order to maximise the size of the dataset therefore, a two-state mixed effects model was fitted in R using the lme4 package to account for the fact that not all measurements were independent of each other - this allowed for estimation of means and standard errors (468).

In total, 56 measurements from *Ae. aegypti* females (35 before pymetrozine exposure, 21 after), 45 measurements from *Ae. aegypti* males (30 before pymetrozine exposure, 15 after), 50 measurements from *An. gambiae* females (33 before pymetrozine, 17 after), 31 measurements from *An. gambiae* males (22 before pymetrozine exposure, 9 after), 50 measurements from *Cx. quinquefasciatus* females (29 before pymetrozine exposure, 21 after) and 54 measurements from *Cx. quinquefasciatus* males (33 before pymetrozine exposure, 21 after) were included in the final analysis.

Tests for statistically significant differences in the apparent antennal mass of conspecific male and female mosquitoes were made using two-tailed t-tests with a significance level of 0.05 in Sigmaplot. Post-hoc calculations of statistical power gave estimates of over 99% power for comparisons made for *An. gambiae* and *Cx. quinquefasciatus* mosquitoes, but below 50% for *Ae. aegypti*. This is a lower statistical power than would usually be recommended and would lead to an increased likelihood of type II errors.

7.2.10. Step recording analysis

As this was the first thorough examination of mechanotransduction in the mosquito auditory system, unless otherwise stated the original two state model of a single transducer population (as detailed in section 6.2.8) was utilised throughout. This fit provided estimates of the number of ion channels, the channel gating force, $K_{INFINITY}$, K_{STEADY} and K_{GS}.

The fitting of mosquito force-displacement data to the two state model of two independent populations (as discussed in section 7.3.3.4) was conducted as described for *Drosophila melanogaster* in section 6.2.8. Statistical comparisons between different model fits were made by calculating the AICc by fitting both models for either individual mosquitoes using Sigmaplot and selecting the fit with the lesser value or to the median dynamical stiffness values for different mosquito groups and selecting the lesser value.

Maximum displacements of ±2000nm were selected both because CAP saturation points were reached at these values for both sexes and to avoid the increased level of noise in the dynamic stiffness of the system observed at greater displacements, which is potentially associated with non-transduction related nonlinearities. The analytical process was identical to that of *Drosophila melanogaster* lines tested in section 6, except for an extra round of run averaging applied to the effective force values for male mosquitoes to counteract the increased noise observed in their force-displacement data sets.

The extent of nonlinearity has been reported to be a key measure of transduction introduced nonlinearities within the *Drosophila melanogaster* auditory system, and is significantly involved in modifying the best frequency of the antennal receiver in response to changes in stimulus intensity (456). An estimate of the extent of auditory nonlinearity was calculated for all mosquitoes tested using a previously reported formula (from (285)):

$$Extent\ of\ nonlinearity = \frac{Nz^2}{(4K_BT * K_{INFINITY})}$$

where N, z, K_B, T and $K_{INFINITY}$ are as defined in section 6.2.8.

In total, 21 *Ae. aegypti* females, 18 *Ae. aegypti* males, 18 *An. gambiae* females, 15 *An. gambiae* males, 17 *Cx. quinquefasciatus* females and 15 *Cx. quinquefasciatus* males were included in the final analysis. Statistical comparisons between different mosquito species were made using ANOVA on ranks tests in Sigmaplot. Statistical tests to determine whether dynamical stiffness estimates were non-negative utilised one sided t-tests with a significance level of 0.05 in Sigmaplot.

7.2.11. White noise stimulus experiments

Male and female mosquitoes between 3 and 7 days old were mounted as previously described in section 7.2.4 and then, via charging electrode insertion, had their electrostatic potential raised to -20V above ground. The force step stimulation programme described previously was then utilised to provide an estimate of the mosquito's auditory function and to calibrate the maximum displacement of the flagellum to approximately ±25000nm. The programme also enabled the calculation of a proportionality coefficient for conversion between different units of force (in this case, volts and Newtons).

A white noise stimulus, programmed in PSV 9.1 (Polytec software, Polytec), was then provided between 1 and 10000Hz, with attenuation to this stimulus provided by an external attenuation system (Electronics workshop, University of Cologne). At first a maximum attenuation of 80dB was applied, which was then lifted in 5db steps until 0dB was reached. At each step free fluctuations of the flagellar response to stimulation were taken, with a final, unstimulated fluctuation being taken at the end of the experiment to help assess whether the maximum force stimulus had damaged the system and caused a loss of antennal function.

The white noise stimulus itself was also recorded at each step, which allowed for calculation of the ratio of the antennal velocity amplitude and stimulus intensity at each frequency and the fitting of a harmonic oscillator model to the resulting data (with no assumptions made during this fitting procedure as to whether the flagellum itself is best modelled as a harmonic oscillator) (283); this enabled calculation of the mechanical sensitivity at each level of stimulus attenuation.

These mechanical sensitivity values for each stimulus attenuation were then fitted using a three parameter-sigmoidal function in the software package Sigmaplot, with all fits that were accepted having R^2 values greater than 0.9. This enabled the estimation of the displacement gain by comparing the values for maximum and minimum attenuations. Figure 29 shows a typical displacement gain plot as well as the sigmoid function fit; for reference the female mosquito used for the figure had a calculated displacement gain of 3.10.

In total, 7 *Ae. aegypti* females, 7 *Ae. aegypti* males, 9 *An. gambiae* females, 7 *An. gambiae* males, 13 *Cx. quinquefasciatus* females and 13 *Cx. quinquefasciatus* males were included in the final analysis. Statistical comparisons were made using Wilcoxon signed rank tests with a significance level of 0.05 in Sigmaplot.

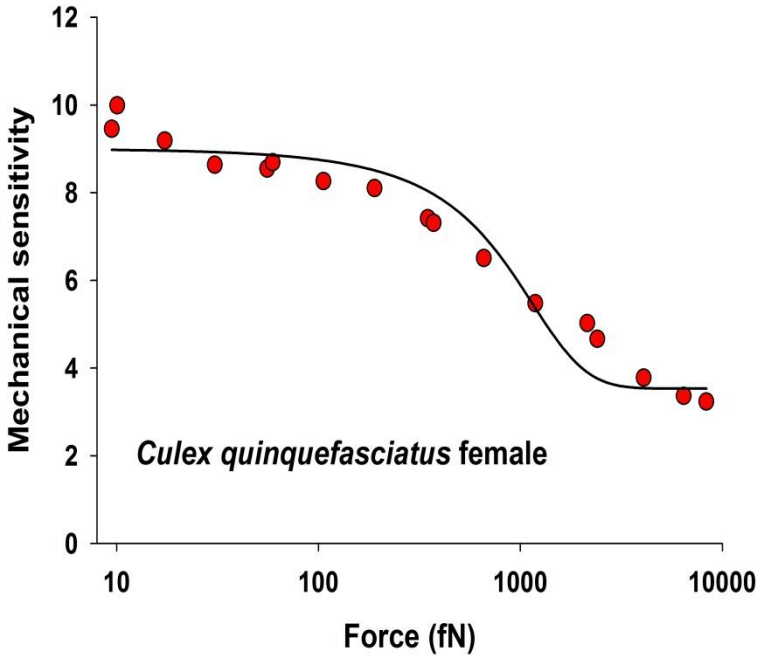


Figure 29. Changes in mechanical sensitivity in response to increases in white noise stimulus intensity for an individual Cx. quinquefasciatus female. Each point represents the mechanical sensitivity at an individual attenuation of the stimulus and the black line represents the best fit of a sigmoid function.

7.2.12. Pure tone sine stimulus experiments

Male and female mosquitoes between 4 and 8 days old were prepared in an identical manner as described for the white noise and force step stimulation experiments (in section 7.2.4), including utilising the force step stimulation protocol to estimate the relevant proportionality coefficient for conversion between different units of force. A recording electrode was also inserted into the mosquito in order to record CAP responses. After this, pure tone sine wave stimuli were used to stimulate the antenna. The stimuli ranged between 15 and 695Hz, in 10Hz intervals. This range was chosen to completely encompass all significant mechanical and nerve responses seen in test run-throughs of the set-up.

Mechanical and nerve responses at higher fundamentals of WBF estimates were found to be negligible when compared to the responses within the above frequency ranges and so were not included in the analysis. At every frequency included, the stimulus lasted continuously for 2.5 seconds before stopping for a further 2.5 seconds; this pattern was repeated 5 times for each frequency tested. After the conclusion of the pure tone stimulation process, a free fluctuation recording was taken to judge the final auditory capabilities of the flagellum.

Figure 30 part A displays the mosquito flagellar displacement in response to pure tone stimuli – there is a short period of constantly increasing displacements before a maximum displacement amplitude is reached. By fitting a sine wave function to a steady segment of the displacement response (after having first applied a DC remove to the data in order to centre the response around the resting position) an estimate of the peak flagellar displacement at each stimulus frequency can be obtained. Applying the same process to the stimulus itself at each frequency tested enabled a ratio of flagellar displacement to stimulus force to be calculated for all frequency values.

Plotting this estimated mechanical sensitivity data over frequency produces figures like that shown in Figure 30 part B. By fitting a Gaussian function to this data set an estimate of the maximum and minimum values can be obtained, with the point of maximum mechanical sensitivity in each mosquito being defined as the best frequency of the flagellum for that individual. Calculating the ratio of the maximum and minimum values obtained from the fit enables an estimation of the displacement gain (using pure tone stimulation) to be made in individual mosquitoes, which can then be aggregated together into groups of interest. The Gaussian function itself is not assumed to model the mosquito flagellum and is only used for estimation purposes.

Crosstalk between the stimulus and the recording electrode set up can lead to reflections of the stimulus amplitude in the nerve response, creating artifacts. These artifacts can cause problems during data analysis by artificially distorting the apparent nerve responses, though fortunately they cannot by themselves provoke nerve responses (285). Crosstalk can be immediately identified in nerve recordings because there is no time delay associated with the phenomenon and as such artifacts become immediately visible in both the stimulus and nerve channels.

One way of counteracting this issue is by taking advantage of the known phase of the artifact, as well as its' distinguishability from the real nerve response because of the lack of a time delay. Assuming that the crosstalk present in the system results in an overlaying of a copy of stimulus into the nerve response then by subtracting this artifact (after accounting for a change of scale) the real data should be revealed.

In order to achieve this, stimulus and nerve data were extracted from spike2 data files and exported to Matlab with only the steady state of the nerve being used for this analysis (i.e. after the initial onset had reached a constant state). After applying a DC remove to both sets of data, the stimulus at each frequency was subtracted from the corresponding nerve response - in order to account for a change in scale between the two channels the stimulus data was first multiplied by a constant which was selected such that the area under the curve of the remaining nerve response would be minimised.

In practice this meant allowing the constant to increase between 0 and 2 in steps of 0.01, subtracting the product of the constant and the stimulus from the nerve data and then calculating the resulting area under the curve. The nerve response at each frequency in the absence of crosstalk could then be estimated from the power spectrum of the residual nerve response, with the median best frequency of the nerve being defined as the frequency at which the nerve response magnitude was greatest.

In total, 8 *Ae. aegypti* females, 10 *Ae. aegypti* males, 7 *An. gambiae* females, 7 *An. gambiae* males, 8 *Cx. quinquefasciatus* females and 8 *Cx. quinquefasciatus* males were included in the final analysis. All statistical comparisons were made using Wilcoxon signed rank tests with a significance level of 0.05 in Sigmaplot.

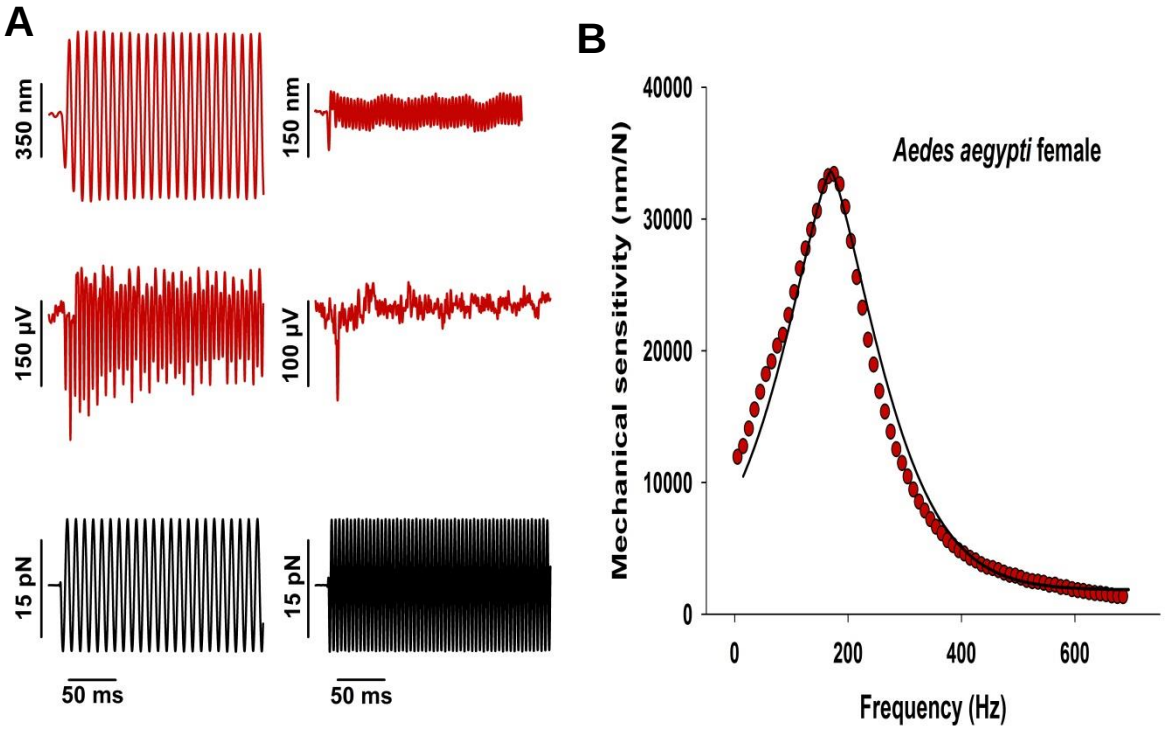


Figure 30. A) Flagellar displacement (top) and CAP amplitude (middle) in response to the corresponding pure tone stimuli (bottom) for Aedes aegypti females - the left pure tone stimulus frequency is set at 185Hz whilst the pure tone stimulus to the right has a frequency of 445Hz.

B) A Gaussian fit (solid black line) to individual sensitivity data points, also for an Ae. aegypti female.

7.2.13. TTX and TeNT injection series

Male and female mosquitoes between 3 and 8 days old were mounted as described in section 7.2.4 before a reference electrode was inserted in order to ground the insect and a free fluctuation of their baseline auditory state was recorded. A ringer control solution was then injected into the thorax of the insect so as to displace the original biological fluids contained within the mosquito. Free fluctuations of the antenna were then taken for 10 minutes at 1-2 minutes intervals in order to assess any changes in auditory function.

Mosquitoes that were judged to be in a suitable condition after this time had elapsed were then injected with either TTX (5µM concentration) or TeNT (20nM concentration). Free fluctuations were then taken every 1-2 minutes over either the next 10 or 25 minutes (for TTX and TeNT injections respectively). More time was allowed for TeNT injections as preliminary investigations suggested that the mechanism of action of the compound, along with the lower concentration used, necessitated a longer wait before potential changes to antennal auditory function could be seen.

After the specified period of time had elapsed, the mosquito was sedated so that a measurement of its passive system could be taken. The mosquito was then allowed to recover, with recovery being assessed by free fluctuation recordings – this sedation process followed the protocol given for CO_2 sedation experiments in section 7.2.5. After the mosquito had been given five minutes to recover from sedation, pymetrozine (10nM) was injected into the thorax. After this final injection was completed, free fluctuations were once again taken at 2 minute intervals for the following 10 minutes. The mosquito was sedated again using CO_2 and a free fluctuation of the passive system was taken. The mosquito was allowed time to recover before a last fluctuation was recorded. After the conclusion of each TeNT experiment, each mosquito was disposed of according to the departmental protocols for toxic waste disposal.

Energy gain calculations were made utilising the same procedure as described in section 7.2.8, with the last free fluctuation recorded for each injection state serving as a baseline for the most stable system state recorded for each injection state. Pymetrozine energy gains were estimated using the second, post-pymetrozine sedated free fluctuation data whilst all other injection states used the first, pre-pymetrozine sedated free fluctuation data as a baseline.

The final analysis included 10 TTX and 11 TeNT injections for *Ae. aegypti* females, 10 TTX and 11 TeNT injections for *Ae. aegypti* males, 12 TTX and 12 TeNT injections for *An. gambiae* females, 7 TTX and 8 TeNT injections for *An. gambiae* males, 14 TTX and 15 TeNT injections for *Cx. quinquefasciatus* females and 15 TTX and 14 TeNT injections for *Cx. quinquefasciatus* males. As all injection experiments (regardless of whether TTX or TeNT was injected) included both ringer and pymetrozine injection, this meant that in total 21 *Ae. aegypti* females, 21 *Ae. aegypti* males, 24 *An. gambiae* females, 15 *An. gambiae* males, 29 *Cx. quinquefasciatus* females and 29 *Cx. quinquefasciatus* males were included in the final analysis of energy gain estimates following exposure to either ringer or pymetrozine.

Tests for significant differences between injection states within a specific mosquito group were done using paired before and after t-tests with a significance level of 0.05 in Sigmaplot. Statistical tests to determine whether energy gain estimates following pymetrozine injection were greater than zero utilised one sided t-tests with a significance level of 0.05. Post-hoc statistical power calculations found that all comparisons made between different injection states for *Ae. aegypti* and *An. gambiae* females had a power <50%, meaning that these statistical comparisons should be considered underpowered.

7.3. Results

7.3.1. Mosquito free fluctuations - CO_2 sedation and energy gain

Table 11 contains median estimates of the flagellar best frequency before and after sedation for male and female *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus*, as well as significance levels for all statistical comparisons made. Sedating female and male *An. gambiae* as well as *Cx. quinquefasciatus* males caused the best frequency of the system to shift; in the females this led to an increase of over 100Hz, whilst for both groups of males there was a significant decrease by approximately the same amount. Male and female *Ae. aegypti* however, along with *Cx. quinquefasciatus* females, did not show shifts in best frequency on this level (with the maximum shift identified for these mosquitoes being 15Hz for female *Cx. quinquefasciatus*).

Examples of these best frequency changes are given in figure 31; whilst the *Ae. aegypti* female shown in part A maintains a constant best frequency estimate across both active and passive states, the sedated *An. gambiae* female in part B show an increase in best frequency of approximately 100Hz when compared to the active state. It also seems that as a result of this shift in frequency the passive system is partially recognisable in the free fluctuations of the active system. The *Cx. quinquefasciatus* male example shown in part C also demonstrates a shift in best frequency of approximately 100Hz, though in this case this is a decrease rather than an increase. The passive system of this male is also observable whilst the mosquito is not sedated.

Table 11. Median values of flagellar best frequency for male and female mosquitoes from each species investigated comparing post-ringer and post-sedation states (numbers in brackets are standard errors) as well as P-values for statistical comparisons between the two states.

Mosquito species/ sex	Best frequency after ringer (Hz)	Best frequency after sedation (Hz)	P-value
Ae. aegypti females (n = 36/ 35/ 36)	198.5 (1.42)	207.5 (3.99)	P <0.01
Ae. aegypti males (n = 25/ 30/ 25)	290.0 (11.91)	297.99 (11.90)	P = 0.706
An. gambiae females (n = 29/ 33/ 26)	215.0 (3.95)	325 (7.44)	P <0.001
An. gambiae males (n = 20/ 22/ 18)	352.0 (13.01)	283.7 (5.70)	P <0.001
Cx. quinquefasciatus females (n = 29/ 29/ 27)	221.6 (5.16)	206.5 (3.26)	P <0.01
Cx. quinquefasciatus males (n = 28/ 33/ 35)	365.6 (6.88)	274.1 (10.81)	P <0.001

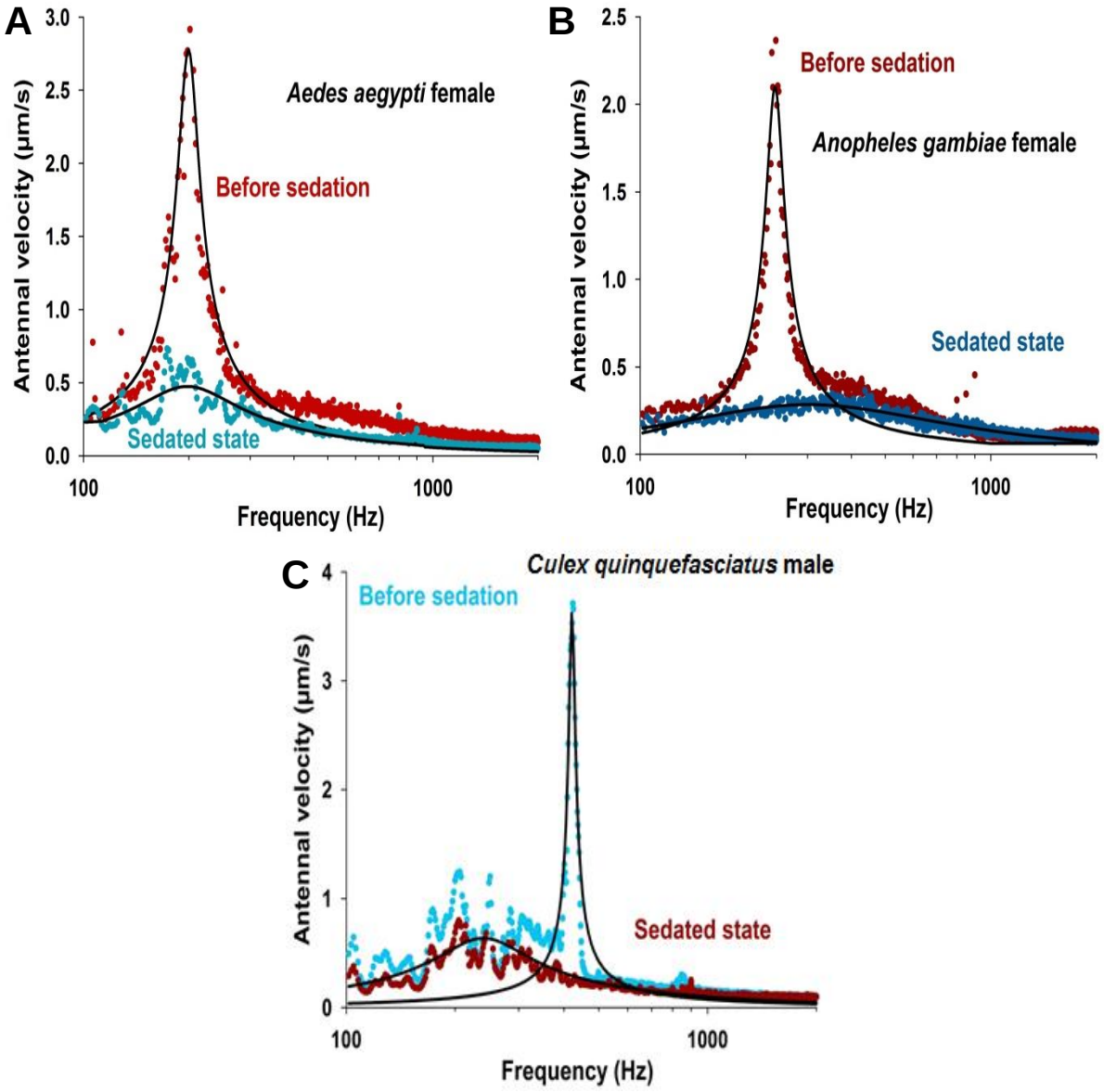


Figure 31. Free, unstimulated fluctuations both before and during sedation over a frequency range of 100 to 2000Hz for A) an Ae. aegypti female, B) an An. gambiae female and C) a Cx. quinquefasciatus male. Individual data points represent Fourier-transformed velocity amplitudes whilst solid black lines show the velocity amplitude function fits for each recording.

Table 12 contains median values for the three parameters obtained from the velocity amplitude fits for free fluctuations (as detailed in section 7.2.7) as well as median estimates for the level of energy gain in all three mosquito species: significant differences in terms of the energy gain estimates were calculated between male and female *Cx. quinquefasciatus* ($p<0.005$) but not for either *Ae. aegypti* or *An. gambiae* ($p>0.05$ in both cases). Energy gain values for all three species are also shown in figure 32.

Table 12. Median velocity fit parameters and energy gain calculated for each mosquito species and sex tested in both the active and sedated states (standard errors are given in brackets; the number of mosquitoes used to calculate the median energy gain value for each group is equal to the size of the relevant passive state group).

	Ae. aegypti females	*Ae. aegypti* males	*An. gambiae* females	*An. gambiae* males	*Cx. quinque-fasciatus* females	*Cx. quinque-fasciatus* males
ACTIVE STATE						
Sample size	52	39	42	35	37	43
F0/m	6.11×10^{-4} (1.8×10^{-5})	6.87×10^{-4} (3.7×10^{-5})	7.71×10^{-4} (3.2×10^{-5})	5.50×10^{-4} (2.6×10^{-5})	7.12×10^{-4} (3.8×10^{-5})	5.70×10^{-4} (2.9×10^{-5})
Best frequency/Hz	203.06 (2.22)	293.83 (11.72)	219.70 (3.55)	336.46 (8.58)	212.96 (2.41)	387.89 (6.60)
Q	3.32 (0.21)	1.59 (0.47)	1.19 (0.24)	2.87 (1.32)	5.76 (2.74)	7.70 (4.34)
PASSIVE STATE						
Sample size	35	29	33	24	28	31
F0/m	5.16×10^{-4} (1.9×10^{-5})	5.78×10^{-4} (4.3×10^{-5})	6.62×10^{-4} (3.2×10^{-5})	5.68×10^{-4} (4.2×10^{-5})	5.41×10^{-4} (2.4×10^{-5})	6.11×10^{-4} (3.0×10^{-5})
Best frequency/Hz	207.47 (3.99)	301.43 (12.32)	325.00 (7.44)	283.55 (5.97)	206.45 (3.32)	263.20 (8.70)
Q	1.04 (0.04)	0.94 (0.06)	0.67 (0.03)	0.91 (0.07)	1.11 (0.04)	1.00 (0.05)
Energy gain (kBT)	3.06 (0.62)	2.81 (1.69)	1.93 (0.25)	2.05 (0.58)	6.26 (2.05)	1.85 (2.40)

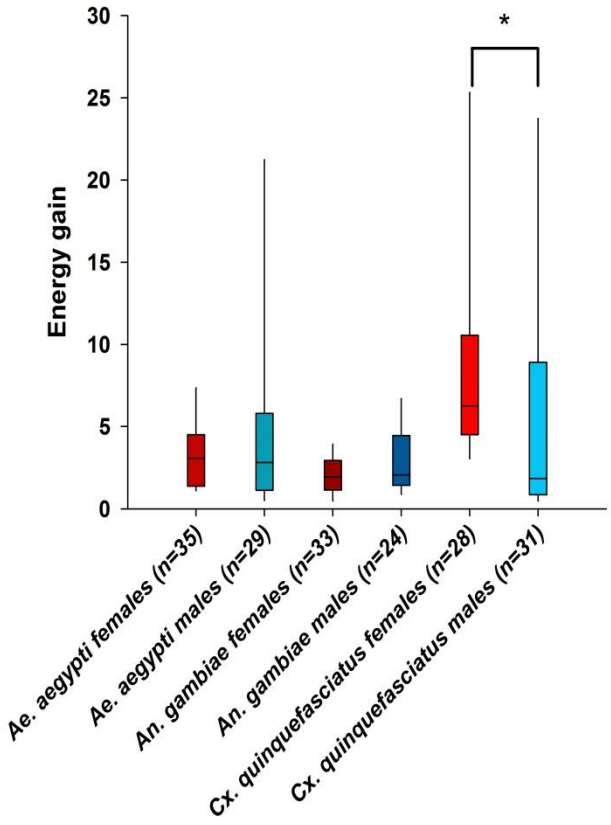


Figure 32. Energy gain estimates calculated for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (sample sizes given in brackets, significant differences between conspecific male and female mosquitoes are starred).

7.3.2. Mosquito free fluctuations - effective stiffness

All male mosquitoes have a higher best frequency (as shown in table 10 above) and effective stiffness than females of the same species in the active state after ringer injection, with the effective stiffness of all mosquito species and sexes significantly increasing whilst in the sedated state (as demonstrated in table 13).

Table 13. Median values of effective stiffness for male and female mosquitoes from each species investigated comparing post-ringer and sedated states (numbers in brackets are standard errors) as well as P-values for statistical comparisons between the two states.

Mosquito species/ sex	Effective stiffness after ringer (µN/m)	Effective stiffness (sedate state) (µN/m)	P-value
Ae. aegypti females (n = 36/ 35/ 36)	13.8 (0.84)	74.0 (7.34)	P <0.001
Ae. aegypti males (n = 25/ 30/ 25)	54.6 (5.05)	148.1 (24.4)	P <0.001
An. gambiae females (n = 29/ 33/ 26)	22.6 (17.8)	157.0 (21.2)	P <0.001
An. gambiae males (n = 20/ 22/ 18)	85.6 (8.51)	162.5 (12.4)	P <0.001
Cx. quinquefasciatus females (n = 29/ 29/ 27)	3.84 (0.5)	58.7 (2.35)	P <0.001
Cx. quinquefasciatus males (n = 28/ 33/ 35)	18.3 (6.06)	107.9 (21.6)	P <0.001

7.3.3. Mosquito apparent antennal mass estimations

Mean values of the mosquito apparent antennal mass are given in table 14 below: male and female *An. gambiae* and *Cx. quinquefasciatus* were found to have significantly different apparent masses ($P < 0.05$ for *An. gambiae* and $P < 0.01$ for *Cx. quinquefasciatus*), whilst no such significant differences were seen between male and female *Ae. aegypti* ($P > 0.05$).

Table 14. Mean values for mosquito apparent antennal mass estimates (numbers in brackets refer to standard errors). Significant differences between conspecific males and females are starred.

Species/ sex	Sample size (before pymetrozine)	Sample size (after pymetrozine)	Mean apparent antennal mass (kg)
Ae. aegypti female	35	21	4.054×10^{-11} (2.592×10^{-12})
Ae. aegypti male	30	15	4.393×10^{-11} (2.995×10^{-12})
**An. gambiae* female	33	17	4.535×10^{-11} (2.638×10^{-12})
**An. gambiae* male	22	9	5.414×10^{-11} (3.460×10^{-12})
**Cx. quinquefasciatus* female	29	21	3.232×10^{-11} (1.435×10^{-12})
**Cx. quinquefasciatus* male	33	21	3.918×10^{-11} (2.201×10^{-12})

7.3.4. Step recordings

7.3.4.1. Mechanical and nerve responses to force steps

The response of both male and female mosquitoes to force step stimulation is similar to that seen for *Drosophila melanogaster* both in section 6.2.8 and in previous reports (285). This includes initial overshooting of the flagellum in response to the stimulus onset, negative recoil following this overshoot (with further flagellar overshoots and recoils taking place after this, particularly if small force stimuli are used as in figure 33 part B) and adaptation of the system to the stimulus such that a steady state displacement is reached. This response to stimulation, especially with regards to the extent of adaptation noted, is a key mechanical signature of the direct gating of transducer channels (and has been noted as such for *Drosophila melanogaster* and vertebrate hair cells for example) (285, 469-471).

Nerve responses to the largest force steps are also produced with a delay on the order of a millisecond (as seen in figures 33 and 34). The recoil action that occurs after stimulus onset for mosquito species takes longer to reach equilibrium than for *Drosophila melanogaster* however, particularly with regards to female mosquitoes, and which is therefore potentially indicative of an overall change in flagellar stiffness.

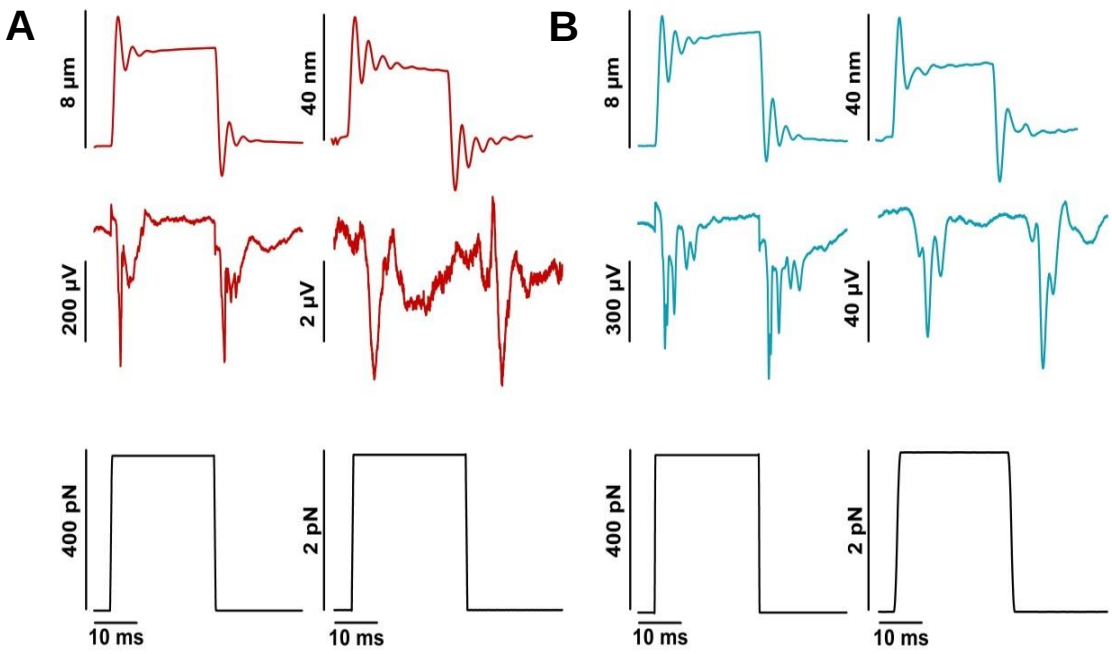


Figure 33. Flagellar displacement (top) and CAP amplitude (middle) in response to the corresponding force steps (bottom) for A) Ae. aegypti females (in red) and B) Ae. aegypti males (in blue).

In both mosquito sexes there is a reproducible delay between receiver displacement and nerve response of approximately 800-900 μs for large force steps (as seen in figure 34 below). The order of magnitude for this time interval is strongly suggestive of direct gating of transducer channels; for example the equivalent time delay for *Drosophila melanogaster* has been estimated at closer to 500 μs for analogous stimuli (which is comparable given the increased length of the mosquito flagellum) and is considered to be within the acceptable time range to indicate direct gating of mechanotransducer channels (285). That the nerve response returns to the baseline established prior to stimulus onset despite the stimulus continuing is also demonstrative of the adaptation of transducer channels (285).

For small force steps, the time delay between the receiver and nerve responses increases considerably in both sexes, which is also in agreement with *Drosophila melanogaster*. The male mosquito nerve response however now takes more than 1 ms longer than the female to appear, as well as being seemingly split into a number of parts (as shown in figure 34, which could be related to potential male-specific neuron populations).

Both male and female *Ae. aegypti* share similarities with regards to maximum CAP response but it is when comparing the smallest steps that the major differences can be identified – the male nerve response is an order of magnitude greater than the female response, potentially indicating the presence of a neuronal subgroup which demonstrates an increased sensitivity to minute displacements. The male nerve response to large stimuli also seems to contain a second spike after the major component has diminished, which may be the result of neuronal firing from this same sensitive population.

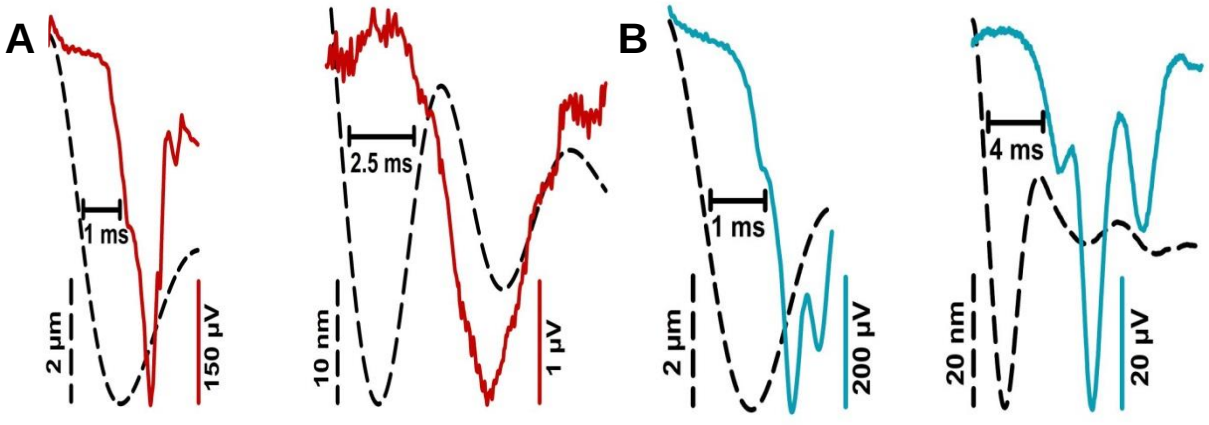


Figure 34. Flagellar displacements (dashed line) and CAP amplitudes (solid line) showing the time delay between flagellar and nerve responses for large (left) and small (right) stimuli for A) Ae. aegypti females and B) Ae. aegypti males (stimulus onset is at start of flagellar displacement and force is constant throughout).

7.3.4.2. Two state model of a single transducer population in mosquitoes

Table 15 gives median values of the parameters estimated by fitting the two state model of a single transducer population to the force-displacement data collected for each mosquito species. No significant differences were calculated for any parameter when comparing female *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes ($p>0.05$ for all tests), whilst significant differences were found between *An. gambiae* females with females from the other two species when comparing every parameter apart from the estimated number of ion channels ($p<0.01$ for all tests apart from the estimated number of ion channels, in which case $p>0.05$).

For male mosquitoes the differences between species appear less clear. In terms of the estimated number of ion channels, male *Cx. quinquefasciatus* had a significantly smaller population than estimated for either male *Ae. aegypti* or *An. gambiae* ($p<0.05$), who were not found to be significantly different ($p>0.05$). However male *Ae. aegypti* had significantly smaller estimates for the ion channel gating force, $K_{INFINITY}$ and K_{STEADY} than males from the other two species ($p<0.05$ for all tests), who were statistically not significantly different from each other ($p>0.05$ for all tests).

Further than this, K_{GS} was found to be significantly larger in male *An. gambiae* than for *Ae. aegypti* and *Cx. quinquefasciatus* males ($p<0.05$), with males from these two species being statistically indistinguishable ($p>0.05$). Finally, no significant differences were calculated between males from any species in terms of the estimated extent of nonlinearity ($p>0.05$). Complete lists of all P-values are available in appendix I.

Table 15. Median values for two state model of a single transducer population parameter estimates for each species and sex, grouped by sex (values in brackets are standard errors). Starred values within a group indicate significant differences between this value and values for the same sex in the other species (all P-values are given in appendix I).

	Number of ion channels	Ion channel gating force (fN)	$K_{INFINITY}$ (μN/m)	K_{STEADY} (μN/m)	K_{GS} (μN/m)	Extent of non-linearity
Ae. aegypti female (n = 21)	943.9 (178.1)	16 (2.9)	74 (1.8)	64 (1.3)	9 (0.7)	0.178 (8.7×10^{-3})
An. gambiae female (n = 18)	982.1 (210.2)	*28 (1.9)	*134 (6.4)	*94 (4.0)	*38 (2.7)	*0.370 (0.018)
Cx. quinquefas-ciatus female (n = 17)	916.7 (205.0)	14 (1.4)	71 (2.7)	59 (1.7)	12 (1.2)	0.189 (0.010)
Ae. aegypti male (n = 18)	298.1 (130.6)	*27 (3.5)	*145 (8.4)	*106 (6.6)	41 (2.6)	0.097 (0.012)
An. gambiae male (n = 15)	189.6 (89.0)	41 (6.0)	181 (9.6)	135 (7.1)	*53 (3.6)	0.121 (0.024)
Cx. quinquefas-ciatus male (n = 15)	*77.2 (15.9)	58 (7.8)	176 (5.8)	145 (4.6)	41 (2.2)	0.097 (0.018)

The extent of nonlinearity in the auditory system (described in section 7.2.10) is an important measure of the nonlinearities introduced into the auditory system via transduction. That it is significantly greater in female *An. gambiae* mosquitoes (as demonstrated in figure 35) as compared to female mosquitoes from the other species investigated is in strong agreement with the aforementioned frequency shifts between active and passive states noted for females from this species, with relationships between nonlinear gating compliance and antennal best frequency having been reported previously (472).

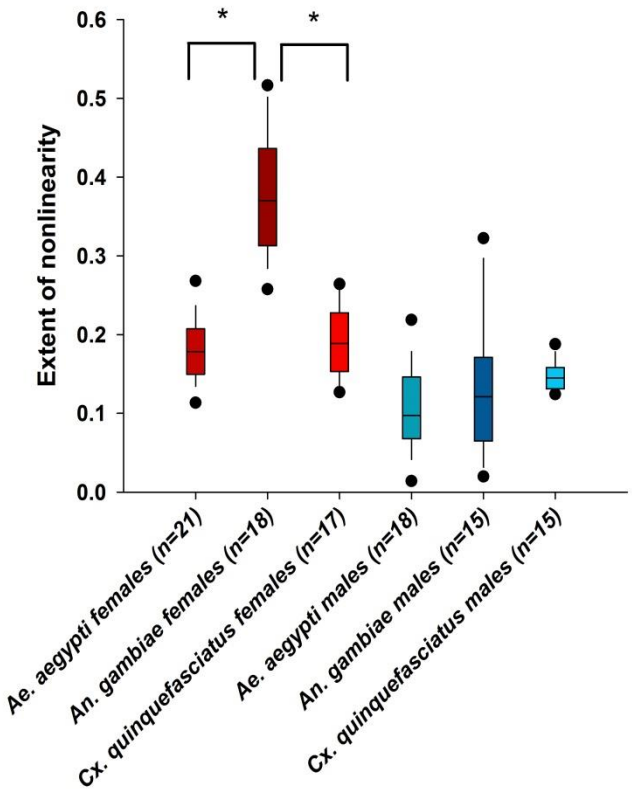


Figure 35. Comparison of estimates for the extent of nonlinearity in the auditory systems of the different mosquito species investigated (sample sizes are given in brackets, with significant differences within a sex are starred and black dots correspond to the 5th and 95th percentiles).

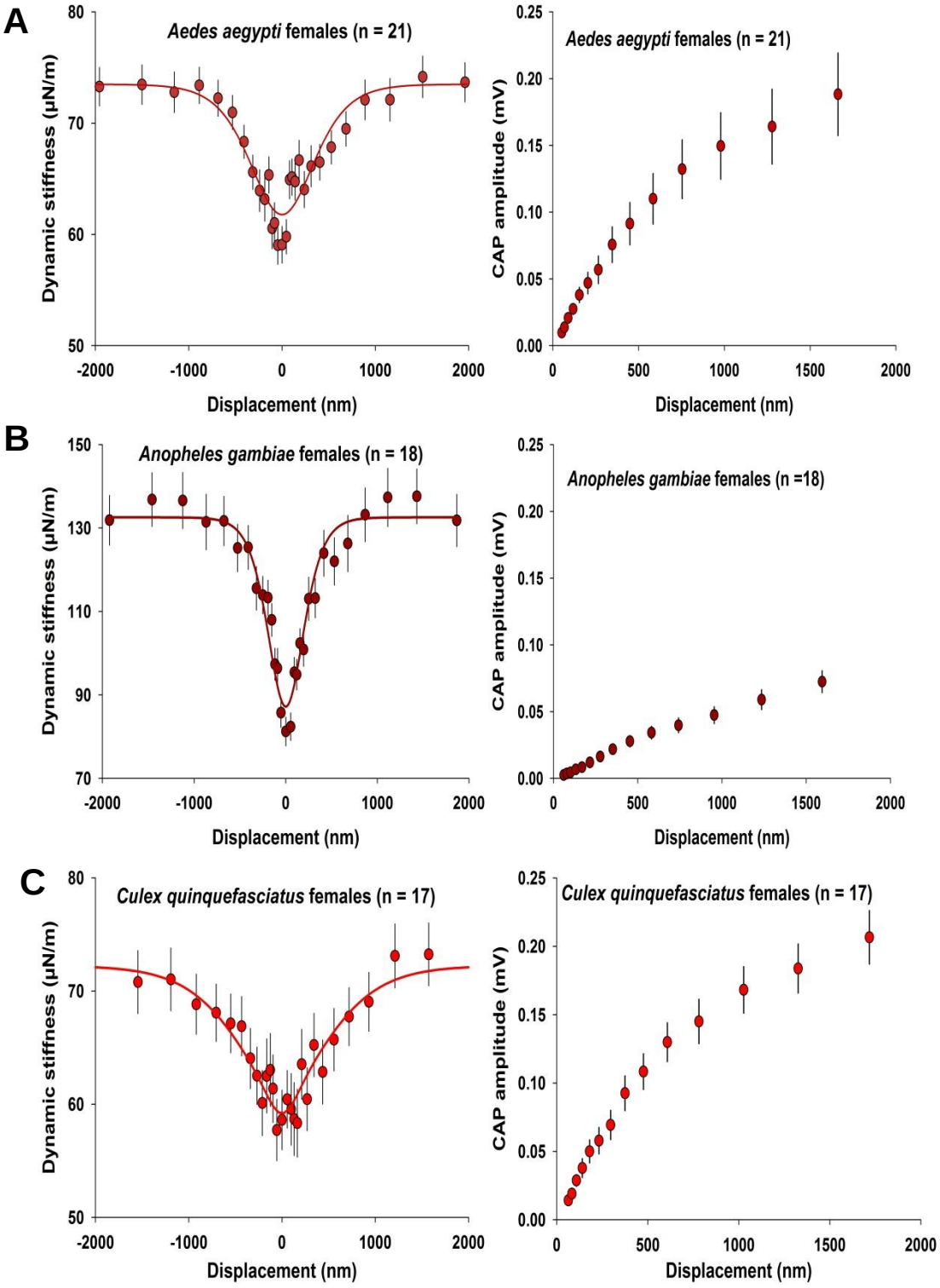
7.3.4.3. Dynamical stiffness and CAP amplitude

As is evident from figure 36, there is considerable variability between not only different sexes but also different mosquito species in terms of both the calculated changes in dynamical stiffness and the CAP responses to stimulation. Broadly however the dynamic stiffness values for all mosquito groups shown demonstrate a decrease in dynamic stiffness around the resting position and the CAP response shows a saturating increase in magnitude as displacement increases.

This increase in compliance is characteristic of mechanotransduction activity (285). In general, female mosquitoes tended to show these decreases once stimuli had become smaller than 1500nm whilst males seemed to only display such drops for very small stimuli (approximately 250-400nm). These contrasting sensitivity levels are indicative of strong differences in the auditory system between the sexes.

Sexually dimorphic responses are shown not just in the dynamical stiffness changes but also in the nerve response; for all males more than half of the total maximum CAP amplitude was produced after displacements of only a few hundred nanometres. The maximum CAP amplitudes produced by male *Ae. aegypti* and *Cx. quinquefasciatus* were slightly larger than for females (though it took much larger displacements for the females to produce similarly sized CAP amplitudes whilst the males demonstrated maximum CAP amplitude saturation much more quickly, suggestive of a shift in working ranges).

The CAP responses of *An. gambiae* males were not only much greater than those produced by males from the other two species but also stood in sharp contrast to the low CAP amplitudes recorded for the females of that species – these CAP amplitude findings proved highly reproducible and are unlikely to be the result of sub-optimal recording electrode insertions.


A
Aedes aegypti females (n = 21)
Dynamic stiffness (μN/m)
Displacement (nm)
Aedes aegypti females (n = 21)
CAP amplitude (mV)
Displacement (nm)
B
Anopheles gambiae females (n = 18)
Dynamic stiffness (μN/m)
Displacement (nm)
Anopheles gambiae females (n =18)
CAP amplitude (mV)
Displacement (nm)
C
Culex quinquefasciatus females (n = 17)
Dynamic stiffness (μN/m)
Displacement (nm)
Culex quinquefasciatus females (n = 17)
CAP amplitude (mV)
Displacement (nm)

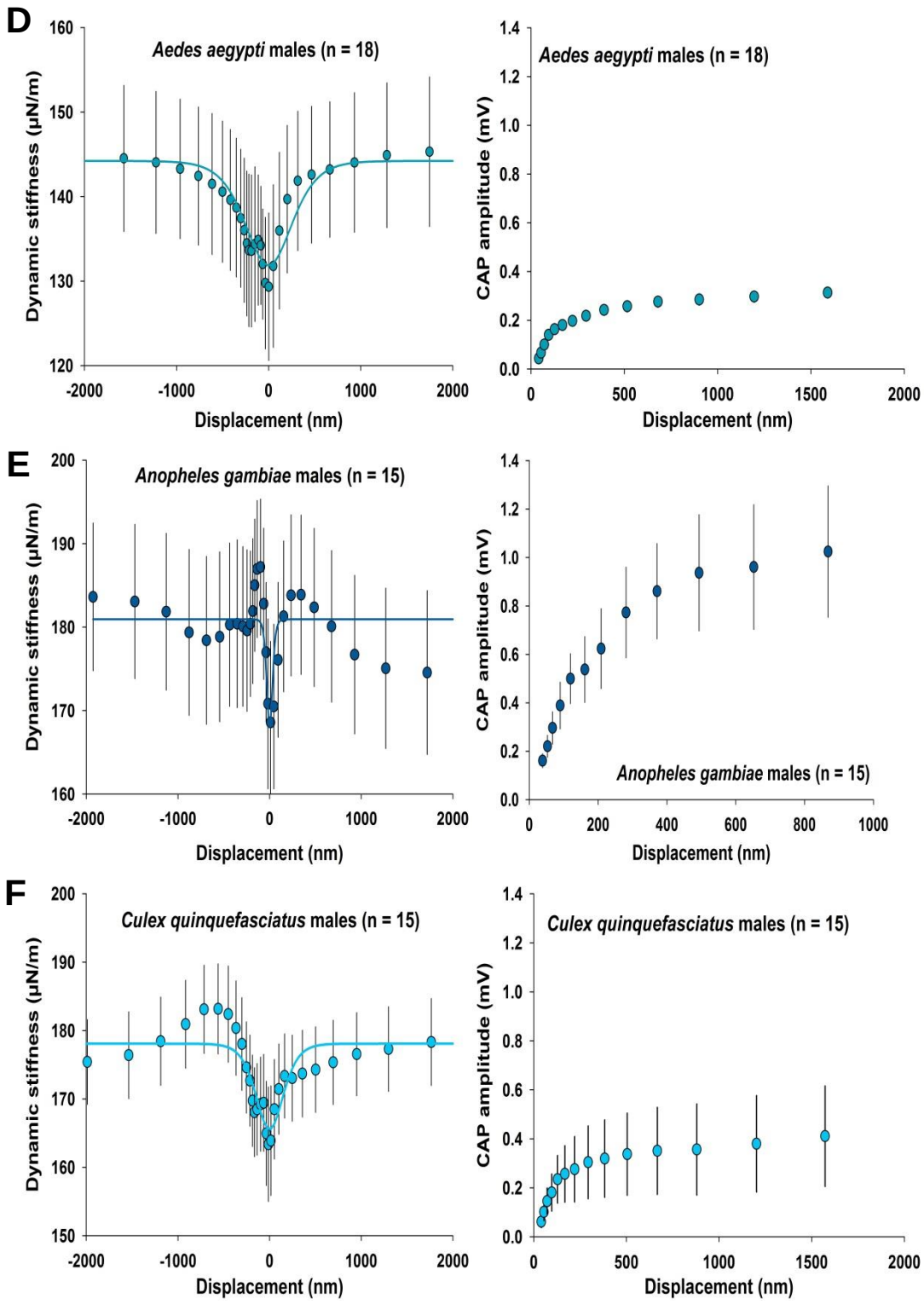


Figure 36. Changes in dynamical stiffness and CAP amplitude in response to changes in antennal displacement to a maximum displacement of ±2000nm for female and male mosquitoes from each species investigated: A) Ae. aegypti females, B) An. gambiae females, C) Cx. quinquefasciatus females, D) Ae. aegypti males, E), An. gambiae males and F) Cx. quinquefasciatus males. The bold lines represent two-state gating spring model (for a single transducer population) fits to the median data (285).

The CAP amplitude plots also display potential saturation points at which different populations of ion channels contribute towards the total CAP response produced. As the biology of the mosquito JO is not fully understood it is difficult to assess exactly at what working ranges these different populations may be working or even how many different independent transducer populations there may be.

The male *An. gambiae* for example appears to have perhaps as many as three saturation points even within 2000nm deflections, at approximately 150, 600 and 1500 nm respectively. These are potentially indicated in the dynamical stiffness plots show above as well, with changes in stiffness gradient noticeable within those displacement ranges. The presence of highly sensitive male-specific neurons, which are highly likely to be involved in locating females given the exclusive focus males place upon courtship, could explain these initial saturation points.

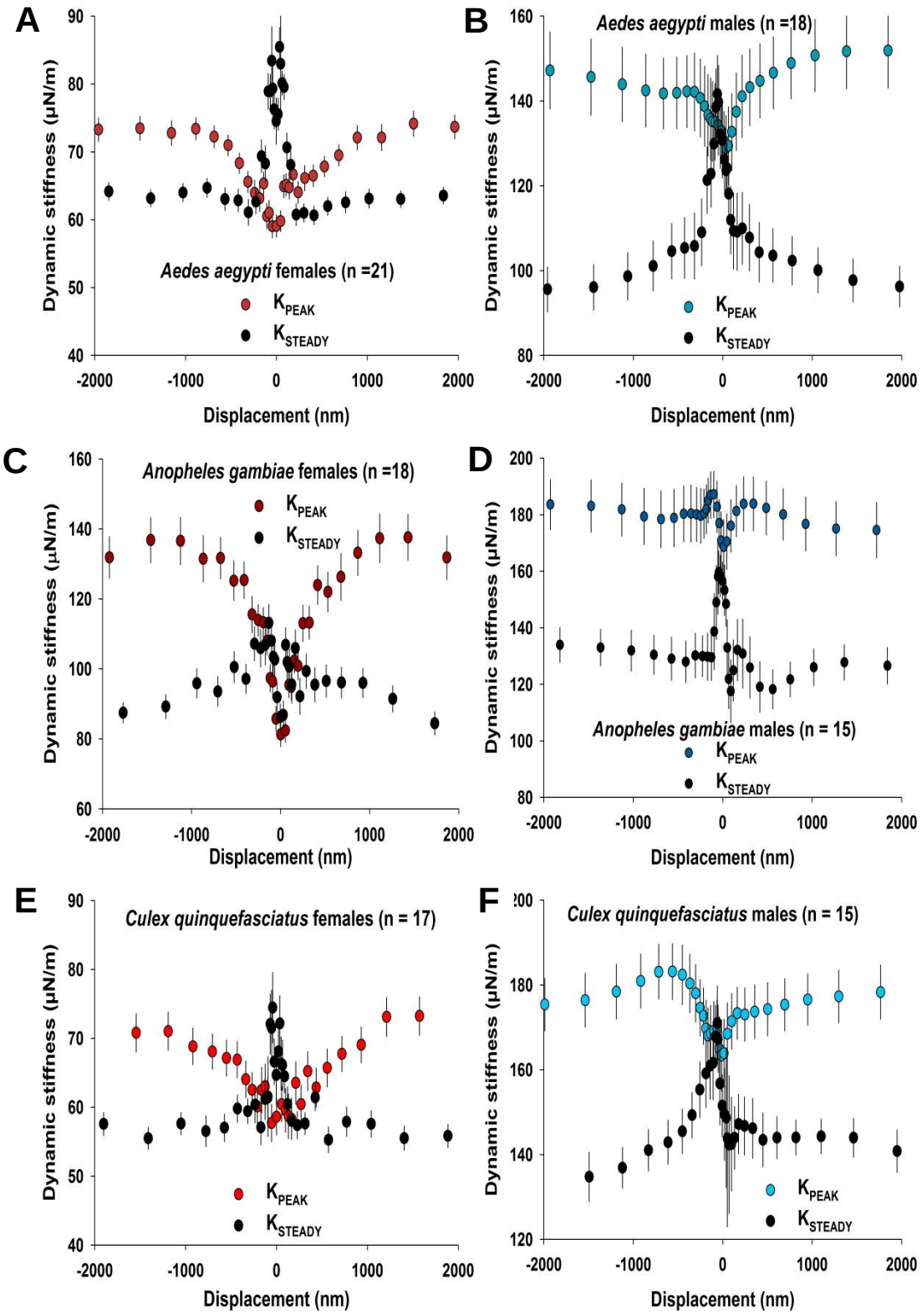
7.3.4.4. Potential mechanisms to maintain constant frequency tuning: compensatory stiffness antagonises the gating compliance?

The dynamic stiffness calculations described so far have all used stiffness values calculated from the peak displacement of the flagellum (X_{PEAK}) after the force stimulus onset (referred to in section 6.2.8 as K_{PEAK}). Using this method, an increase in compliance is seen around the resting position. This is not the only stiffness present in the system however, with the steady state stiffness (calculated using X_{STEADY} values and referred to in section 6.2.8 as K_{STEADY}) also potentially playing a role in stiffness changes.

K_{STEADY} was originally assumed to be constant across all displacement magnitudes as X_{STEADY} scales linearly with force. The dynamical stiffness plots in figure 37 however show the full extent of the changes seen in K_{STEADY} across the whole displacement range for both mosquito species and *Drosophila melanogaster*.

For *Drosophila melanogaster*, calculating dynamical stiffness using values from either the peak or steady displacement data leads to similarly shaped plots – the increase in compliance around the resting position appears far less pronounced but still visible over the same range of displacements that produce the characteristic inner part of the nonlinearity predicted by the two state gating spring model. This also seems to be the case for female *An. gambiae*, with both K_{PEAK} and K_{STEADY} matching closely at displacements smaller than ±500nm. This continues to support the previous evidence (including nonlinearity estimates, two state model fitting and CAP amplitude saturation points) that these females share remarkable similarities with *Drosophila melanogaster* in terms of their antennal responses to displacement force steps.

For all other groups of mosquitoes investigated however the K_{STEADY} values show increases in dynamic stiffness as displacement size decreases. *Cx. quinquefasciatus* and *An. gambiae* males display this behaviour to some extent but for *Cx. quinquefasciatus* females and both *Ae. aegypti* sexes these increases are much more evident, with a high degree of anti-symmetry between the K_{PEAK} and K_{STEADY} values.


A
Dynamic stiffness (μN/m)
Aedes aegypti females (n =21)
K PEAK
K STEADY
Displacement (nm)
B
Aedes aegypti males (n =18)
C
Anopheles gambiae females (n =18)
D
Anopheles gambiae males (n = 15)
E
Culex quinquefasciatus females (n = 17)
F
Culex quinquefasciatus males (n = 15)

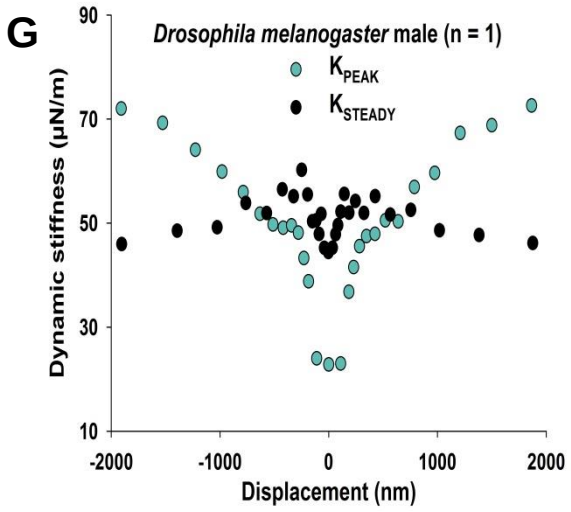


Figure 37. Dynamical stiffness changes in response to changes to displacement calculated using either values calculated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}) for A) Ae. aegypti females, B) Ae. aegypti males, C) An. gambiae females, D) An. gambiae males, E) Cx. quinquefasciatus females, F) Cx. quinquefasciatus males and G) a Drosophila melanogaster male (CantonS strain).

These increases in dynamic stiffness could be reflective of a compensatory mechanism within the auditory system that prevents the system from becoming too compliant and attempts to maintain the best frequency of the active and passive systems within the very narrow frequency ranges that are necessary for successful mosquito courtship. Both of these suggestions imply some other kind of mechanism, possibly active, that somehow provides stiffness as the flagellum becomes compliant.

If the system is indeed active then it should be physiologically vulnerable to CO_2 sedation, which removes the ability to breakdown ATP, and so should change during sedation – these changes are shown in figure 38 below for an *Ae. aegypti* female and for a male *Ae. aegypti* mosquito, as well as male and female *Cx. quinquefasciatus* in appendix J.

Whilst the mosquito is sedated, auditory CAP responses are completely abolished - this is unsurprising given the removal of ATP breakdown from the system. The sharp increase in compliance seen in K_{PEAK} in the active state for displacements less than ±500nm disappears during sedation, meaning the characteristic nonlinearity that was clearly present in the mosquito shown in figure 38 whilst awake is no longer evident during sedation; the remaining peak and trough in the sedated state could be due to the gas flow in the chamber.

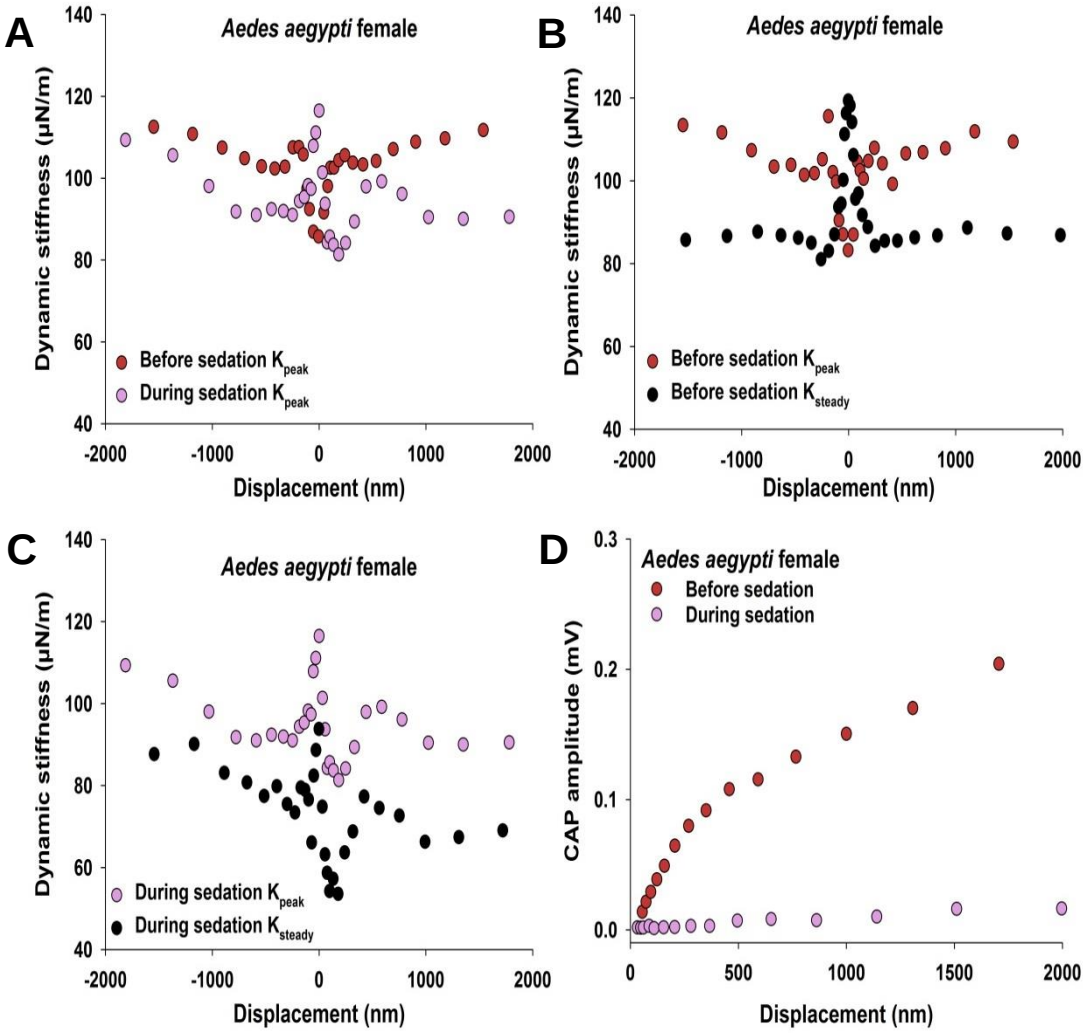


Figure 38. Changes in dynamical stiffness in response to changes to displacement, calculated for an Ae. aegypti female using either values estimated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}); A) K_{PEAK} values for both active and sedated states, B) K_{PEAK} and K_{STEADY} comparison for the active state and C) K_{PEAK} and K_{STEADY} for the sedated state.

D) Changes in CAP amplitude responses in response to changes in displacement calculated for an Ae. aegypti female in both active and sedated states.

Assuming that the overall dynamic stiffness of the antennal receiver system is composed of these two active, counteracting components (with a compensatory increase in steady state stiffness around the resting position to balance out the increase in compliance seen in K_{PEAK}), it could potentially be the case that the previously calculated dynamic stiffness plots did not represent the full extent of the gating compliance present in the mosquito auditory system.

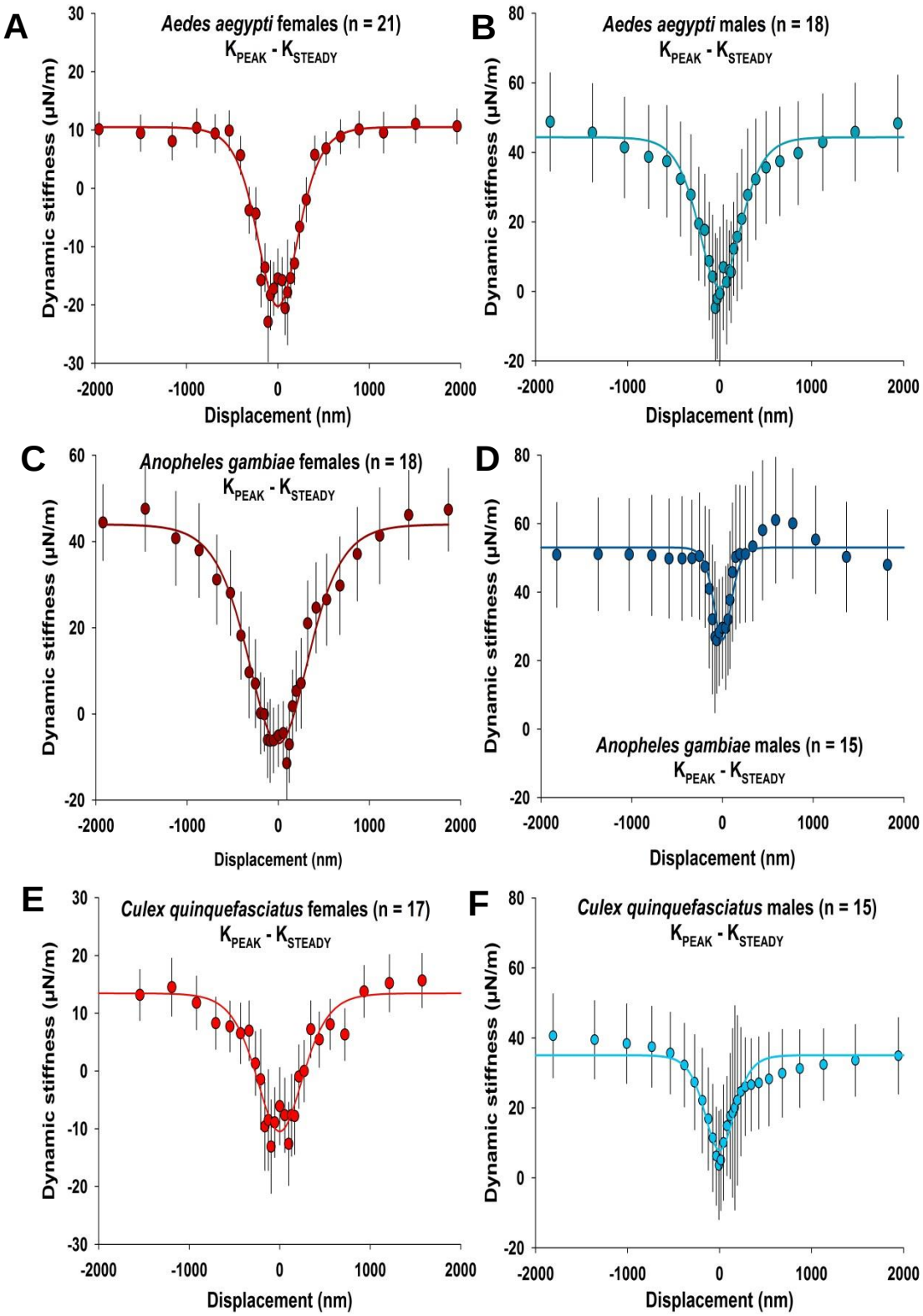
In order to calculate a more precise estimate of the entire scope of the gating compliance therefore it may be necessary to subtract the steady state stiffness from the peak stiffness (i.e. $K_{PEAK} - K_{STEADY}$), thus removing any compensatory processes from the system. Attempting this calculation for *Drosophila melanogaster* (shown below in figure 39) leads to dynamical stiffness values below 0 μN/m around the resting position, which should thus result in spontaneous oscillations of the antenna (as can also be seen in Nanchung and Inactive mutants (27)).

Applying this same principle to mosquito species reveals similar results (shown in figure 39 with a full list of parameter estimates contained in table 16); for all three mosquito species, female mosquitoes show negative dynamical stiffness values once the extra stiffness provided by K_{STEADY} has been removed from the system. Dynamic stiffness estimates calculated for male *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes around the resting position are also negative, though equivalent values for male *An. gambiae* remain significantly greater than zero across all displacement ranges investigated ($p>0.05$).

Following the subtraction of the steady state stiffness from the system, the extent of nonlinearity substantially increased across all species and sexes tested (as can be seen in table 16), indicating an increase in auditory transduction related nonlinearities in the system.

Table 16. Parameter estimates obtained fitting the two-state model of a single transducer population to male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after steady state stiffness has been subtracted from the system.

	Number of ion channels	Ion channel gating force (fN)	K_{GS} (μN/m)	Extent of nonlinearity
Ae. aegypti females (n = 21)	692.1	27	11	2.963
An. gambiae females (n = 18)	2512.6	18	44	1.170
Cx. quinquefasciatus females (n = 17)	699.2	24	13	1.800
Ae. aegypti males (n = 18)	975.2	27	44	0.987
An. gambiae males (n = 15)	111.4	63	53	0.515
Cx. quinquefasciatus males (n = 15)	346.3	35	35	0.753


A
Aedes aegypti females (n = 21)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)
B
Aedes aegypti males (n = 18)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)
C
Anopheles gambiae females (n = 18)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)
D
Anopheles gambiae males (n = 15)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)
E
Culex quinquefasciatus females (n = 17)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)
F
Culex quinquefasciatus males (n = 15)
K_PEAK - K_STEADY
Dynamic stiffness (µN/m)
Displacement (nm)

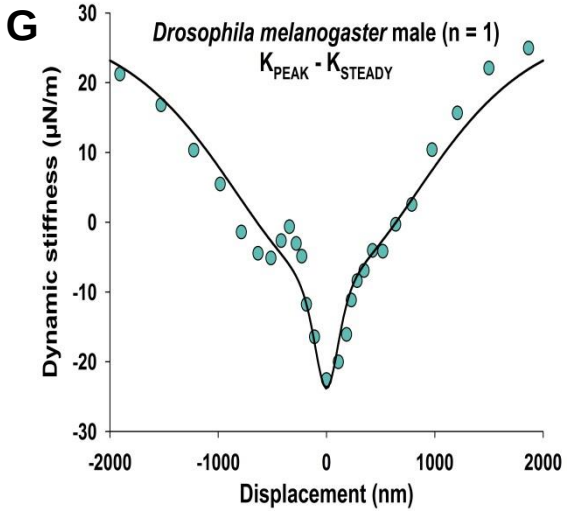


Figure 39. Changes in dynamical stiffness calculated by subtracting K_{STEADY} values from K_{PEAK} values for A) Ae. aegypti females, B) An. gambiae females, C) Cx. quinquefasciatus females, D) Ae. aegypti males, E), An. gambiae males, F) Cx. quinquefasciatus males and G) Drosophila melanogaster males (CantonS line). Solid lines represent two-state gating spring model (for a single transducer population) fits.

7.3.4.5. Effects of hypothesised multiple ion channel populations

The single population ion channel open probability fits (which have previously been reported on for use in this context (285, 394)) are produced from the assumption that at zero displacement the probability of an ion channel being open is equal to the probability of it being closed i.e. $P_O = 0.5$, and that there is a single population of ion channels producing the entirety of the CAP response – once all channels have become open then the maximum CAP amplitude should be produced.

The ion channel open probability fit included in figure 40 part A for *Drosophila melanogaster* includes a larger displacement range than in parts B or C because the second ion channel population is considered more insensitive than the first and so requires larger aristal deflections to become noticeable (394). Using these fits as an example (as the *Drosophila melanogaster* system is known to have more than one transducer population present (394)), the single channel fit displayed in figure 40 part A underestimates the minimum displacement necessary for the maximum CAP response as it assumes all channels have the same open probability and effective displacement ranges. Using a two-sigmoidal model however produces a significantly better fit, with one saturation point emerging at around 2500nm and another at around 6000nm.

Similarly, the single ion channel population fits for mosquitoes drastically reduce the complexity of the system – for the *An. gambiae* males shown in figure 40 part C for example every ion channel is predicted to open at displacements greater than 200nm. A two population model however suggests that whilst there is a population of ion channels that is completely open at this level of force stimulus, there is another group that requires a much greater relative force to open. The first saturation points for *An. gambiae* females (shown in figure 40 part B) do not emerge until flagellar deflections reach almost 1000nm when using a two population model, closely matching the dynamic stiffness changes seen earlier in figure 36.

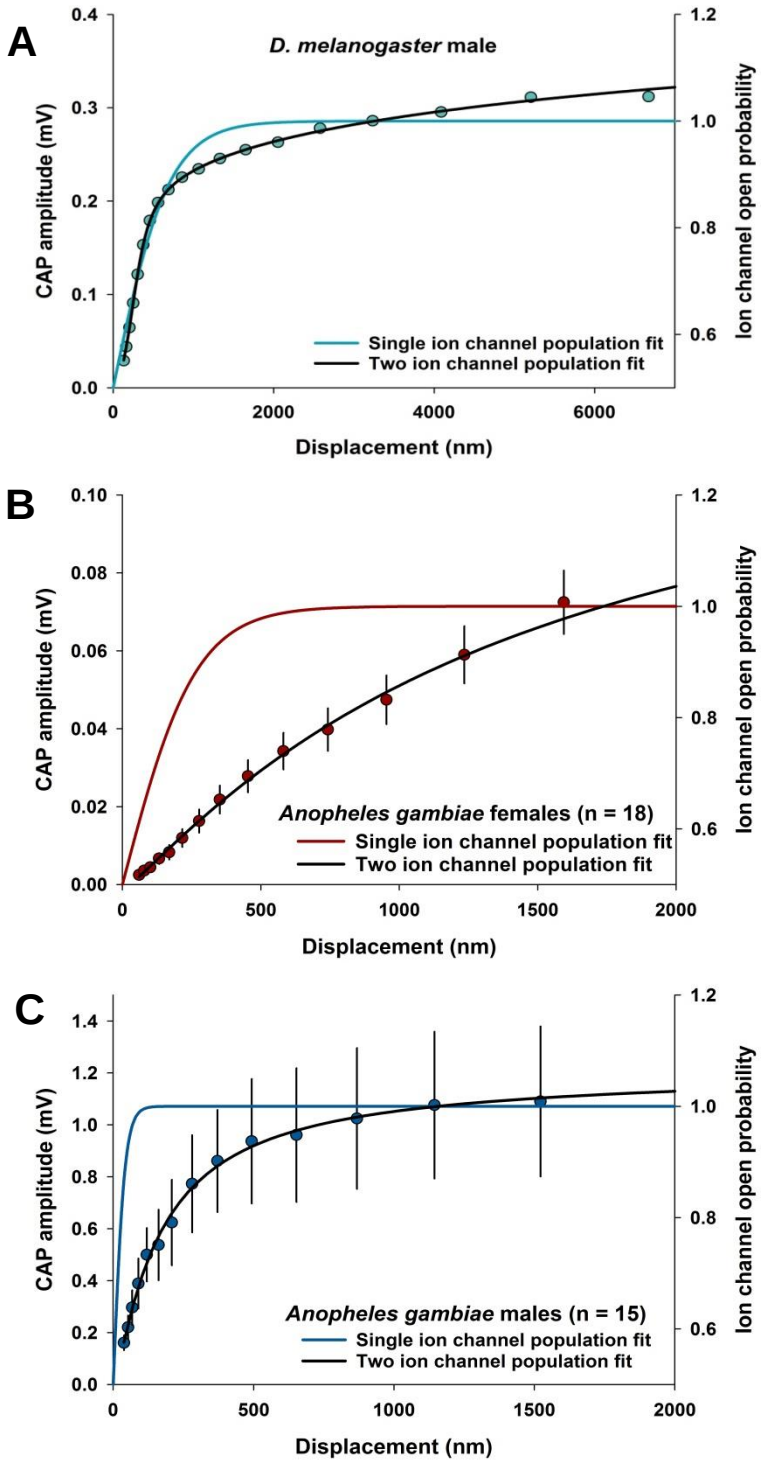


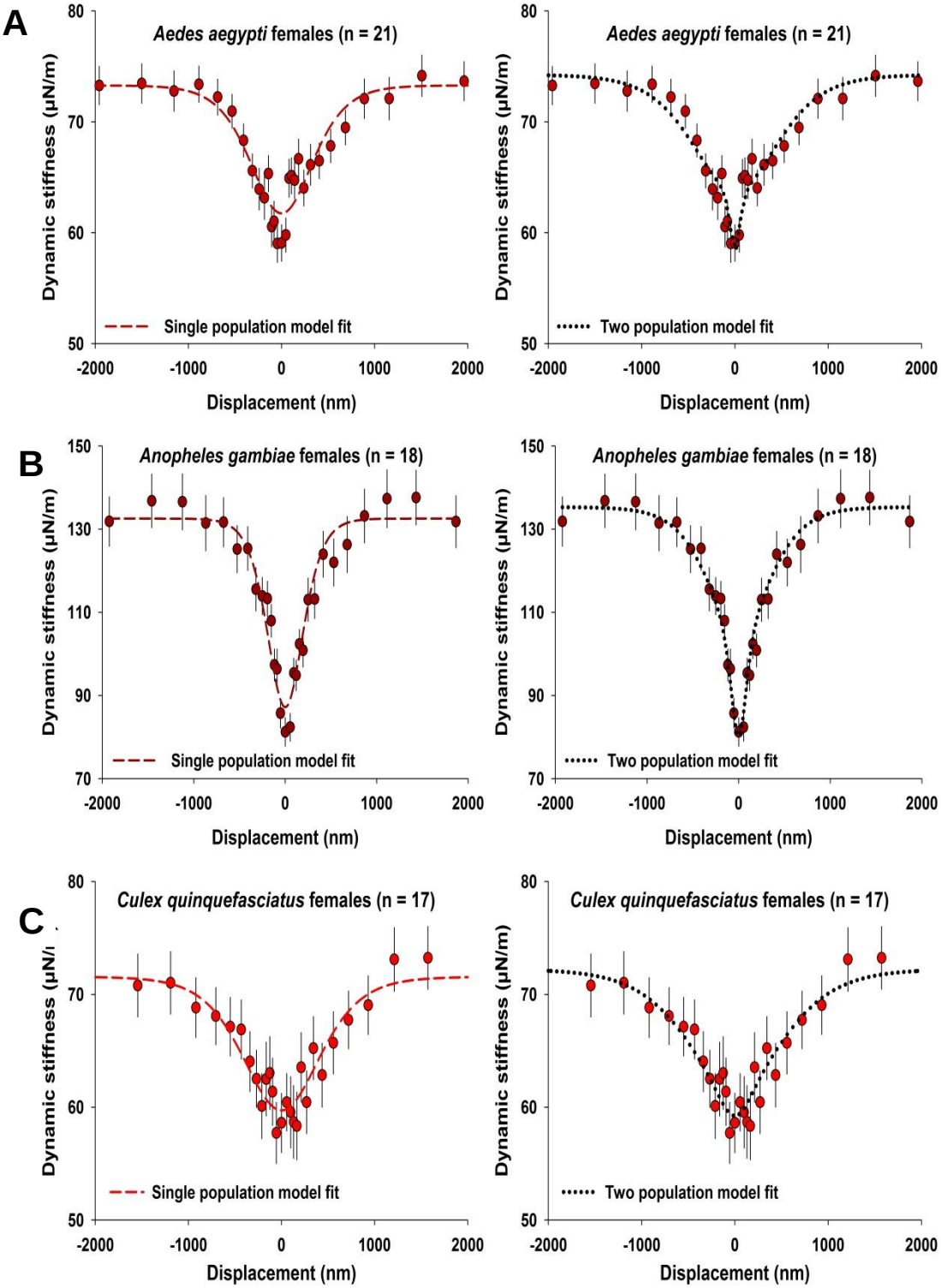
Figure 40. CAP amplitude (left axis) and ion channel open probability (right axis) as functions of displacement for A) a Drosophila melanogaster male, B) An. gambiae females and C) An. gambiae males. The points represent CAP response amplitude at the relevant force displacement (standard errors where possible are included as black lines) whilst the different fit types are labelled within each image.

There is therefore some evidence that models which account for multiple ion channel populations should be used in future analyses. Figure 41 shows the differences seen between using single population fits and two independent population fits as a starting point for increasing the model complexity – median model fit parameters are included in table 17, with all fits being calculated assuming that $X_0 = 0$.

The calculated AICc values provided in table 41 in appendix K for fits to the two models for male *Cx. quinquefasciatus* and *An. gambiae* mosquitoes suggest that increasing the complexity of the model did not significantly increase the quality of the model; for all other mosquito types however using a two state model for two independent transducer populations produced a better fit than using a single population model even after applying a cost to utilising extra parameters. Both *Ae. aegypti* and *An. gambiae* females in particular show an improved fit, with the full extent of the increase of compliance for the smallest force stimulation steps now being captured.

Table 17. Parameter estimates obtained by fitting the two-state model for two independent transducer populations to both sexes for Ae. aegypti, An. gambiae and Cx. quinquefasciatus (sample sizes are given in brackets).

	Ae. aegypti females (n = 21)	*An. gambiae* females (n = 18)	*Cx. quinq-uefasciatus* females (n = 17)	*Ae. aegypti* males (n = 18)	*An. gambiae* males (n = 15)	*Cx. quinq-uefasciatus* males (n = 15)
Number of first population ion channels	6.0	79.7	37.8	87.4	-	-
Number of second population ion channels	936.7	1504.1	1243.0	2337.7	189.6	77.2
First population gating force (fN)	122	74	36	48	-	-
Second population gating force (fN)	14	18	12	7.3	41	58
$K_{INFINITY}$ (µN/m)	74	135	72	150	181	176
K_{STEADY} (µN/m)	64	94	59	106	135	145
K_{GS} (µN/m)	10	41	13	44	53	41


A
Aedes aegypti females (n = 21)
Dynamic stiffness (μN/m)
Displacement (nm)
Single population model fit
Two population model fit
B
Anopheles gambiae females (n = 18)
C
Culex quinquefasciatus females (n = 17)

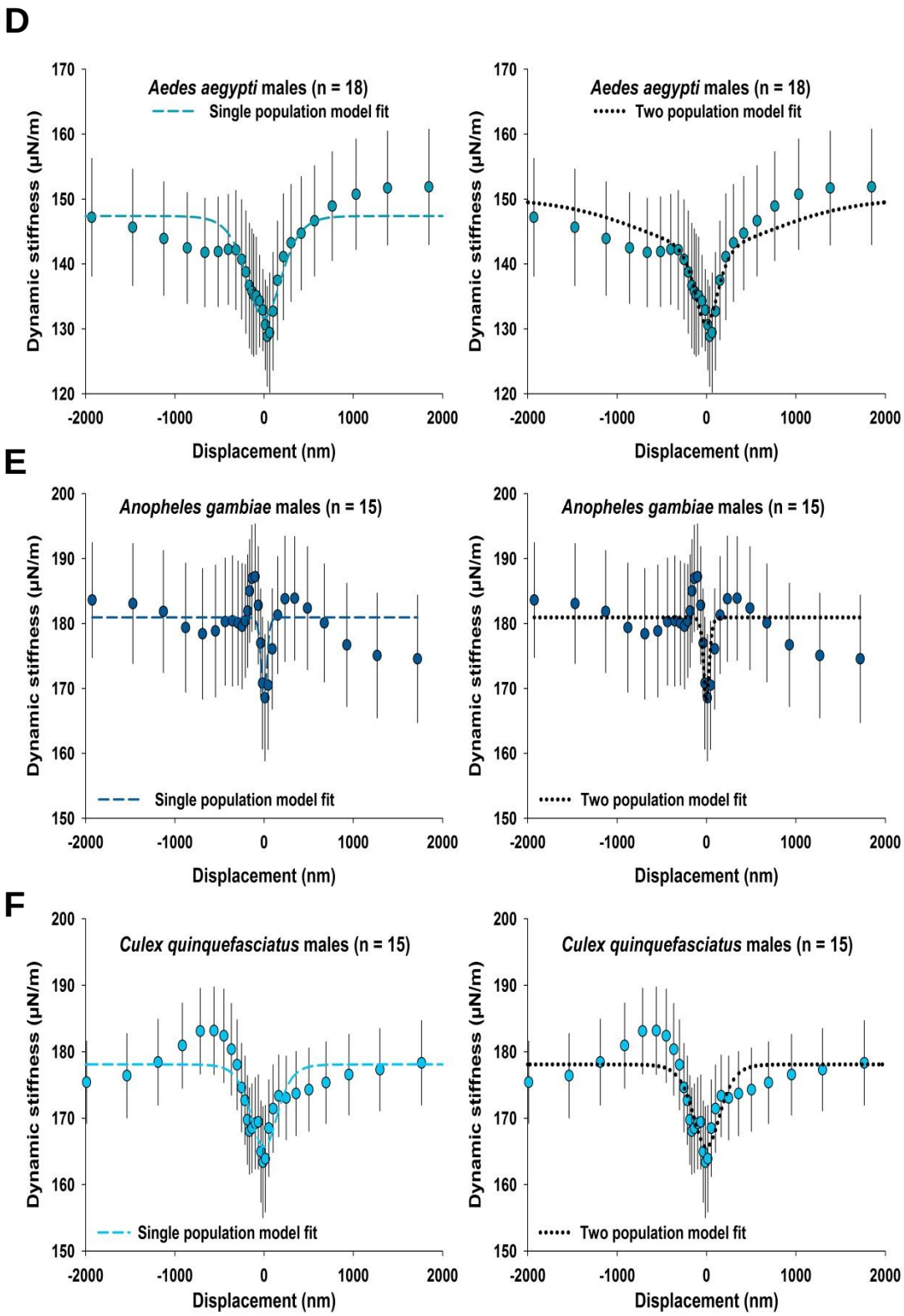


Figure 41. Changes in dynamical stiffness in response to changes in antennal displacement to a maximum displacement of ±2000nm plotted using two-state gating spring model (for either one or two independent transducer populations) fits to the median data for: A) Ae. aegypti females, B) An. gambiae females, C) Cx. quinquefasciatus females, D) Ae. aegypti males, E), An. gambiae males and F) Cx. quinquefasciatus males.

7.3.5. White noise stimulus displacement gain experiments

Decreased attenuation (i.e. increased stimulus force size) led to greater antennal velocities but decreased sensitivity for all mosquitoes investigated (demonstrated in figure 42 parts A and B). Interestingly however different species and sexes showed distinct changes in the best frequency of their flagella as the stimulus intensity increased (shown in figure 42 parts C and D) – males showed a clear decrease whilst females either showed an increase (in the case of *An. gambiae*) or demonstrated a small (~5%) decrease followed by a suitably compensatory increase. The shifts in best frequency did not occur until a threshold stimulus intensity was reached.

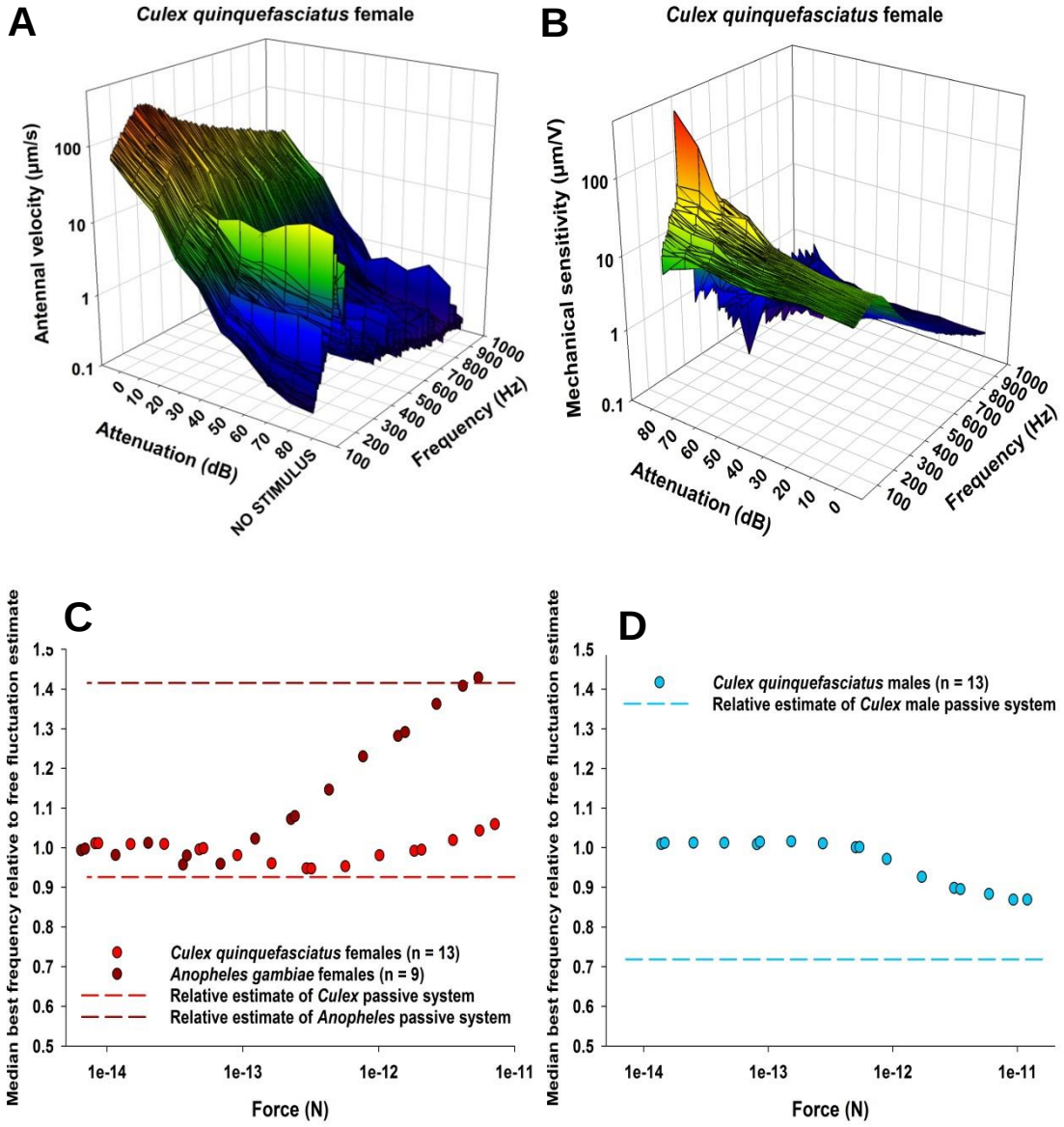


Figure 42. A) Changes in antennal velocity magnitude seen in response to changes in the relative stimulus force attenuation for an individual Cx. quinquefasciatus female (shown here in dB).

B) Corresponding changes in sensitivity at each attenuation for the same female (N.B. attenuation axis is reversed here compared to A)).

C) Changes in the median best frequency of the flagellum relative to the unstimulated best frequency as the stimulus force increases for An. gambiae and Cx. quinquefasciatus females (dashed lines represent median best frequency of the passive systems).

D) Changes in the median best frequency of the flagellum relative to the unstimulated best frequency as the stimulus force increases for male Cx. quinquefasciatus mosquitoes (dashed lines represent median best frequency of the passive systems).

Similar to the results of the absolute energy gain calculations shown earlier, *Cx. quinquefasciatus* males demonstrate a significantly smaller displacement gain in response to white noise stimulation than females (with displacement gain in this sense being defined in section 7.2.11). Now however this difference has also been extended to both *Ae. aegypti* and *An. gambiae* males when compared to females of their own species – table 18 contains median values of estimated white noise displacement gains as well as significance values for all statistical comparisons made, whilst figure 43 shows the range of calculated displacement gains for each mosquito species and sex.

Table 18. Median displacement gains estimated using white noise stimulation for all mosquito species and sexes investigated (standard errors are shown in brackets). Significance levels between different sexes within a single species are shown next to the sex that was estimated as having the significantly greater level of energy injection.

Species/ sex	Median displacement gain (white noise)	Significance level
Ae. aegypti female (n= 7)	2.28 (0.58)	P = 0.038
Ae. aegypti male (n= 7)	1.51 (0.31)	-
An. gambiae female (n= 9)	2.14 (0.13)	P = 0.026
An. gambiae male (n= 7)	1.19 (0.37)	-
Cx. quinquefasciatus female (n= 13)	3.67 (0.54)	P = 0.031
Cx. quinquefasciatus male (n= 13)	2.22 (0.80)	-

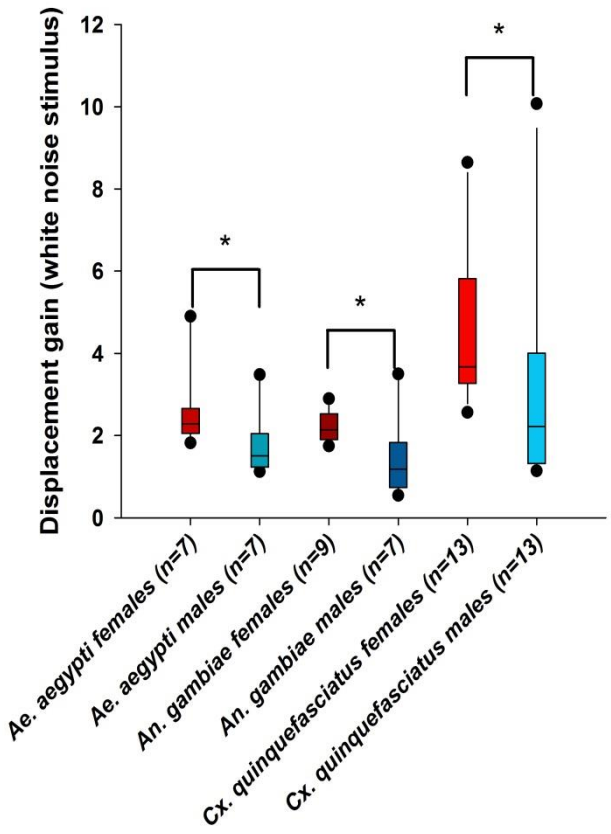


Figure 43. Displacement gain values estimated using white noise stimulation for each species and sex (sample sizes are given in brackets, with significant differences between male and female mosquitoes from the same species starred; black dots correspond to the 5th and 95th percentiles).

7.3.6. Pure tone stimulus displacement gain experiments

Significant differences can be seen in the flagellar and JO best frequency estimates in table 18 between male and female mosquitoes from the same species. Significant differences can also be seen between males from different species; *Anopheles* males have significantly lower best frequency values for both peak mechanical and nerve responses than *Ae. aegypti* males ($p<0.05$ in both cases) – *Cx. quinquefasciatus* males also have a significantly lower peak nerve response best frequency than *Aedes* ($p <0.01$). The only such difference seen throughout the females was a significantly lower frequency for the peak nerve response in *Cx. quinquefasciatus* females than in *Ae. aegypti* ($p <0.01$).

The best frequency tuning of the female flagellum and the male JO has previously been noted to be equal to the two major distortion products included in table 19; the cubic distortion product and the difference tone respectively. Whilst the median best frequency of the male *Ae. aegypti* JO was approximately 20Hz greater than would expected from the difference tone estimation, for all other mosquito types there was close agreement between the values approximated from calculating the distortion products of WBF measurements and those that were directly found in the pure tone experimental results.

There was almost no difference between the values estimated for the two relevant distortion products in *Cx. quinquefasciatus* (197 Hz for the cubic distortion product as compared to 193Hz for the difference tone) meaning that it may not be clear for this species as to which distortion product is relevant for the exact best frequency tuning – this was not an issue in the other species however as there were far greater differences in frequency between the two distortion products.

Table 19. Median values of the best frequency of the flagellum and nerve responses to pure tone stimulation (numbers in brackets refer to standard errors). Female fundamental WBF and the best frequency estimates for the flagellum from the velocity fits are provided to act as reference values. Two of the most prominent distortion products are also shown, with the difference tone being equal to the difference between the male and female WBF and the cubic distortion product being equal to the difference between the male WBF and double the female WBF.

	Ae. aegypti female (n= 8)	*Ae. aegypti* male (n= 10)	*An. gambiae* female (n= 7)	*An. gambiae* male (n= 7)	*Cx. quinque-fasciatus* female (n= 8)	*Cx. quinque-fasciatus* male (n= 8)
Median flagellum best frequency (from pure tone stimulation)/ Hz	195.0 (5.49)	360.0 (12.31)	205.0 (7.51)	325.0 (4.36)	205.0 (6.66)	335.0 (7.43)
Flagellar free fluctuations best frequency / Hz	203.1 (2.22)	384.4 (20.5)	219.7 (3.55)	332.0 (5.81)	213.0 (2.41)	311.2 (5.52)
Cubic distortion product / Hz	185.5	-	210.0	-	197.0	-
Female fundamental WBF/ Hz	405.0 (22.0)	-	384.0 (16.7)	-	390.0 (10.5)	-
Median best frequency of nerve response/ Hz	175.0 (6.66)	245.0 (10.62)	155.0 (3.69)	185.0 (9.69)	125.0 (7.43)	185.0 (5.90)
Difference tone/ Hz	-	219.5	-	174.0	-	193.0

Figure 44 provides a representation of the best frequency tuning of the flagellum and the JO in the three mosquito species studied with figure 45 allowing for species specific comparisons of flagellar sensitivity and maximum nerve response between conspecific males and females. One of the most notable sex specific differences shared between all species is that the magnitude of the male nerve response was significantly greater than the magnitude of the female nerve response in all three species, especially when considering *An. gambiae*.

Female *Ae. aegypti* and *Cx. quinquefasciatus* however demonstrated much greater maximum flagellar sensitivity estimates than males from these two species, which agreed with the earlier presented results in figures 40 and 44 with regards to the energy gain and the displacement gain estimated using white noise stimulation. In contrast to this however the flagellar sensitivity of *An. gambiae* males was much greater than that of females when a pure tone stimulus was utilised.

From both the nerve and mechanical data, *Ae. aegypti* and *Cx. quinquefasciatus* males appear very similar after accounting for some frequency shifts. Both the maximum nerve response and peak mechanical gain values are similar across species, with this also being true for the females from these two species.

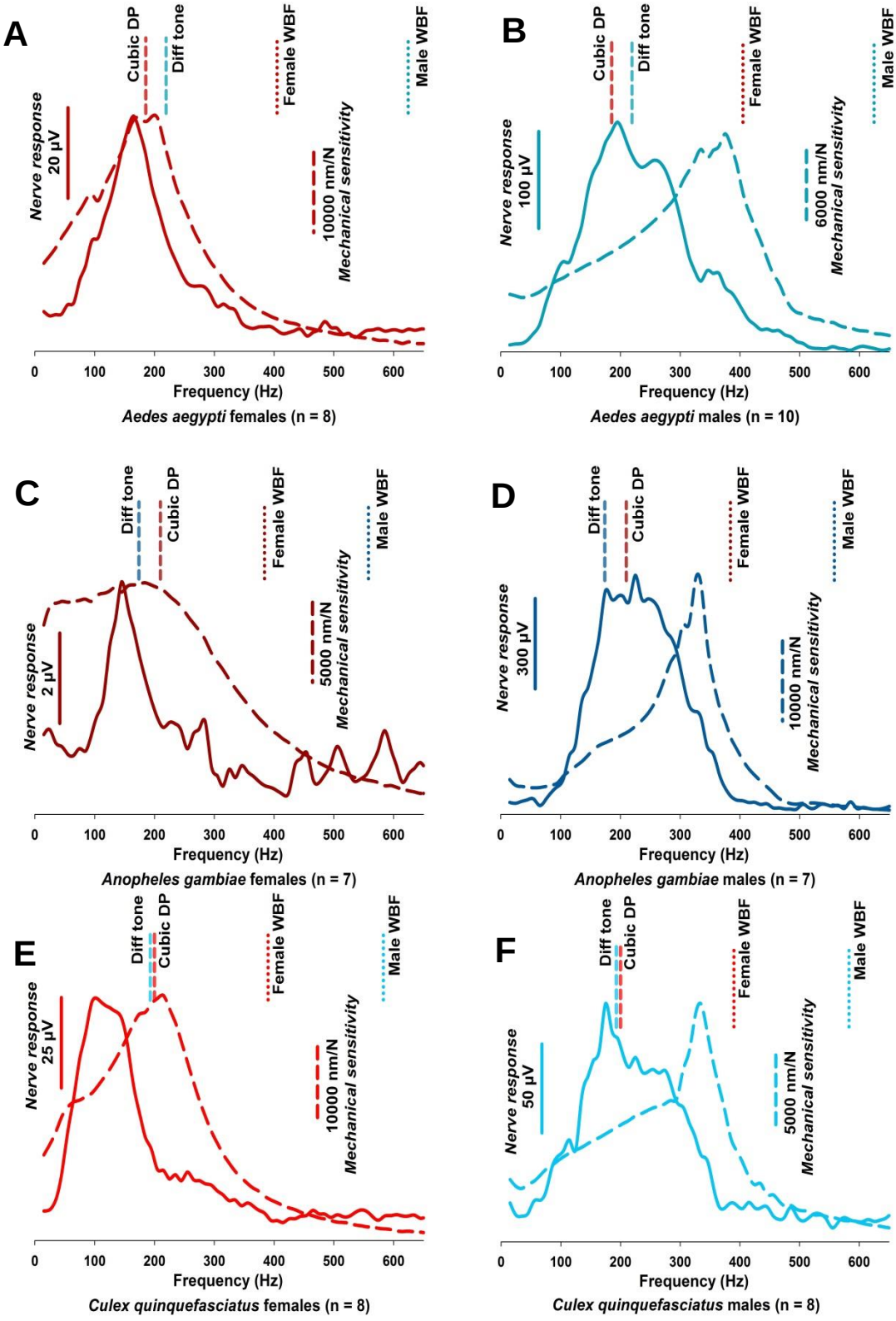


Figure 44. Sensitivity (dashed line) and nerve response (solid line) changes in response to changes in stimulus frequency for A) Ae. aegypti females, B) Ae. aegypti males, C) An. gambiae females, D) An. gambiae males, E) Cx. quinquefasciatus females and F) Cx. quinquefasciatus males. Fundamental WBFs are indicated as well as the difference tone and cubic distortion product (labelled 'diff tone' and 'cubic DF').

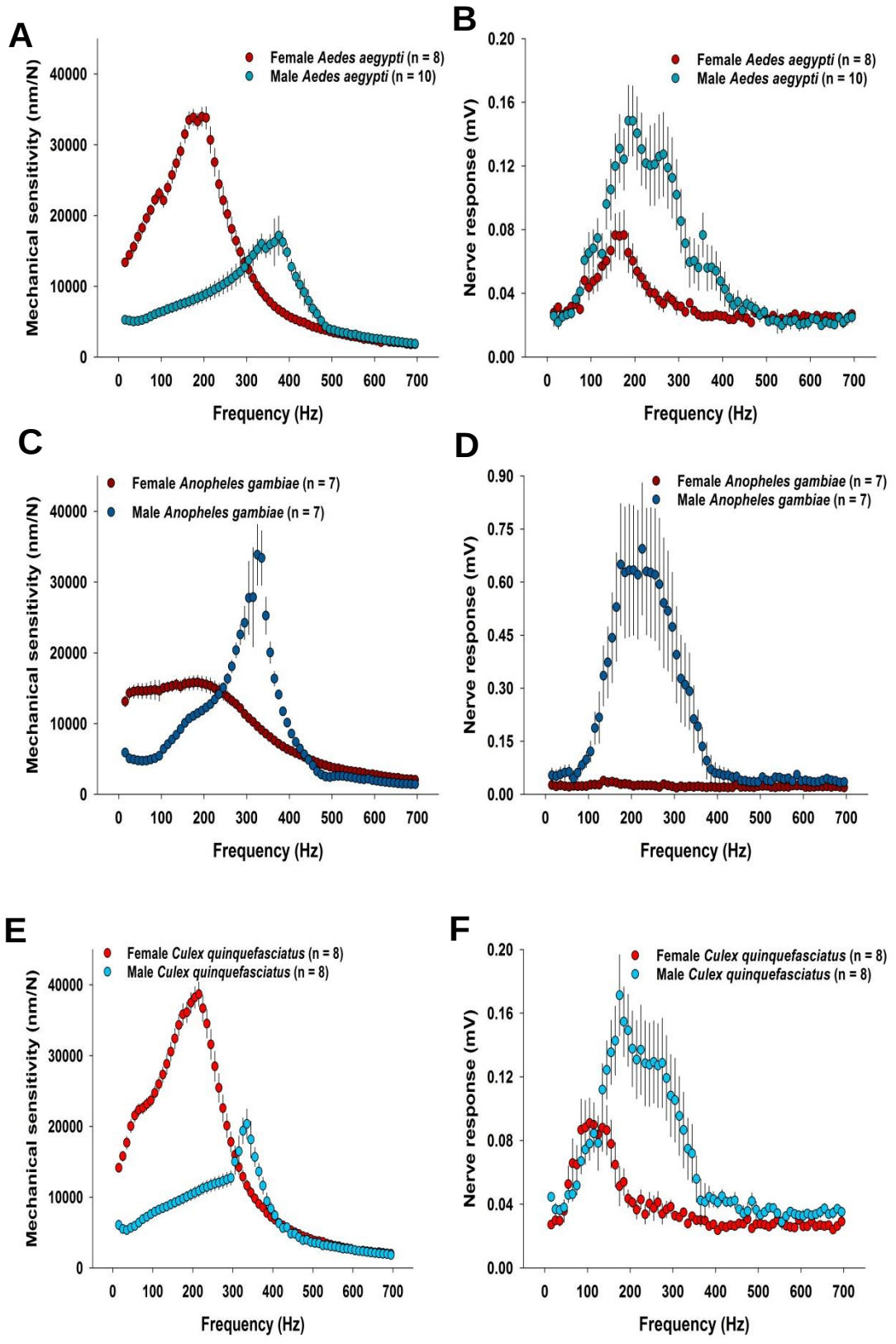


Figure 45. Sensitivity (left) and nerve response (right) in response to pure tone stimulation at different frequencies for male and female A) Ae. aegypti B) An. gambiae and C) Cx. quinquefasciatus. All points represent median values of individual experimental values at that frequency and bars signify standard errors at that point.

Table 20 includes the median displacement gains for all mosquito groups tested (estimated using pure tone stimulation) and demonstrates the sex-specific significant differences seen across all three species. *Cx. quinquefasciatus* females demonstrate significantly larger displacement gain values than males, with *Ae. aegypti* females also having significantly greater displacement gain values than males from that species (with all P-values included in table 20). Male *An. gambiae* however have significantly greater displacement gains than conspecific females. Figure 46 illustrates the range of median displacement gains for each mosquito species and sex investigated.

Table 20. Median displacement gains estimated using pure tone stimulation for all male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (standard errors are shown in brackets) as well as P-values for comparisons between male and female mosquitoes from the same species

Species/ sex	Median displacement gain (Pure tone)	P-value
Ae. aegypti female (n= 8)	14.75 (0.55)	P = 0.00009
Ae. aegypti male (n= 10)	8.24 (0.79)	
An. gambiae female (n= 7)	6.76 (0.33)	
An. gambiae male (n= 7)	11.92 (0.64)	P = 0.00003
Cx. quinquefasciatus female (n= 8)	16.52 (0.89)	P = 0.00215
Cx. quinquefasciatus male (n= 8)	10.08 (1.06)	

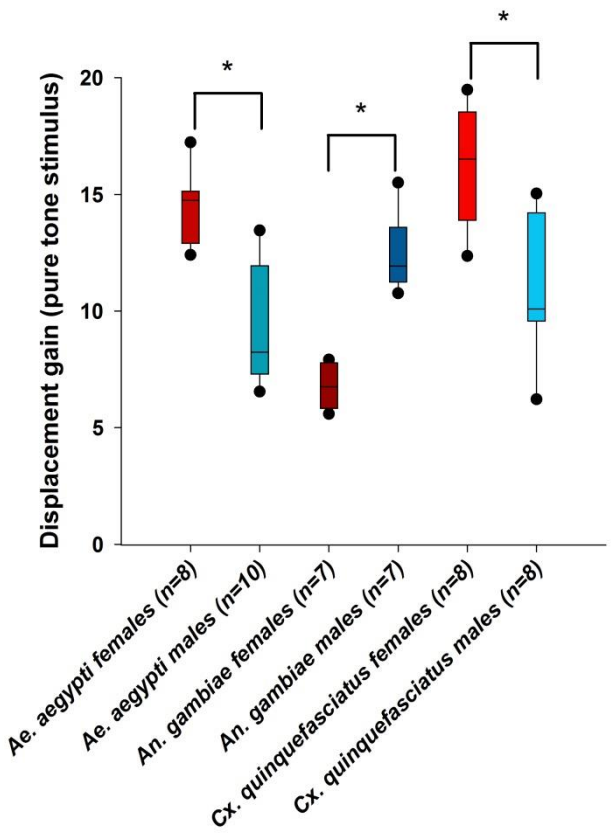


Figure 46. Displacement gain values estimated using pure tone stimulation for each mosquito species and sex (significant differences between males and females from the same species are starred, with black dots corresponding to the 5th and 95th percentiles).

7.3.7. TTX and TeNT injection series

Male and female mosquitoes across all three species showed significantly different responses to injection with either TTX or TeNT: figure 47 contains examples of free fluctuations from both male and female *Cx. quinquefasciatus* mosquitoes comparing both and after TeNT injection states. Whilst TeNT injection resulted in a significant increase in energy gain for the female *Cx. quinquefasciatus* (which was not the case for female *Ae. aegypti* or *An. gambiae* mosquitoes), the extent of this gain was significantly smaller than that seen in the male following injection (which began spontaneously oscillating). Injection with TTX also produced these sexually dimorphic responses in all three species.

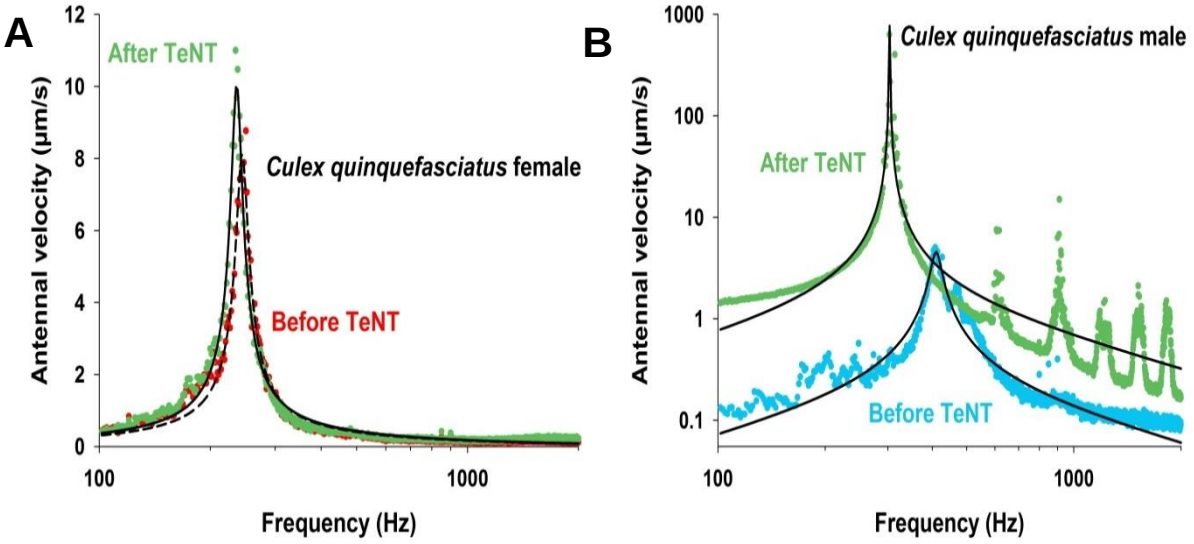


Figure 47. Free, unstimulated fluctuations both before and after TeNT injection over a frequency range between 100 to 2000Hz for A) a Cx. quinquefasciatus female (linear scale) and B) a Cx. quinquefasciatus male (logarithmic scale). Individual data points represent Fourier-transformed velocity amplitudes whilst solid black lines show the velocity amplitude function fits for each recording.

Whilst both TTX and TeNT injection into male mosquitoes resulted in spontaneous oscillations of the flagellum, the length of time necessary for these oscillations depended to some extent on the compound type. The injection time courses contained in figure 48 show the extended length of time required for TeNT and TTX to cause spontaneous oscillations in an *An. gambiae* male as compared with a *Cx. quinquefasciatus* male – both compounds had an effect in the *Cx. quinquefasciatus* male almost immediately, whilst TeNT took far longer to have an effect on the *An. gambiae* male than TTX (which was almost instantly effective).

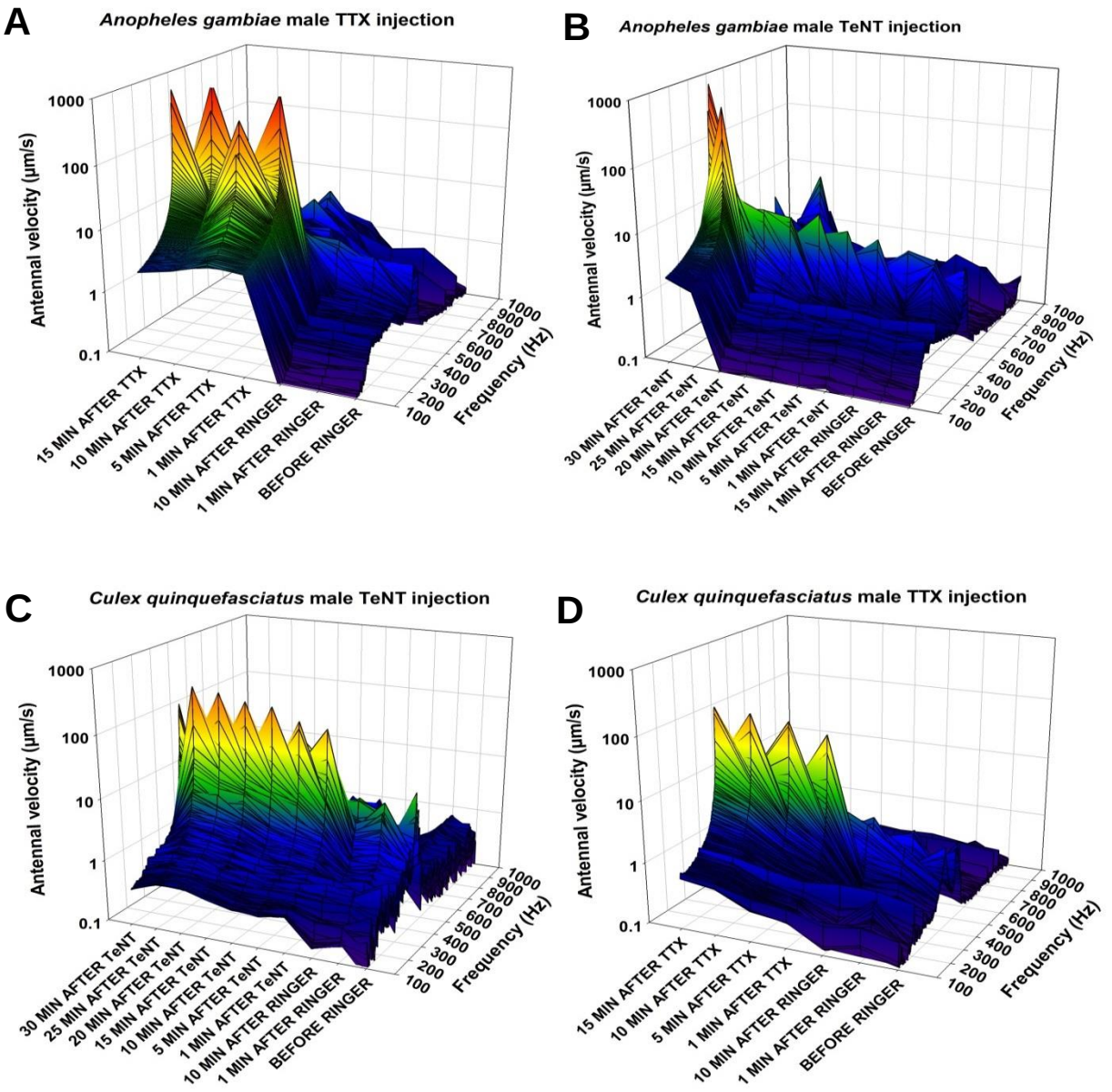


Figure 48. A and B) Time series of representative antennal velocities before and after ringer injection for An. gambiae males compared to TTX (A) or TeNT (B) injection. C and D) Time series of representative antennal velocities before and after ringer injection for Cx. quinquefasciatus males compared to TTX (C) or TeNT (D) injection.

The maximum antennal velocity reached, whilst still considerable when compared to the quiescent state, was far smaller for the *Cx. quinquefasciatus* male than for the *An. gambiae* male – flagellar parameter values obtained by fitting the velocity amplitude function to the free fluctuation data (as described in section 6.2.6) are provided in appendix H. Both mosquito species were able to maintain the level of energy injection required for such large oscillations for extended periods of time. Examples of the nerve and mechanical responses to TTX and TeNT injection in male and female mosquitoes are contained in appendix L.

Increases in energy gain for male mosquitoes (which could signify the removal of inhibitory feedback mechanisms) are shown in Figure 49 - full list of median values are included in tables 33 and 34 in appendix H. *Ae. aegypti* and *An. gambiae* female mosquitoes showed no significant change in energy gain following either TTX or TeNT injection when compared to the post ringer injection state ($p>0.05$ for all tests). The only significant change in energy gain for female mosquitoes from these two species was following pymetrozine injection, which resulted in the calculation of a significantly lower energy gain than for any other state ($p<0.001$ for all tests).

Female *Cx. quinquefasciatus* mosquitoes however were calculated to have significantly increased energy gains following injection by either TTX or TeNT ($p<0.01$ in both cases) – a significant decrease in energy gain was also calculated following pymetrozine injection ($p<0.001$ for all tests).

Simililarly, males from all three mosquito species had significantly increased energy gains following injection by either TTX or TeNT as well significantly decreased energy gains after pymetrozine injection ($p<0.01$ in all cases tested). The amount of energy gain however was much greater for all male mosquitoes regardless of species than for *Cx. quinquefasciatus* females. No female mosquito from any species was determined as having a spontaneously oscillating flagellum whilst all males that were injected with either TTX or TeNT were identified as having spontaneously oscillating flagella. Significance levels for all comparisons made are included in table 21.

Table 21. P-values for statistical comparisons between the different injection states for male and female mosquitoes from each species investigated.

	Ae. aegypti females	*An. gambiae* females	*Cx. quinq-uefasciatus* females	*Ae. aegypti* males	*An. gambiae* males	*Cx. quinq-uefasciatus* males
Before and after ringer	P = 0.065	P = 0.619	P <0.01	P = 0.011	P = 0.001	P <0.001
After ringer and after TTX	P = 0.232	P = 0.394	P <0.001	P <0.001	P <0.001	P <0.001
After ringer and after TeNT	P = 0.966	P = 0.266	P <0.001	P <0.001	P <0.001	P <0.001
After ringer and after pymetrozine	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001
After TTX and after pymetrozine	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001
After TeNT and after pymetrozine	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001

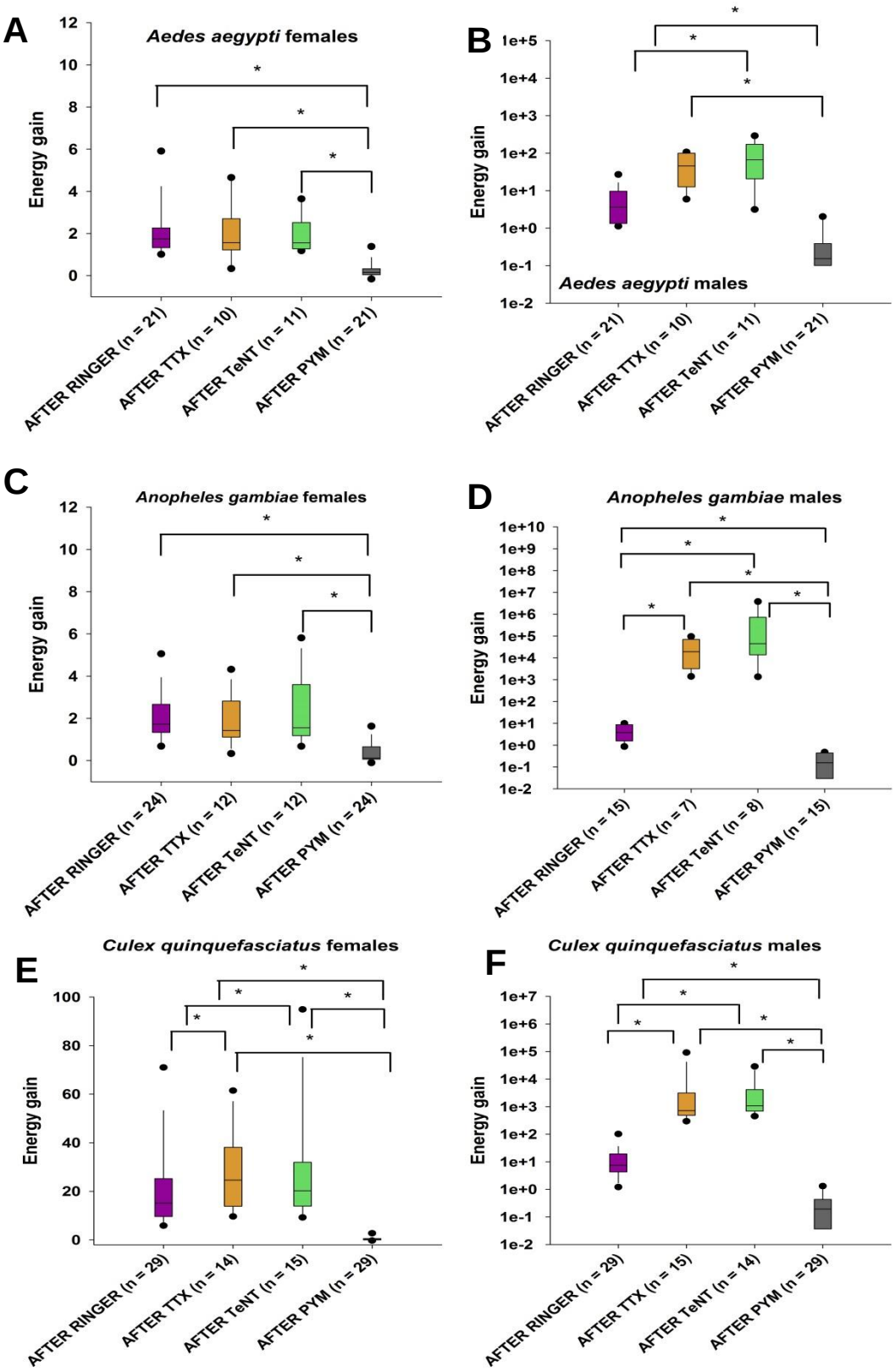


Figure 49. Energy gain values calculated for different injection states for A) Ae. aegypti females, B) Ae. aegypti males, C) An. gambiae females, D) An. gambiae males, E) Cx. quinquefasciatus females and F) Cx. quinquefasciatus males (significant differences are starred, with black dots corresponding to the 5th and 95th percentiles).

7.4. Discussion

7.4.1. Mosquito auditory systems as measured by free fluctuations

The passive antennal receiver of *Drosophila melanogaster* has a best frequency of approximately 800Hz, whilst the best frequency of the active system is between 150 – 250Hz (283). The difference in best frequency between the two states is indicative of the active mechanotransduction processes taking place within the insects' auditory system (472). These active processes are important in order for *Drosophila melanogaster* to tune their antennal receiver to courtship songs but are linked to the development of nonlinearities.

In contrast to this, one of the most notable results shown in tables 12 and 13 is that the difference between active and passive states in terms of the best frequency of the flagellum of female *Cx. quinquefasciatus* mosquitoes is less than 15 Hz – this is also true for *Ae. aegypti* females. Such shifts are statistically significant but are unlikely to be biologically relevant because of the small absolute frequency change (473). *An. gambiae* females were alone amongst females in shifting from 219 to 325Hz in a sedated state. In addition to this, male *Ae. aegypti* did not show any significant changes in their best frequency when comparing active and passive states, although males from the other two species tested demonstrated a decrease in best frequency following sedation.

That these shifts were all either relatively small or non-existent suggests that frequency specificity is highly important for the mosquitoes. All female mosquitoes have similar best frequencies in the active state. This could imply that species specific differences may reside instead in the male auditory system, which seems to contain a greater level of complexity given the data presented in section 7.3.3.2 and previous reports (34, 289).

The flagellar best frequency estimates for female and male *Ae. aegypti* have previously been reported as approximately 230 and 380Hz respectively, both of which are higher than the equivalent values calculated in this thesis of approximately 200 and 280 Hz (363). These differences could be the result of changes in environmental conditions or variance between strains (in a similar manner to the differences noted in section 5.4.5 for differences between the reported and calculated values of mosquito WBF) (355, 442).

However, the best frequency of the male *Ae. aegypti* flagellum whilst it was spontaneously oscillating was estimated to be 380 Hz, exactly the same as the previous report (363). In addition to this, the best frequency of spontaneously oscillating male *Cx. quinquefasciatus* was estimated to be 311 Hz, which is similar to values reported previously

for this species – this similarity was not found for female *Cx. quinquefasciatus* however with best frequency estimates approximately 100Hz lower than previously published (308).

Male *An. gambiae* on the other hand were calculated to have a distinctly different best frequency in this thesis to that previously reported in either the fibrillae extended or collapsed state, although the flagellar tuning of females from this species was similar to the reported value (approximately 220 Hz as opposed to the reported estimate of 209 Hz) (353).

Flagellar effective stiffness increases following sedation (or pymetrozine) are in line with the results of section 6, as well as previous reports for *Drosophila melanogaster* using equivalent experiments, and are likely the result of a cessation of active processes which contribute to determining the stiffness of the flagellum due to CO_2 exposure (283).

In the non-stimulated, quiescent states used for the energy gain estimates, male *Ae. aegypti* and *An. gambiae* mosquitoes were not statistically significantly different from their female counterparts (which may be the result of an underpowered statistical comparison and so would require a larger sample size to confirm), whilst female *Cx. quinquefasciatus* had a significantly greater level of energy gain than males. These results suggest that in the absence of an biologically relevant stimulus, mosquitoes of both sexes inject comparatively low levels of energy into their auditory systems; wildtype *Drosophila melanogaster*, whose JO is considerably smaller than that of both mosquito sexes, typically exhibit energy gains of approximately 4.6 for example (283).

That the energy gain calculated in section 7.3.1 for mosquito species should be on the same scale as *Drosophila melanogaster* despite their JOs containing at the very least 10 times as many neurons may be the result of the configuration of the mosquito antennal receiver system (271, 277, 278). Whilst the one-dimensional auditory system in *Drosophila melanogaster* means that half the total number of ion channels can be opened by sufficiently large stimuli in a single direction (with the other half requiring stimulation in the opposite direction), the male mosquito flagellum is attached to between 60 to 70 radial prongs - this corresponds to approximately 6º rotational precision (287).

The LDV measurements used to estimate the energy gain values are only taken in the plane of one pair of prongs and so do not account for other planes, which could have a significant effect on the energy gain calculation. For example, assuming there are 60 radial prongs and an approximately equal distribution of about 15000 neurons, this would mean there are approximately 250 neurons per direction (76). This is very similar to the estimated number of neurons in the *Drosophila melanogaster* JO, which are equally distributed into two separate groups to cover both possible displacement directions (271).

7.4.2. Mosquito apparent antennal mass estimations

There are no prior reports of mosquito apparent antennal mass values and as such there are no comparisons available to assess the accuracy of the estimates given in table 14. Whilst estimations of 276 ng have been reported for the effective mass of *Cx. quinquefasciatus* males (i.e. approximately 7 times greater than the reported apparent mass calculated in section 7.3.3), it is unfortunately difficult to compare effective and apparent mass estimates; not only are apparent mass estimates dependent on the measurement point chosen for recordings but also not all of the contents of the antennal system contribute towards auditory function as measured by the LDV protocol (308).

Of the three mosquito species investigated in section 7, significant differences were calculated between male and female mosquitoes for *An. gambiae* and *Cx. quinquefasciatus*, but not for *Ae. aegypti*. Given the plumose nature of the male mosquito's flagellum, it could be assumed that males from all mosquito species tested should have greater apparent antennal mass values than females. In *Drosophila melanogaster* however, apparent antennal mass and total body size are thought to be strongly correlated (laboratory data, unpublished). Whilst mosquito antennal systems show a greater level of sexual dimorphism than *Drosophila melanogaster*, it could be the case that body mass also plays a significant role in determining the apparent mass of the antenna for mosquito species as well.

Ae. aegypti males have been previously reported to be significantly smaller than females, with *Ae. albopictus* males also found to be significantly smaller than females from that species (335, 474). This could in turn help to explain why no significant differences are seen between the apparent antennal mass estimates for *Ae. aegypti* males and females; if females mosquitoes from that species are considerably larger than the males, this could compensate for the increased density of fibrillae attached to the male flagellum, meaning that the mass estimates for both sexes become approximately equal.

On the other hand, given the low value of the post hoc statistical power calculation given in section 7.2.9 for the comparison between male and female *Ae. aegypti* it is reasonable to believe that this comparative test is insufficiently powered to detect a significant difference. Greater sample size numbers would be necessary in order to sufficiently power the experiment and check the underlying cause of this lack of significance. Regardless of the significant differences (or lack of) seen, the estimation of these apparent mass values allows for the use of the previously reported gating spring model in order to analyse mosquito force-displacement data obtained from force step electrophysiology (285).

7.4.3. Mosquito auditory systems as measured by force step stimulation electrophysiology

Interpretation of the model results shown in table 15 must be done cautiously as it is unclear what the estimates for the number of ion channels and relevant gating forces mean for mosquito species – for example, assuming a standard 60-fold symmetrical arrangement of radial prongs linked to the male mosquito flagellar shaft with only two of these prongs (within the same plane) being affected by force-displacement steps, it could be suggested that the estimated number of channels in males should be multiplied by a constant to obtain a better estimate of the total number of ion channels within the male auditory system (76).

Significant differences identified between female *An. gambiae* and either *Ae. aegypti* or *Cx. quinquefasciatus* female mosquitoes could be indicative of the relative level of genetic similarity between *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes as compared to *An. gambiae* – such a level of similarity was not observed for male mosquitoes from these species however. Statistical comparisons between mosquitoes of the same sex but of a different species were also made more difficult to interpret because of a lack of statistical power for some comparisons; post-hoc power calculations indicated that these comparisons (such as those made for the number of ion channels in female mosquitoes) tended to have below 50% statistical power and as such should be considered underpowered. Larger sample sizes would be needed to categorically determine whether the lack of statistically significant differences is real or the result of these relatively underpowered comparisons.

The estimated values provided in table 15 can still provide some interesting results with regards to parameters that are more readily comprehensible across species. It is immediately clear that $K_{INFINITY}$, K_{STEADY} and K_{GS} are significantly greater within each mosquito species in the males as compared to the females for example, indicating that the male antennal system is significantly less compliant than the equivalent female system.

This maintenance of stiffness (with only narrow displacement windows of small increases in compliance) for male mosquitoes is likely due to the extreme importance of maintaining frequency selectivity, with the nonlinearities connected with gating compliances being reported to result in changes in the antennal best frequency of *Drosophila melanogaster* (and as previously discussed in section 6.1) (456).

The high levels of stiffness of the mosquito flagellum can be well demonstrated by comparing the proportional increases in compliance typically noted. For healthy *Drosophila melanogaster*, dynamic stiffness tends to decrease from a maximum of 70 to around 15 μN/m for large and small displacements respectively, which equates to a greater than 4.5 fold difference in stiffness (as shown in figure 27 in section 6.3.2 and as has been reported previously) (285, 394). This change in stiffness is directly related to the differences in best frequency observed between passive and active states as stiffness and best frequency are proportional (as shown in section 7.2.9) (472).

By comparison, *An. gambiae* females (as shown in figure 36) regularly demonstrated maximal decreases in dynamic stiffness from 130 to 80 μN/m, a reduction of less than 50%. Females from the other two mosquito species showed proportional decreases of just over 20% whilst estimates for males from all species were even smaller than this, although the baseline stiffness of the male mosquito flagellum is considerably higher in mosquitoes than for *Drosophila melanogaster*.

Despite this, the presence of a mechanical nonlinearity was clear for all mosquito sexes and species tested (as judged using previously reported methods and with data presented in figure 35), which is highly suggestive of the presence of the direct gating of mechanotransduction channels (285).

Only CAP recordings propagated by the axonal projections of neurons within the auditory system were able to be recorded via insertion of a recording electrode into the mosquito's antennal nerve because it was not possible to access any compound currents within the mosquito's auditory neurons. This means that whilst it was still possible to obtain some assessment of the auditory nerve response to stimulation, there are unfortunately time delays associated with spike generation and action potential propagation that are unavoidable using this recording method; these problems can be further compounded by differences associated with potential independent neuronal populations (285, 394).

Further than this, it seems unlikely that multiple populations of ion channels would be present in *Drosophila melanogaster* (denoted as sensitive and insensitive for this species) but not in mosquito species and therefore a single population model should not be sufficient to describe the additional layers of system complexity that are present (271, 394). This two state model of a single transducer population is used here however as a first step to quantify mechanotransduction with the mosquito auditory system (and specifically avoids including data that may lead to the analysis of non-auditory nonlinearities), and not as a definitive model of the mosquito mechanotransduction system; it can thus be further built upon in the future.

7.4.4. Systems of compensatory stiffness maintenance in mosquito species

The maintenance of an auditory system tuned within a specific frequency range involves active transduction-related processes which are associated with essential auditory nonlinearities (279). Nonlinear gating compliances and adaptation motors shift the best frequency of the antennal receiver in response to stimulation of different intensities (279, 456). In order to maintain such a flagellar tuning therefore the mosquito auditory system could seek to minimise these auditory mechanotransduction-based nonlinearities. Compensatory stiffness mechanisms could provide a method to reduce these non-linearities by ensuring that compliance changes are minimised. That such changes in K_{STEADY} are seen across all three mosquito species tested strongly supports this theory.

During sedation the sharp increase in K_{STEADY} seen around the resting position for the female *Ae. aegypti* mosquito included in figure 39 before sedation is no longer present. Instead both K_{PEAK} and K_{STEADY} appear identical apart from a constant background level increase in K_{PEAK}. This suggests that the additional stiffness provided in the steady state could have an active component. All sedation experiments across different species show similar conclusions – loss of nonlinearity, lack of auditory CAP response to stimulation and an absence of stiffness increases in K_{STEADY} around the resting position.

Given the low number of mosquitoes involved in the sedation experiments however, it is difficult to judge exactly how repeatable this loss of increases in K_{STEADY} is, in spite of the promising preliminary results. It may be the case that instead of an active process that leads to a change in stiffness occurring, viscoelastic elements within the auditory system provide compensatory mechanisms in a passive manner; such systems have previously been described in other situations (475, 476).

Within each individual recording taken whilst a mosquito was sedated, K_{PEAK} and K_{STEADY} seem almost identical aside from a constant vertical shift. This could be indicative of a systematic bias in the recording process or data analysis procedure, although the fact that K_{PEAK} and K_{STEADY} have distinctly different trends between different mosquito species and sexes suggests that this is perhaps not the case. The sharp peaks and troughs in dynamic stiffness around the resting position seen in both K_{PEAK} and K_{STEADY} are potentially the result of insufficient data averages being calculated for small displacements (for whom the effect of a constant flow of CO_2 is likely to be most important in terms of affecting displacement measurements); that these sudden changes in stiffness are observable for both K_{PEAK} and K_{STEADY} suggests that this phenomenon is truly the result of gas flow and could be removed by including more data into the averaging procedure.

Fitting the two state model for a single transducer population to the difference between K_{PEAK} and K_{STEADY} for male mosquitoes (as shown in section 7.3.4.4) provided a better fit in some respects than fitting to just K_{PEAK}; it may be necessary therefore to remove the effect of K_{STEADY} from K_{PEAK} before conducting the force step stimulation analysis.

However the mosquito flagellar system is comprised of many interlocking systems designed to maintain a precise focus on narrow frequency ranges, whilst also exploiting distortion products in a manner possible only in a sharply tuned system (34). The removal of this potentially compensatory mechanism therefore could have substantial negative consequences on the entire auditory system; for example this counterbalancing stiffness might be necessary to prevent the system from constantly spontaneously oscillating.

One noticeable difference between male and female mosquitoes is the asymmetry evident in the dynamical stiffness estimates presented throughout section 7.3., with male (particularly *An. gambiae* and *Cx. quinquefasciatus*) mosquitoes displaying asymmetrical dynamic stiffness estimates around the resting position in response to positive and negative displacements of equal absolute magnitude. This may be due to differences between male and female mosquitoes in terms of the analysis procedure, with males undergoing a greater amount of data smoothing than females; this is unlikely to explain the significant differences seen however given that the smoothing should act to reduce outlying estimates.

The asymmetries could therefore be reflective of real differences in dynamical stiffness calculations for positive and negative displacements, which would thus depend on the orientation and positioning of the mosquito relative to the LDV experimental setup. Such asymmetries have not been noted for *Drosophila melanogaster* (285, 394).

These sexually dimorphic responses likely reveal the differing objectives of the male and female auditory systems; females may aim to prevent spontaneous oscillations whilst males, for whom spontaneous oscillations can naturally occur, focus on preventing increases in nonlinearity within the system (with male *An. gambiae* in section 7.3.4.4 for example having non-negative calculated dynamic stiffness estimates across all flagellar displacements tested) (289, 308).

Maintenance and minimisation of nonlinearities in the auditory system is essential for male mosquitoes. Whatever the mechanism by which steady state stiffness is maintained, removing this compensatory behaviour could result in a severe impairment on mosquito copulation attempts as the insects would no longer be able to maintain such a finely tuned system. The exact systems which maintain stiffness are therefore of great potential interest, and require further exploration.

7.4.5. Multiple transducer populations

The existence of only a single ion channel population in the auditory system of mosquitoes is, conceptually at least, unlikely given the complexity of the mosquito JO and the multiple populations reported for *Drosophila melanogaster* (271). There are unfortunately no available investigations reported as yet into these potential populations, which is why a two-state model for a single transducer population has been fitted throughout section 7. Investigating the changes in dynamical stiffness with increasing force steps (as shown in section 7.3.4.3) however clearly reveals a number of stiffness regimes present throughout, in a similar manner to that seen for *Drosophila melanogaster*. This is also visible from the CAP amplitude changes seen in figure 41, with several apparent changes in slope observable.

The improvement in ion channel open probability fit shown in section 7.3.4.5 for both male and female *An. gambiae* when a two transducer population was assumed was substantial but still requires modification. Given the low maximum CAP responses observed for female *An. gambiae* it may be more suitable to use another species, such as *Cx. quinquefasciatus*, for those fitting attempts as the maximum CAP response was more similar between males and females from this species.

Although the comparison of the relative quality of the single transducer population and two transducer population model fits using AICc values did suggest that the two population model described the force-displacement data more accurately for all female mosquitoes investigated, as well as male *Ae. aegypti*, no such improvement in quality was found for male *An. gambiae* and *Cx. quinquefasciatus* mosquitoes. This could indicate that only a single transducer population exists in these types of mosquitoes, though this would seem unlikely given the existence of multiple populations in *Drosophila melanogaster* and the indication of the existence in the other mosquito groups investigated (271).

The differences between the two fit types in terms of the AICc values were not particularly large even for those mosquito groups where the two ion channel population model was recommended, and as such this analysis does not provide solid evidence for the existence of a second transducer population. It therefore seems more likely that the lack of an improvement in fit quality results from a lack of understanding of the data and suggests that more research is necessary into the transducer populations before any conclusions can be drawn.

This fuller understanding is vital as there are serious issues that can result from incorrectly using more complex models; for example additional populations would require the inclusion and investigation of additional resultant nonlinearities, which if not fully accounted for could result in misleading conclusions. These issues are also important when considering the ion channel open probability fits; whilst incorporating a second population into the fit function (as described in section 6.2.8) enables a seemingly more accurate description of the mosquito data because of an increase in the number of degrees of freedom associated with two populations, without a solid biological understanding of the mosquito system it could potentially lead to inaccurate assessments being made (394).

Thus it is important that the number of transducer populations in the mosquito system be properly estimated, potentially by the same mechanisms used to identify the auditory and wind/ gravity populations in *Drosophila melanogaster*, which will become possible hopefully in the foreseeable future as genetic manipulations of the mosquito genome become more widespread (271, 280).

Considering the extent of the sexual dimorphism between male and female mosquitoes (particularly with regards to auditory systems, such as the distribution of scolopidia populations) it may not be surprising if the two sexes also contained a different number of transducer populations (76). This is particularly relevant when taking into account the substantial CAP responses seen for even the smallest force steps for male mosquitoes from all three species, which are not present for female mosquitoes from any species tested.

7.4.6. Extent of sexual dimorphism in mosquito auditory systems with regards to displacement gain estimates

The best frequency of the peak mechanical sensitivity calculated from the pure tone stimulation data for each mosquito species and sex tested was similar to the estimates obtained by fitting the velocity amplitude fit function to free fluctuations of the flagellum (as shown in table 18 in section 7.3.6), The peak mechanical sensitivity of male mosquitoes from any species however did not well match the conspecific fundamental female WBF, in contrast to previous reports regarding tuning (358). There was a consistent difference of approximately 50Hz across all species, which could be the result of reported changes to the flagellar mechanical tuning due to temperature (308).

Both the peak mechanical sensitivity and nerve response to pure tone stimulation occurred at higher frequencies for male mosquitoes than for females across all three species tested, which is in agreement with prior reports (353, 358). Comparing these values with estimates of the difference tone and cubic distortion product for each species suggested various conclusions: whilst for *Ae. aegypti* both the peak male nerve response and the peak female mechanical sensitivity were approximately equal to the relevant calculated distortion products (as detailed in section 2.10.2), the equivalent tuning of responses for *An. gambiae* were too broad to reach any firm conclusions.

In addition to this, although *Cx. quinquefasciatus* males and females appeared to tune to the relevant calculated distortion product frequencies, the values calculated for the difference tone and the cubic distortion product were so similar that in practice it was not possible to distinguish which distortion product was relevant for which sex. This stands in contrast to previous reports for this species, in which the two distortion products could be individually distinguished (358).

This could be the result of imprecise estimates of the male and female WBF for each species (as discussed in section 5.4.5). Further experiments investigating the peak frequencies of mechanical sensitivity and nerve response to pure tone stimulation for these mosquito species would first require a more precise estimate of the WBF so that distortion products can also be calculated with greater accuracy. The sample size of the mosquito groups tested using pure tone stimulation should also be increased in order to investigate potential differences between female mosquitoes from different species in the best frequency of the peak mechanical sensitivity.

The mechanical tuning of the flagellum of male and female mosquitoes from all three species was discussed in section 7.4.1; the frequency of the peak nerve response differed significantly between the sexes as well as between *Ae. aegypti* males and males from the other two species. The frequency of the maximum nerve response of male *An. gambiae* was closely in line with that reported previously for this species whilst the tuning of male *Cx. quinquefasciatus* at a considerably lower value that than available in the literature (353, 358). Males from both these species had an estimated frequency tuning of 185Hz for their peak nerve response: given the resolution of the pure tone stimuli used in section 7.3.6 (which was presented in 10Hz steps) it may prove difficult even with a larger sample size to identify significant differences between the two species, should such differences exist.

Estimates of the displacement gain using white noise stimulation relied on fitting a sigmoidal model to the data obtained at each stimulus intensity and then calculating the ratio of the maximum and minimum values of mechanical sensitivity. The sharp decrease in sensitivity once the stimulus intensity had reached a threshold value however may be indicative of deeper underlying auditory mechanics. It may be the case therefore that modelling this sudden decrease in sensitivity as stimulus intensity increases requires the utilisation of a Hopf bifurcation model in order to account for the fact that mosquito auditory systems exist on the boundary of oscillatory instability (279, 290, 477).

The changes in best frequency seen for some types of mosquitoes during the white noise stimulation experiments (as shown in figure 42) strongly corresponded with the relative best frequencies of the active and passive systems for distinct mosquito sub-sets. Increased force caused the system to no longer require active gain mechanisms to function and as such the system tended towards the passive state and correspondingly tuned towards the best frequency of that state.

As such, females whose passive and active systems share identical frequency values showed almost no change in best frequency, whilst female *An. gambiae* demonstrated significant shifts which almost completely disappeared following pymetrozine injection. That the final frequency did not fully reach the estimated value for the completely passive system is likely the result of the force stimulus intensity not being powerful enough to reach a completely saturated state.

Previous reports on the mechanical sensitivity of the mosquito flagellum have generally indicated that male mosquitoes are more sensitive than females from the same species (289, 352, 354). This stands in sharp contrast to the displacement gain estimates calculated for both white noise and pure tone stimuli in sections 7.3.5 and 7.3.6 respectively, in which female mosquitoes were typically found to have higher gain estimates than conspecific males. These differences could be the result of different experimental methodologies (such as the specific focal point of the laser on the flagellum) or different mosquito lines used for analysis.

All sexes and species had significantly greater displacement gains when estimating the displacement gain using pure tone stimuli than with white noise stimuli. This could imply that pure tone stimulation is more biologically relevant for male and female mosquitoes from all species tested than broad-tone, lower intensity stimuli whose energy dissipates over a broad range of frequencies that are outside the frequency ranges that mosquito auditory systems are tuned to. This may therefore result in reduced white noise displacement gain estimates being calculated for both male and female mosquitoes due to interference with frequency-specific amplification mechanisms.

The evidence presented throughout section 7 has demonstrated repeatedly the considerable extent of sexual dimorphism in the auditory systems of mosquito species, both with regards to the changes in compliance and nerve responses to force step stimulation (shown in section 7.3.4.3) and also in the displacement and energy gain estimates.

Whilst the increased magnitude of CAP responses to stimulation (both in terms of force steps and pure tone) in male mosquitoes compared to females for all species tested could potentially simply be the result of the much larger JO in male mosquito, the finding that female mosquitoes in general have displacement gains that are either not significantly different to, or are significantly larger than, the corresponding estimates for males suggests that the female mosquito auditory system is potentially just as complex as the male. This is somewhat surprising given that female mosquitoes had previously been thought to possibly be deaf and it is only within the past few decades that female mosquito auditory function has been recognised as highly sensitive (363, 478, 479).

7.4.7. Extent of sexual dimorphism in mosquito auditory systems with regards to compound injection designed to sever efferent feedback loops

Injection of either TTX or TeNT into a mosquito should sever any efferant and afferant feedback mechanisms that exist within the auditory system and therefore potentially reveal the underlying system usually kept in check by constant damping feedback loops. Currently efferent feedback has only been reported in the auditory system of male *Cx. quinquefasciatus* mosquitoes, with some evidence also published alongside this suggesting that females from this species could potentially use efferent control loops to modulate auditory function (314). The results presented in section 7.3.7, with both TTX and TeNT injection leading to spontaneous oscillations in male mosquitoes, suggest that such systems exist in all male mosquitoes tested (including non-*Cx. quinquefasciatus* species) as well as potentially for female *Cx. quinquefasciatus*.

The impact on mosquitoes of injection with either TeNT or TTX is broadly sex specific, with only males exhibiting spontaneous oscillations in addition to corresponding extremely significant increases in energy gain after compound injection whilst females demonstrate much lower, or non-existent, levels – this is in line with previous literature indicating the sex-specificity of spontaneous oscillations (289, 308). This sex-specificity can be further picked apart: neither *An. gambiae* nor *Ae. aegypti* females showed any significant differences in energy gain between post-ringer and post TTX/TeNT states, though *Cx. quinquefasciatus* females showed a relatively small (compared to male mosquitoes) but significant increase when comparing these two states.

Of the three groups of female mosquitoes investigated, only *Cx. quinquefasciatus* females showed a significant difference. This could be suggestive of a potential dilution of neurotransmitters that occurs in the immediate aftermath of ringer injection, with increases in energy gain slowly being reversed until the system returns to a pre-ringer state after sufficient time has elapsed (though the time scale required is unclear given that energy gain estimates were calculated at least 10 minutes following compound injection). That such neurotransmitters could potentially exist only in *Cx. quinquefasciatus* females reflects the differences observed between female mosquitoes from different species.

On the other hand, the changes in male energy gain following TTX/ TeNT injection were much greater than those seen in females, with some *An. gambiae* males being calculated as having energy gains in the hundreds of thousands of kBT (contrasted with a post-ringer gain of less than 10).

Although all three male groups showed significant differences, the level of energy gain was considerably lower for *Ae. aegypti* males than for *Cx. quinquefasciatus* males, which was then in turn much smaller than that of *An. gambiae* males. This could be related to the extent of sexual dimorphism present in each species, with *An. gambiae* having being previously noted throughout sections 7.3 and 7.4 as exhibiting far greater differences between males and females in terms of auditory function than the other two species tested.

For all sexes and species tested, pymetrozine injection led to significant decreases in energy gain when compared to every other injection type. Whilst the energy gain remained above zero for almost all cases (and was tested and found to be significantly greater than zero), it resolutely remained at fractions of $1K_BT$, in agreement with displacement gain reports previously published (27).

The absence of spontaneous oscillations in female mosquitoes following TTX or TeNT injection clearly distinguishes the effects of TTX and TeNT on the antennal system from DMSO for example; DMSO injection may cause the antennal receivers of both male and female mosquitoes to spontaneously oscillate (289) but it also has this effect in *Drosophila melanogaster,* whose auditory systems do not contain efferent systems (310). Although TTX and TeNT have distinct mechanisms of action, the overall effect of compound injection should be to sever all afferent and efferent loops (should they be present) by preventing either voltage-gated sodium ion channels from firing or by blocking the release on neurotransmitters – that this causes spontaneous oscillations in male, but not female, *Cx. quinquefasciatus* suggests different functions for efferent networks in the two sexes.

The absence of energy gain in *Ae. aegypti* and *An. gambiae* females following TTX or TeNT injection could indicate that efferent feedback loops are not present in these two types of female mosquito or that these loops are present but serve a different purpose. It could also mean that the statistical power of the study is not great enough, although even if the power were to be increased and significant differences found, the scale of these differences would be minor compared to those calculated for male mosquitoes from any species or even *Cx. quinquefasciatus* females.

Identification of potential efferent control loops would require experiments similar to those reported for confirming the presences of such loops in males and as such all that can be drawn from the energy gain estimates is that female mosquitoes from all species tested do not contain efferent feedback control mechanisms within their auditory systems that operate in a similar manner to those seen in male mosquitoes (314). Given that only male mosquitoes demonstrate spontaneous oscillations in the absence of compound injection this could be indicative of the role that efferent feedback control plays in this phenomenon.

8. Mosquito pymetrozine vibrometry and electrophysiology

8.1. Introduction

Although section 5 included some behavioural experiments examining the impact of pymetrozine on mosquitoes, those assays were not as broad as the experiments conducted for *Drosophila melanogaster* in section 4. In addition to this, section 6 included detailed examinations of the auditory system of various *Drosophila melanogaster* lines both before and after pymetrozine injection (which were highly promising in terms of the ablation of ChO mechanosensory function) but did not include any mosquito species. Therefore it is important that the impact of pymetrozine on the mosquito auditory system is assessed in order to confirm whether the compound acts in a similar manner on both Dipteran species.

Previous data analysis presented in this thesis in section 6 utilised injection of pymetrozine in order to judge the impact of pymetrozine on the auditory system of *Drosophila melanogaster* (in line with previous reports (27)). The usual method of compound exposure in the field however is oral ingestion (as used throughout section 4, with free fluctuations of pymetrozine exposed *Drosophila melanogaster* provided in appendices A-D) (26). Comparisons between pymetrozine uptake via ingestion or via injection can enable analysis of potential significant differences between the exposure methods.

Whilst ensuring that pymetrozine ingestion is able to affect auditory function as measured by LDV is vital (as if pymetrozine is relatively ineffective after oral uptake it is not suitable for mosquito control programmes), changes from the expected auditory state observed in a mosquito after pymetrozine feeding exposure could not be unambiguously ascribed to the compound itself as there would exist no record of the state of auditory function prior to pymetrozine ingestion. Thus repeating the experiments completed in section 6 for *Drosophila melanogaster* will enable comparisons to be made within individual mosquitoes as well as between groups, in addition to enabling the determination of pymetrozine as the direct cause of any changes observed in auditory function.

This includes not only analyses of force step stimulation experiments but also free fluctuation recordings. The results of velocity amplitude fits to free fluctuation data were presented for mosquito species in section 7.3.1 and demonstrated that for changes in the best frequency of the mosquito flagellum following sedation did not correspond to changes seen for *Drosophila melanogaster* (both in section 6 and in previous reports (283)). Contrasting the changes observed following pymetrozine injection and those visible during sedation could allow for insight into the flagellar frequency tuning mechanisms.

The earlier investigations into the potential compensatory role that K_{STEADY} could fulfil within the auditory system (in section 7.3.4.4) suggested that this steady state stiffness could be the result of an active process because sedation of a mosquito prevented increases in dynamical stiffness observed in K_{STEADY} for small force stimuli from occurring. For all *Drosophila melanogaster* studied in section 6, pymetrozine was able to ablate ChO mechanosensory function and reduce increases in compliance related to K_{PEAK} around the resting position. Pymetrozine could thus also have a significant effect on relative changes in K_{STEADY}. This analysis can be completed in the same manner as in section 7.3.4 and could thus provide support to the theory of compensatory stiffness should K_{STEADY} change significantly following pymetrozine exposure.

Similar to the experiments conducted in section 6 using *Drosophila melanogaster* lines that demonstrated various forms of insecticidal resistance, the possible existence of cross-resistance between already existing insecticide resistance mechanisms in mosquitoes could reduce the potential of pymetrozine for future use as an insecticide.

Two *An. gambiae* strains, referred to as Ngusso and Tiassale, have been reported to exhibit significant levels of insecticide resistance, with the Ngusso line being reported as resistant to the organochloride class of insecticides and the Tiassale line resistant to compounds from all four major insecticide classes (described in section 2.3.1) (480, 481). Investigating whether pymetrozine is still able to ablate ChO mechanosensory function for these lines therefore could help provide information as to the potential usefulness of the compound for targeting mosquito populations in which there is a high prevalence of resistance to already existing insecticides.

The development of insecticidal resistance has been thought to be linked with relative reductions in competitive fitness (228, 482, 483). Before assessing the impact of pymetrozine on these resistant *An. gambiae* lines therefore it is important to judge the baseline, pre-pymetrozine auditory systems in order to compare the results with those seen for the susceptible *An. gambiae* Kisumu line tested in sections 5 and 7 and thus potentially identify any significant differences between the strains.

Finally, the energy gain analysis in section 7.3.7 provided further support for the existence of efferent feedback loops in male *Cx. quinquefasciatus*, which have been suggested to assist the male in locating suitable females for copulation (314). Male flagellar displacement modulation in response to pure tone stimuli (at frequencies which should mimic female flight tones) could be indicative of these feedback loops, and the potential impact of pymetrozine exposure on these loops could therefore be of interest in terms of identifying if pymetrozine can prevent male mosquitoes from identifying conspecific females.

8.2. Materials and methods

8.2.1. Mosquito rearing

Unless otherwise noted, all mosquito species were reared in an identical manner as described in section 5.2.1: all *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* (Muheza) mosquitoes used for experiments were provided by Shahida Begum from the London School of Hygiene and Tropical Medicine. *An. gambiae* (Ngusso and Tiassale strains) were provided by the lab of Dr Gareth Lycett from the Liverpool School of Tropical Medicine. All mosquitoes were reared using a 12 hr: 12hr LD cycle at 26°C and 75% relative humidity and were fed using a 10% glucose mixture.

8.2.2. Compound preparation

Ringer: Preparation of the ringer solution was identical to that described in section 6.2.2 for injection experiments in *Drosophila melanogaster*.

Pymetrozine: Preparation of pymetrozine was identical to that described in section 4.2.2 for feeding experiments in *Drosophila melanogaster.*

8.2.3. Compound exposure methods – feeding and injection

Feeding and injection experiments in mosquitoes entailed the same protocols as described in section 5.2.3 for pymetrozine ingestion and section 7.2.3 for pymetrozine injection. Pymetrozine exposure via ingestion was investigated in section 8.3.1 whilst all other sections included in the results utilised injection procedures for pymetrozine exposure.

8.2.4. Analysis of force step stimulation electrophysiology experiments following pymetrozine exposure via ingestion

Three *Cx. quinquefasciatus* female mosquitoes between 3 and 4 days old were transferred into individual vials and deprived of all food overnight. Following this they were provided with a glucose food source doped with pymetrozine (at a concentration of 1000ppm) for 24 hours. The female mosquitoes were then mounted and prepared for electrophysiological experiments in the same manner as described in section 7.2.4.

Electrophysiological force step recordings (as described in sections 6.2.8 and 7.2.10) were completed for all three female mosquitoes, with the data being analysed using the two state model for a single transducer population (previously described in section 6.2.8 and as used in section 7).

All three female *Cx. quinquefasciatus* mosquitoes were included in the final analysis. No formal power calculations were made for this experiment and as such it offers purely descriptive data as to CAP production and dynamical stiffness properties in response to force step stimulation in three individual *Cx. quinquefasciatus* female mosquitoes.

8.2.5. Analysis of electrophysiological force step experiments before and after pymetrozine exposure via injection for *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus*

Male and female mosquitoes aged between 3 and 8 days old from each species investigated (*Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus*) were mounted and prepared for electrophysiological experiments in an identical manner as described in section 7.2.4. After the mounting procedure had been completed, free fluctuations of the flagellum were recorded and electrophysiological force step recordings (as described in sections 6.2.6 and 7.2.4) were completed in three separate states – before ringer injection, after ringer injection and after pymetrozine injection. Flagellar free fluctuation measurements were taken at the beginning and end of each of the three force step recordings taken per individual mosquito, as well as immediately after each compound injection. Compound injection was conducted in the same manner as described in section 7.2.3.

In total, 7 *Ae. aegypti* females, 6 *Ae. aegypti* males, 9 *An. gambiae* (Kisumu) females, 7 *An. gambiae* (Kisumu) males, 9 *Cx. quinquefasciatus* females and 9 *Cx. quinquefasciatus* males were included in the final analysis.

Analysis and model fitting of the free fluctuation data was completed as described in section 7.2.7 - this allowed for the fitting of the velocity amplitude function fit described in section 6.2.6 and thus the estimation of three key parameters described in that section (namely F_O/m, ω_0 and Q, with aggregated data for *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* mosquitoes being given in appendix H). Statistical comparisons between before and after pymetrozine states (as well as comparisons between sedated and post-pymetrozine injection states) were made using paired before and after t-tests with a significance level of 0.05 in Sigmaplot. Post-hoc calculations of statistical power found that all comparisons made had a power >80%, except for *Ae. aegypti* males for whom the power was below 50% (indicating an underpowered comparison).

Analysis of the force-displacement utilised the two state model for a single transducer population described in sections 6 and 7 (specifically section 7.2.10). Statistical comparisons between after ringer and after pymetrozine states for $K_{INFINITY}$, K_{STEADY} and K_{GS} for *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* were made using paired before and after t-tests with a significance level of 0.05 in Sigmaplot. Post-hoc calculations of statistical power found that these comparisons had a power of less than 50% (with the exception of comparisons made for *Ae. aegypti* males, where the power was calculated to be more than 90%) and such had should be considered statistically underpowered.

8.2.6. Analysis of electrophysiological force step experiments before and after pymetrozine exposure via injection for *An. gambiae* (Ngusso and Tiassale)

Male and female *An. gambiae* (Ngusso and Tiassale) mosquitoes aged between 3 and 10 days old were mounted and prepared for free fluctuation and electrophysiological experiments in an identical manner as described in section 7.2.4.

Once an individual mosquito had been mounted, a recording of the free fluctuations of the flagellum was taken to assess the health of the auditory system. Following this electrophysiological force step recordings were completed in three different states (before and after ringer injection, and after pymetrozine injection). Free fluctuations of the mosquito flagellum were recorded before and after each separate injection. Injection of both the ringer and pymetrozine solutions followed the protocol described in section 7.2.3.

In total, recordings from 2 *An. gambiae* (Ngusso) females, 3 *An. gambiae* (Ngusso) males, 6 *An. gambiae* (Tiassale) females and 5 *An. gambiae* (Tiassale) males were included in the before ringer injection analysis. The after ringer injection and after pymetrozine injection analyses each contained 1 *An. gambiae* (Ngusso) female, 1 *An. gambiae* (Ngusso) male, 3 *An. gambiae* (Tiassale) females and 3 *An. gambiae* (Tiassale) males.

Statistical comparisons between after ringer injection and after pymetrozine injection states used before and after Wilcoxon rank sum tests with a significance level of 0.05. Post-hoc tests of statistical power found that whilst comparisons for *An. gambiae* (Ngusso) males had a power of over 80%, all other comparisons had statistical power estimates <50%, meaning that the comparisons should be considered underpowered.

8.2.7. Analysis of pure tone stimulation experiments following pymetrozine exposure via injection

Two male and two female *Cx. quinquefasciatus* mosquitoes between 5 and 6 days old were mounted and prepared for electrophysiological pure tone stimulation experiments in the same manner as described in section 7.2.4. These pure tone stimulation experiments (as described in section 7.2.12) were then repeated in each of the three injection states for all individuals studied (i.e. before ringer injection, after ringer injection and after pymetrozine injection). Analysis of this data was then completed in the same manner as described in section 7.2.12. These experiments were intended to be purely descriptive and as such no formal statistical power calculations were made.

8.3. Results

8.3.1. Step recordings – pymetrozine feeding

A complete elimination of CAP production in response to stimulation was observed for all three female *Cx. quinquefasciatus* female mosquitoes tested (as is evident in figure 50 below). Changes in dynamical stiffness for small stimuli (as seen in female *Cx. quinquefasciatus* not exposed to pymetrozine in section 7.3.4.3) are also no longer noticeable following pymetrozine ingestion. No comparisons to a before pymetrozine state were possible because of the compound exposure type used, which did not allow for prior electrophysiological recordings to be made.

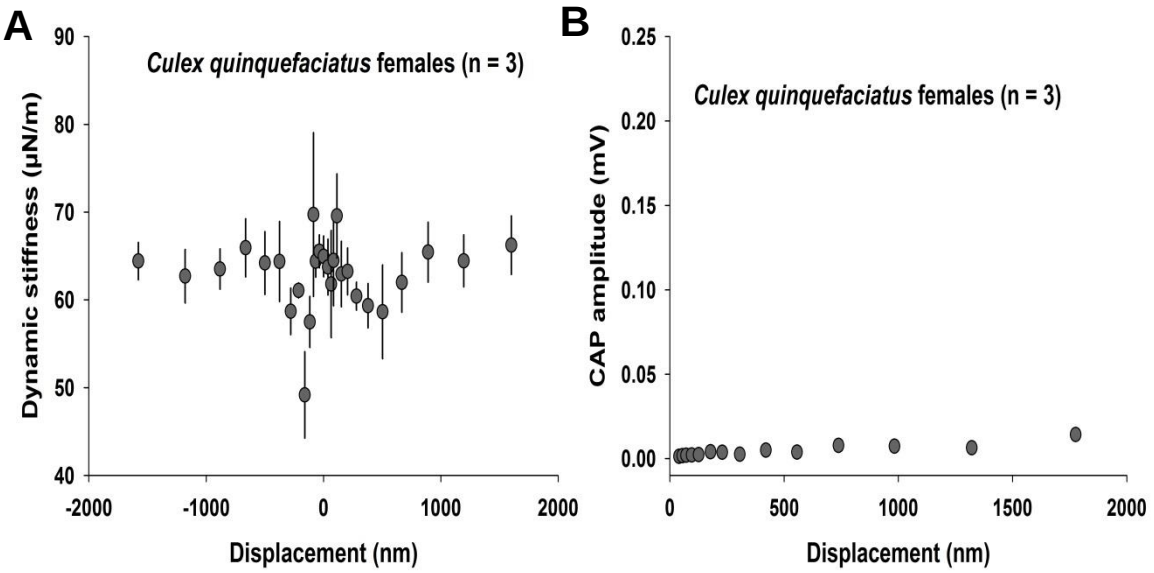


Figure 50. Changes in dynamical stiffness (A) and CAP amplitude (B) in response to changes in displacement for Cx. quinquefasciatus female mosquitoes exposed to pymetrozine via ingestion of the compound (median values were taken from three separate individuals, with vertical black bars representing standard errors).

8.3.2. Changes observed in antennal free fluctuation recordings following pymetrozine injection

Pymetrozine injection did not lead to shifts in best frequency on the scale of those observed for *Drosophila melanogaster* tested in section 6 (as is demonstrated in figure 51). *Ae. aegypti* males showed no significant change in their flagellar best frequency and whilst *An. gambiae* and *Cx. quinquefasciatus* males demonstrated significant decreases in best frequency these shifts were far smaller than the minimum increase of 300Hz seen for *Drosophila melanogaster*. The significant differences observed for *Ae. aegypti* and *Cx. quinquefasciatus* females showed changes in best frequency of less than 13 Hz each.

Only *An. gambiae* females displayed increases in flagellar best frequency that could be considered comparable to those seen previously for *Drosophila melanogaster* lines – table 22 contains a full list of best frequency estimates for all mosquito groups both after ringer injection and after pymetrozine injection.

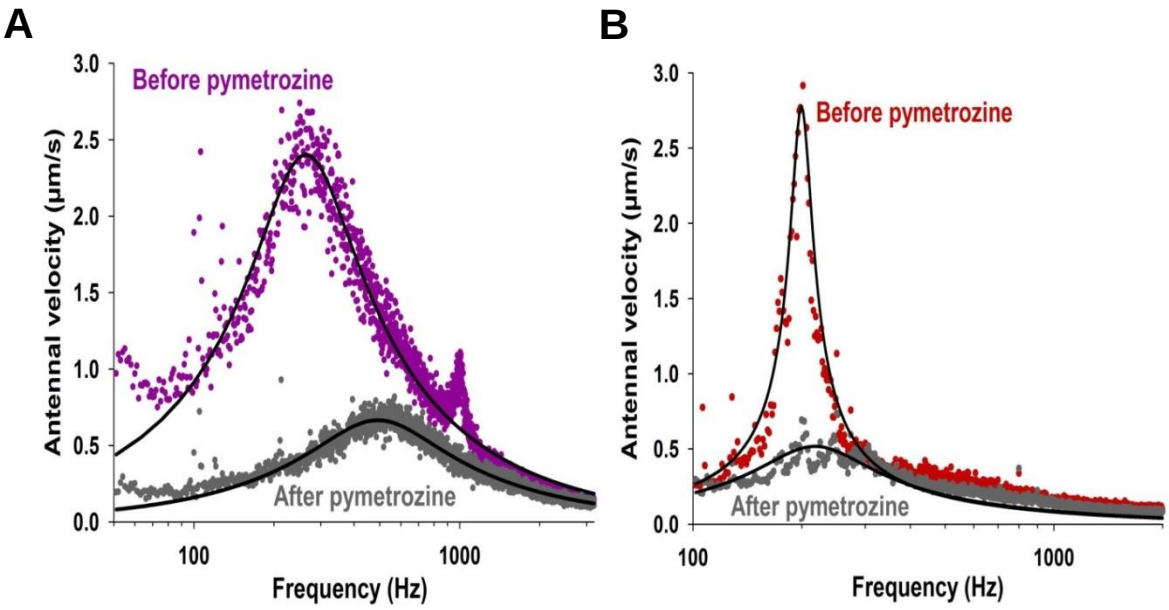


Figure 51. Free fluctuations for a Drosophila melanogaster male (A) and an Ae. aegypti female (B) showing both before and after pymetrozine states. The frequency ranges for A) and B) are 51 - 3200Hz and 100 - 2000Hz respectively. Individual data points represent Fourier-transformed velocity amplitudes at each frequency whilst solid black lines represent velocity amplitude function fits.

Table 22. Median values of flagellar best frequency for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (standard errors are given in brackets, with sample sizes for after ringer and after pymetrozine groups provided respectively below the species and sex type) as well as P-values for statistical comparisons between the two states

Mosquito species/ sex	Best frequency after ringer (Hz)	Best frequency after pymetrozine (Hz)	P-value
Ae. aegypti females (n = 36/ 36)	198.5 (1.42)	210.2 (3.57)	P <0.01
Ae. aegypti males (n = 25/ 25)	290.0 (11.91)	292.7 (11.49)	P = 0.706
An. gambiae females (n = 29/ 26)	215.0 (3.95)	306.0 (7.07)	P <0.001
An. gambiae males (n = 20/ 18)	352.0 (13.01)	319.1 (10.65)	P <0.01
Cx. quinquefasciatus females (n = 29/ 27)	221.6 (5.16)	208.9 (1.90)	P <0.01
Cx. quinquefasciatus males (n = 28/ 35)	365.6 (6.88)	258.7 (8.65)	P <0.001

The best frequency estimates calculated following pymetrozine exposure are statistically indistinguishable from those calculated for mosquitoes whilst in the sedated state – table 44 in appendix M contains comparisons of these two states. Figure 52 shows comparisons between different states for both a female *An. gambiae* and a male *Cx. quinquefasciatus* mosquito in order to illustrate the high level of similarity between the sedated and pymetrozine exposed states in terms of the best frequency.

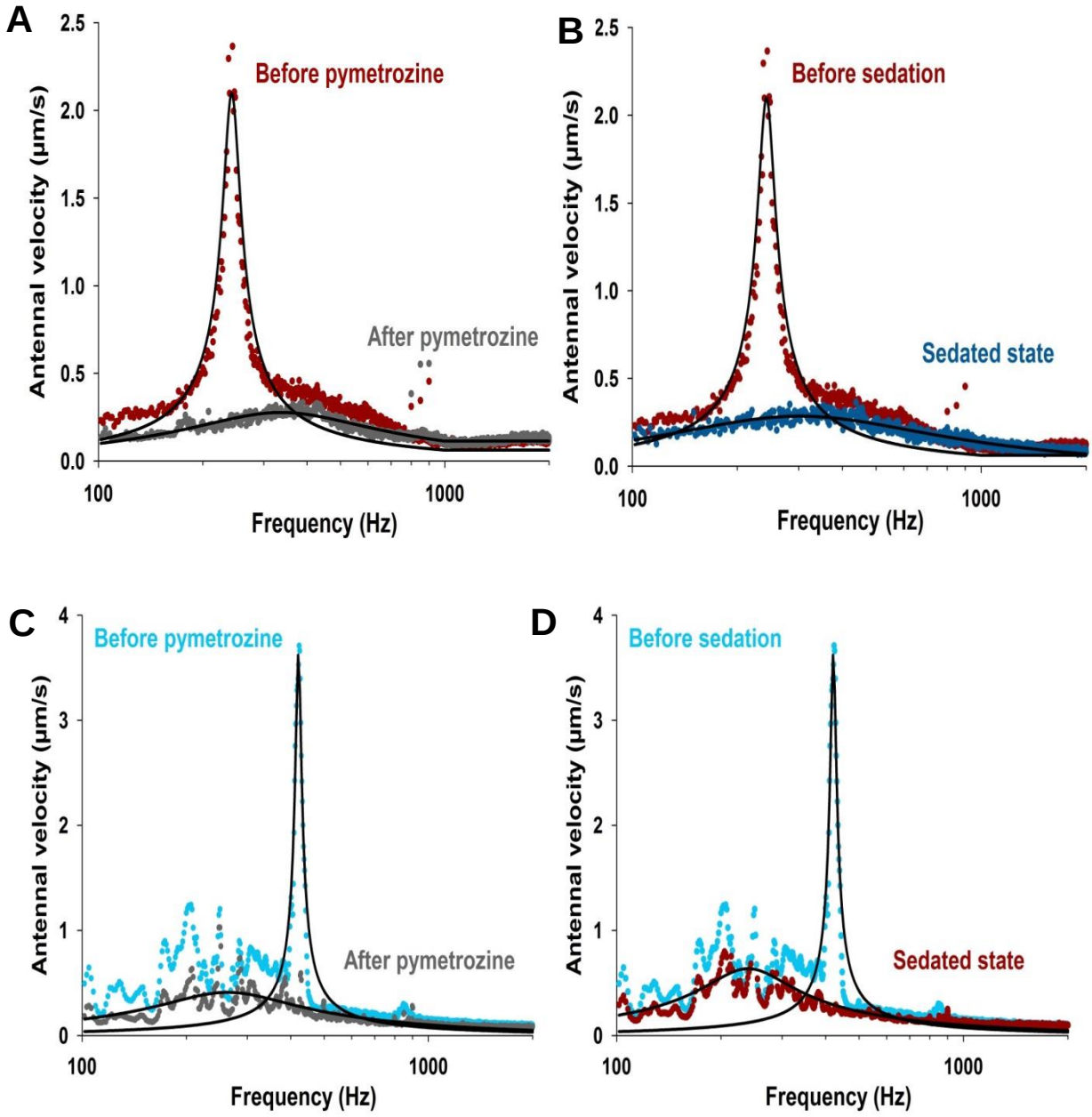


Figure 52. A and B) Free fluctuation recordings an An. gambiae female before and after pymetrozine (A) and before and during sedation (B).

C and D) Free fluctuation recordings a Cx. quinquefasciatus male before and after pymetrozine (C) and before and during sedation (D).

The frequency range for all fits was between 100 and 2000Hz. Individual data points represent Fourier-transformed velocity amplitudes at each frequency whilst solid black lines show the velocity amplitude function fits for each recording.

Following pymetrozine exposure, the flagellar effective stiffness increases significantly for male and female mosquitoes from all three species investigated (as is evident in table 23). In a similar manner to the best frequency estimates following pymetrozine injection, these increases in effective stiffness are statistically indistinguishable from those calculated for mosquitoes in the sedated state (with table 44 in appendix M containing a full list of comparisons); the only exception is a significant difference in the effective stiffness calculated for *Ae. aegypti* females between sedated and pymetrozine injected states. Figure 53 shows the extent of this similarity, particularly in comparison to the post ringer state for both the best frequency and the effective stiffness estimates.

Table 23. Median values of flagellar best frequency for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (standard errors are given in brackets, with sample sizes for after ringer and after pymetrozine groups provided respectively below the species and sex type).

Mosquito species/ sex	Effective stiffness after ringer (µN/m)	Effective stiffness after pymetrozine (µN/m)	P-value
Ae. aegypti females (n = 36/ 35/ 36)	13.8 (0.84)	40.5 (5.04)	P <0.001
Ae. aegypti males (n = 25/ 30/ 25)	54.6 (5.05)	91.7 (27.1)	P <0.001
An. gambiae females (n = 29/ 33/ 26)	22.6 (17.8)	125.9 (13.5)	P <0.001
An. gambiae males (n = 20/ 22/ 18)	85.6 (8.51)	182.6 (22.6)	P <0.001
Cx. quinquefasciatus females (n = 29/ 29/ 27)	3.84 (0.5)	42.3 (2.63)	P <0.001
Cx. quinquefasciatus males (n = 28/ 33/ 35)	18.3 (6.06)	86.6 (8.36)	P <0.001

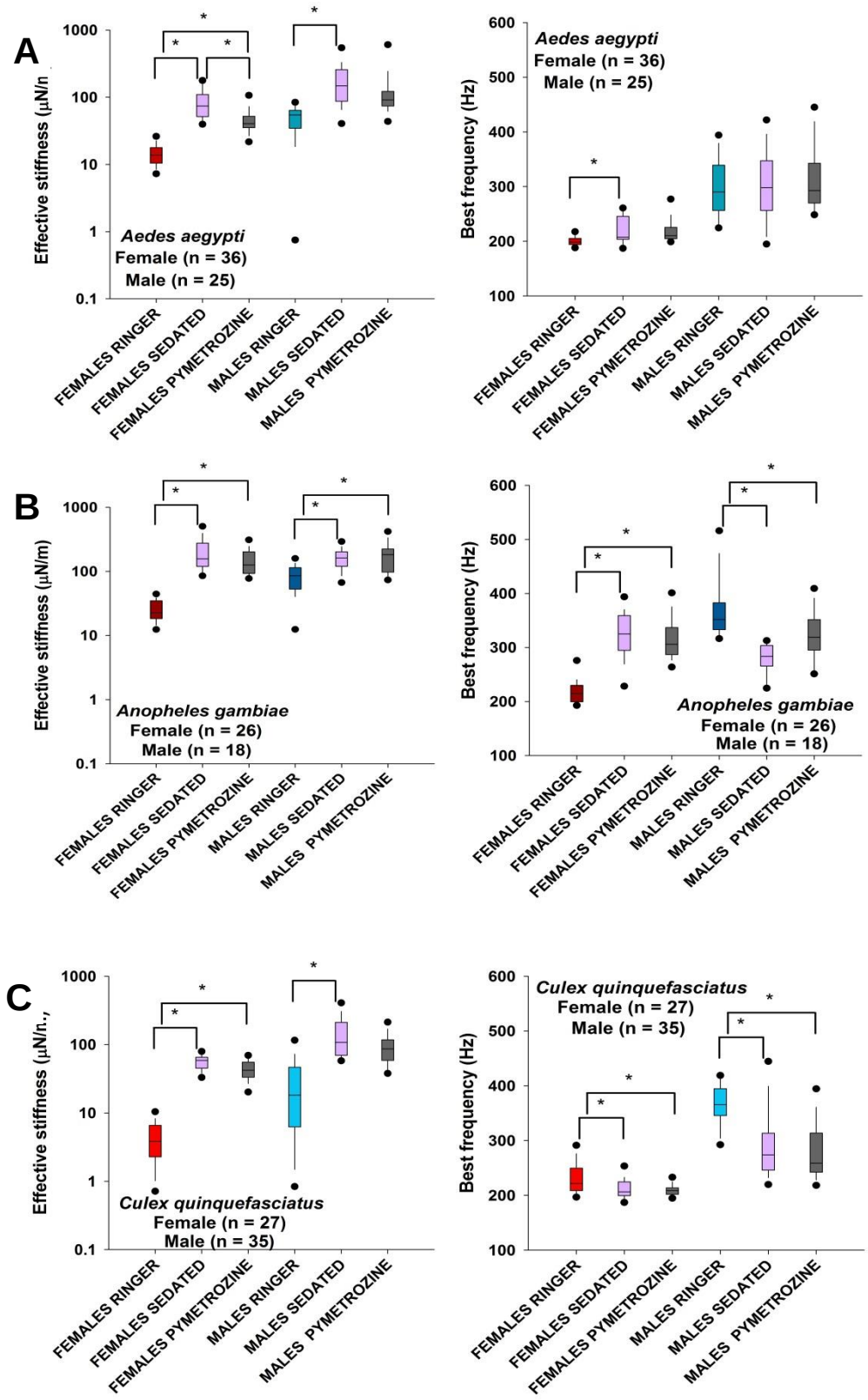


Figure 53. Best frequency and effective stiffness values after ringer injection, sedation or pymetrozine injection for male and female mosquitoes from A) Ae. aegypti, B) An. gambiae and C) Cx. quinquefasciatus, with significant differences between different states within a sex starred (black dots correspond to the 5th and 95th percentiles).

8.3.3. Force step recordings – pymetrozine injection

8.3.3.1. Mechanical and nerve responses to force steps

Male and female mosquito flagellar displacements and CAP production in response to force step stimulation in the absence of pymetrozine injection were previously displayed in figure 33 in section 7.3.4.1 (with equivalent responses for *Drosophila melanogaster* males both before and after pymetrozine injection shown in section 6.2.8 in figure 23). Figure 54 shows flagellar displacements and CAP responses to force step stimulation following pymetrozine injection for female and male *Ae. aegypti* mosquitoes.

In addition to the complete ablation of CAP response to force step stimulation of any size, the flagellar displacement response is also greatly changed – the initial overshoot is now far less prominent than for mosquitoes not exposed to pymetrozine and the recoil stage of the response to stimulation, though still present, undergoes far fewer corrections before reaching a steady state displacement.

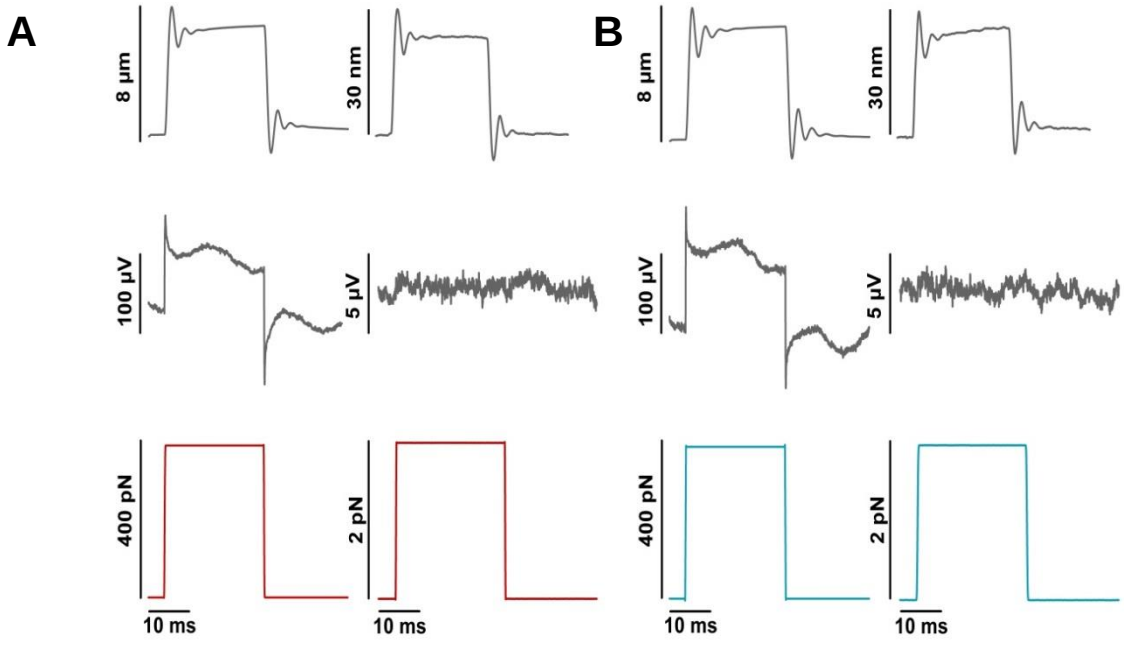


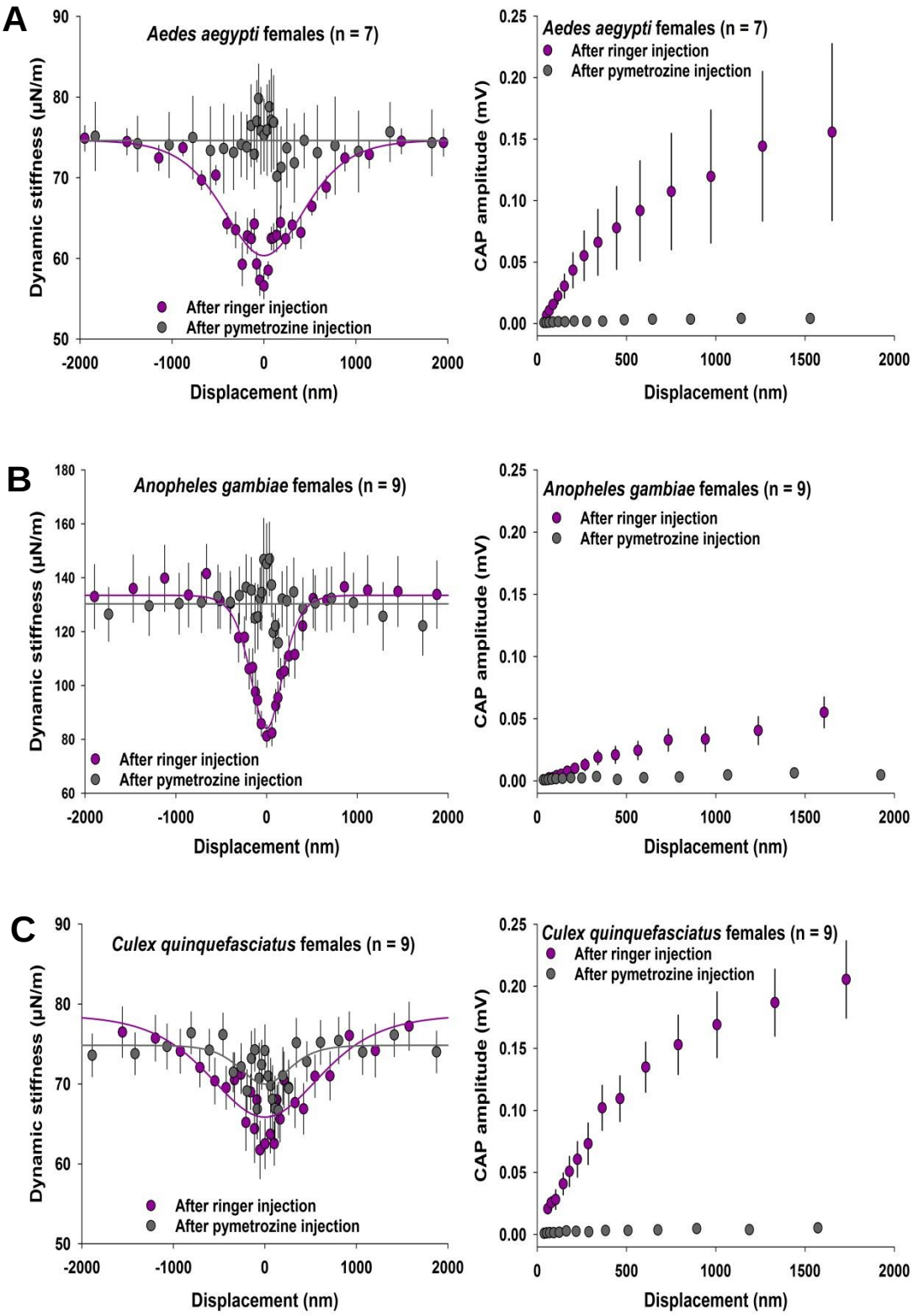
Figure 54. Flagellar displacement (top) and CAP amplitude (middle) in response to the corresponding force steps (bottom) after pymetrozine exposure for A) Ae. aegypti females and B) Ae. aegypti males – N.B. an artifact (as the result of crosstalk between the stimulus and electrodes) is present in the nerve response for the larger force step.

Figure 55 includes data showing that mosquitoes of both sexes from all species investigated demonstrated a complete abolition of CAP production following pymetrozine injection. This occurred in tandem with a significant reduction in the decreases in dynamical stiffness observed around the resting position. As was the case for *Drosophila melanogaster* however, some of the mosquitoes tested (namely *Ae. aegypti* males and *Cx. quinquefasciatus* females, as evidenced in figure 55) seemed to retain an increased level of compliance for small displacements.

Almost all *Drosophila melanogaster* lines tested in section 6.3.2 showed reduced estimates for K_{STEADY}, $K_{INFINITY}$ and K_{GS} following pymetrozine injection. In contrast to this, only a few significant differences were calculated for either male of female mosquitoes (with table 24 providing a full list of median stiffness parameters and significance values): from the males, only *Ae. aegypti* showed a significant decrease in $K_{INFINITY}$ and K_{GS} following pymetrozine exposure, whilst females from both *An. gambiae* and *Cx. quinquefasciatus* species showed significant decreases in K_{STEADY} and significant increases in K_{GS}. No other significant differences were seen for any stiffness parameter.

Table 24. Median values for dynamic stiffness parameters before and after pymetrozine injection for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus (numbers in brackets refer to standard errors and P-values below 0.05 are highlighted in bold).

	Ae. aegypti females (n = 7)	*An. gambiae* females (n = 9)	*Cx. quinq-uefasciatus* females (n = 9)	*Ae. aegypti* males (n = 6)	*An. gambiae* males (n = 7)	*Cx. quinq-uefasciatus* males (n = 7)
$K_{INFINITY}$ after ringer (µN/m)	75 (1.5)	135 (12.4)	76 (3.1)	136 (4.9)	181 (9.4)	175 (6.2)
$K_{INFINITY}$ after pymetrozine (µN/m)	77 (4.2)	131 (10.9)	76 (2.3)	122 (6.0)	225 (22.5)	162 (12.4)
Significance value	P>0.05	P>0.05	P>0.05	**P<0.02**	P>0.05	P>0.05
K_{STEADY} after ringer (µN/m)	66 (1.0)	103 (7.8)	63 (2.0)	98 (2.6)	137 (7.2)	139 (3.8)
K_{STEADY} after pymetrozine (µN/m)	62 (5.6)	94 (5.6)	56 (1.5)	91 (5.6)	175 (17.7)	135 (10.2)
Significance value	P>0.05	**P<0.001**	**P<0.001**	P>0.05	P>0.05	P>0.05
K_{GS} after ringer (µN/m)	9 (0.7)	37 (4.8)	15 (1.4)	37 (2.9)	48 (4.2)	32 (2.7)
K_{GS} after pymetrozine (µN/m)	13 (2.0)	45 (5.7)	18 (0.8)	31 (2.0)	63 (6.0)	28 (2.6)
Significance value	P>0.05	**P<0.04**	P**<0.001**	**P<0.02**	P>0.05	P>0.05


A
Aedes aegypti females (n = 7)
Dynamic stiffness (µN/m)
Displacement (nm)
After ringer injection
After pymetrozine injection
CAP amplitude (mV)
B
Anopheles gambiae females (n = 9)
C
Culex quinquefasciatus females (n = 9)

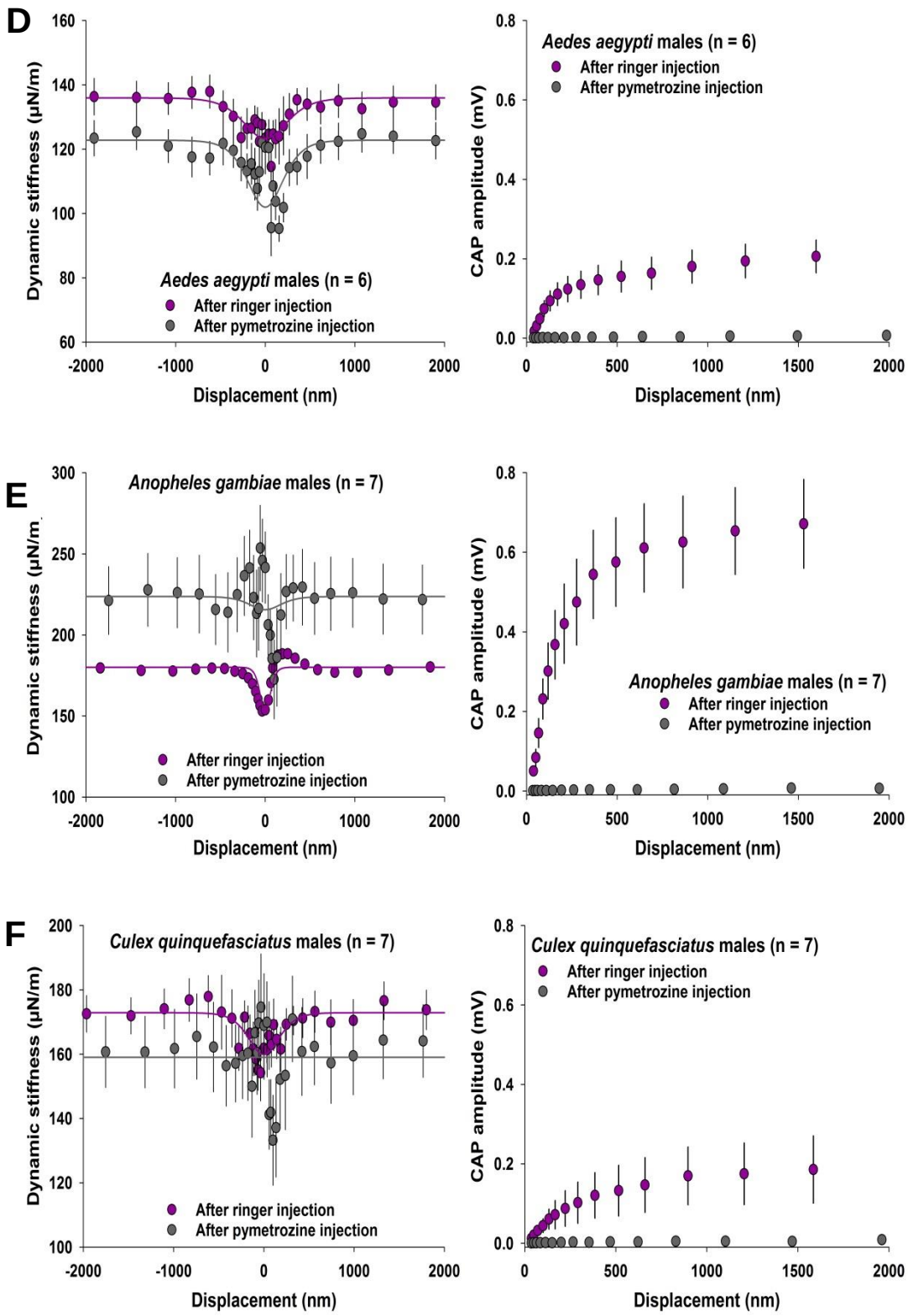
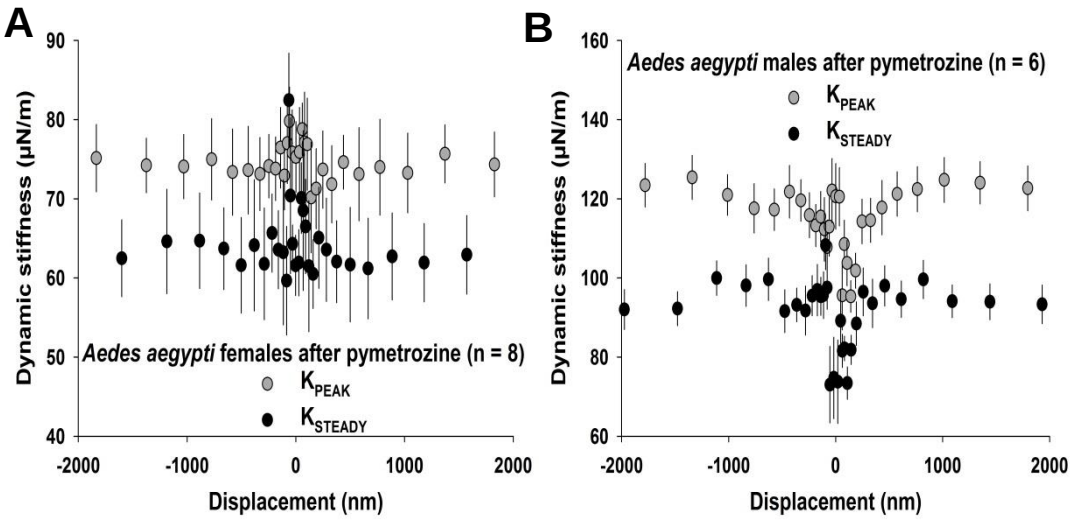


Figure 55. Changes in dynamical stiffness and CAP amplitude in response to changes in antennal displacement to a maximum displacement of ±2000nm for each of the mosquito species and sexes investigated both after ringer and after pymetrozine: A) Ae. aegypti females, B) An. gambiae females, C) Cx. quinquefasciatus females, D) Ae. aegypti males, E), An. gambiae males and F) Cx. quinquefasciatus males.

8.3.3.2. Changes to dynamical stiffness properties following pymetrozine injection

As can be seen directly from figure 56, pymetrozine injection significantly reduced dynamical stiffness changes in not only K_{PEAK} but also K_{STEADY}; this is equivalent to the results obtained from the earlier sedation experiments discussed in section 7.3.4.4.

This reduction was different between male and female mosquitoes however – whilst K_{PEAK} and K_{STEADY} became almost completely linear following pymetrozine injection for female mosquitoes from all three species investigated, K_{STEADY} estimates for all three male mosquito types seemed to show a systematic increase in compliance around the resting position. This increase in compliance for K_{STEADY} was completely different however to the changes seen for this stiffness parameter before pymetrozine however, with figure 37 from section 7.3.4.4 showing that for all male mosquitoes tested there was a significant increase in dynamic stiffness for K_{STEADY} for small stimuli. Indeed, this increase in dynamic stiffness formed the basis for the discussion regarding the potential compensatory mechanisms of stiffness to prevent changes in flagellar frequency tuning in section 7.4.4.



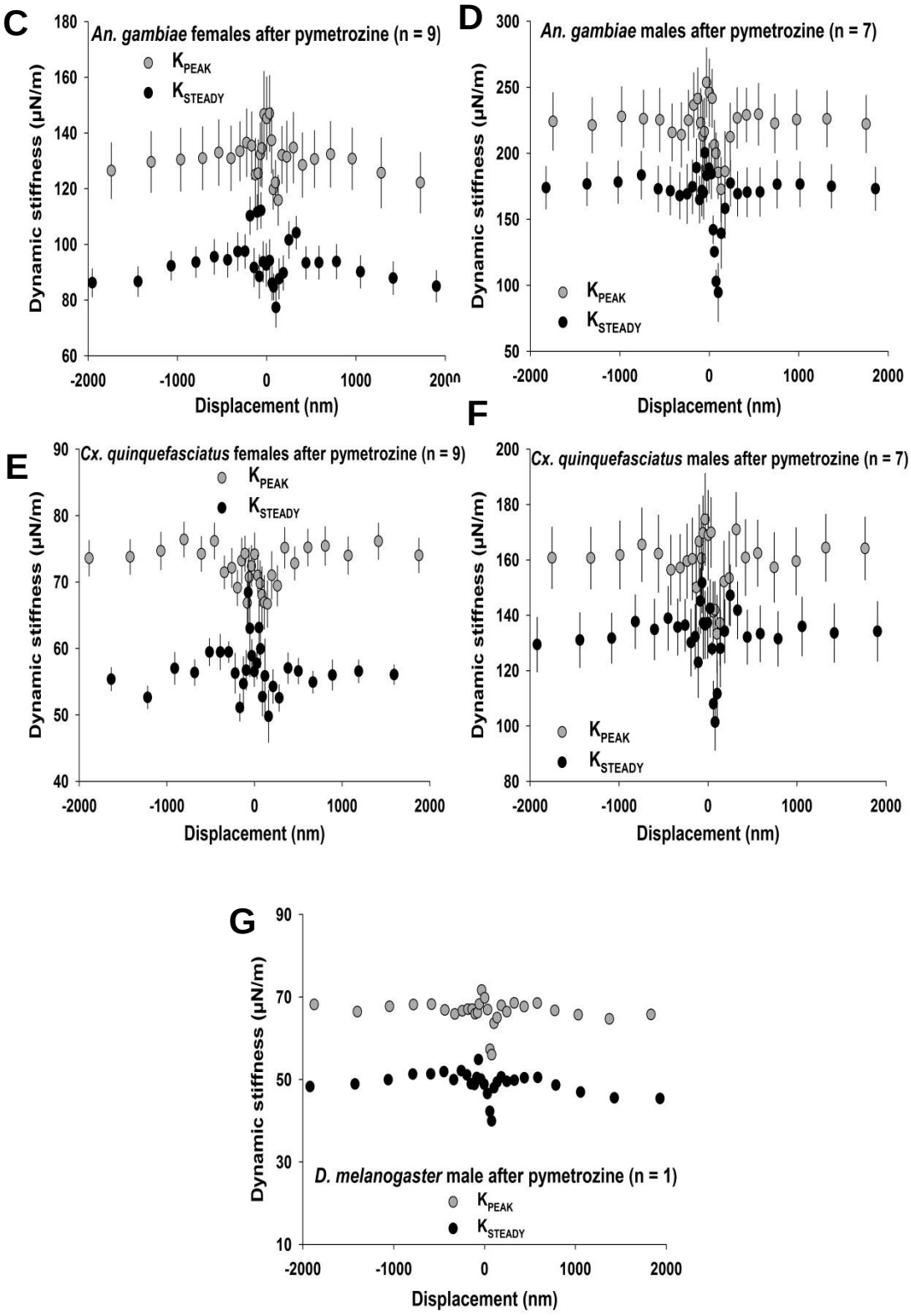


Figure 56. Changes in dynamical stiffness in response to flagellar displacement after pymetrozine injection plotted using either values calculated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}) for: A) Ae. aegypti females, B) An. gambiae females, C) Cx. quinquefasciatus females, D) Ae. aegypti males, E), An. gambiae males, F) Cx. quinquefasciatus males and G) a Drosophila melanogaster male.

8.3.4. Step recordings – pymetrozine injections with insecticide resistant lines

8.3.4.1. Force step electrophysiological recordings for insecticide resistant mosquitoes before pymetrozine exposure

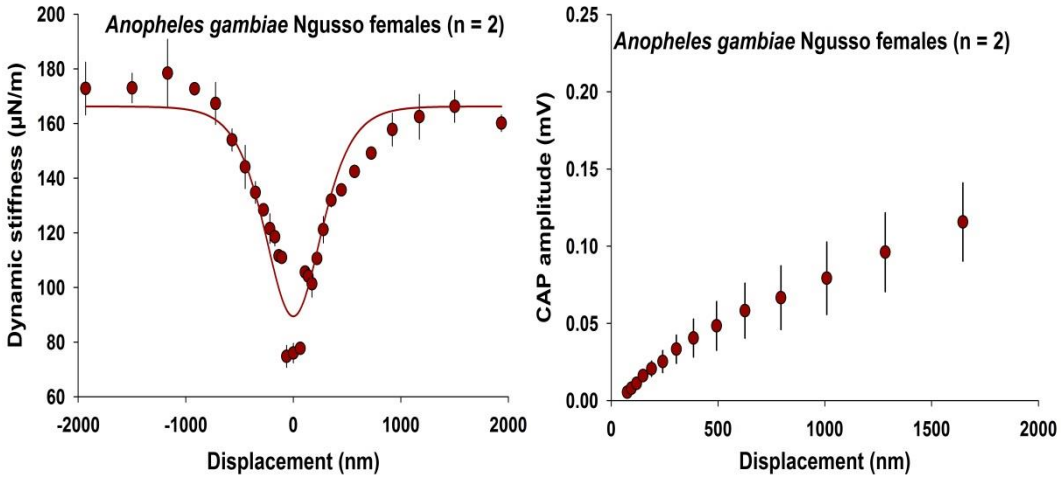
The two insecticide-resistant strains of *An. gambiae* (Ngusso and Tiassale) have broadly similar nerve responses and flagellar dynamical stiffness changes as the susceptible Kisumu strain (figure 57 shows the full range of dynamics stiffness changes and CAP production whilst table 25 contains the median two-state model parameters for a single transducer system).

Male mosquitoes from both strains again demonstrated significantly greater CAP responses to stimulation and seemed much more sensitive (in terms of compliance increases) to small displacements than females. The dynamical stiffness changes in response to stimulated displacements for female *An. gambiae* mosquitoes from both strains fall much more in line with previous studies in *Drosophila melanogaster* than the male results, as was also the case for the Kisumu strain, with the nonlinearity estimates for females being almost three times greater than for males.

Table 25. Median values for two state model of a single transducer population parameter estimates for both An. gambiae Ngusso and Tiassale strains, grouped by sex (values in brackets are standard errors).

	Number of ion channels	Ion channel gating force (fN)	$K_{INIFNITY}$ (µN/m)	K_{STEADY} (µN/m)	K_{GS} (µN/m)	Extent of non-linearity
Ngusso female (n = 2)	2320.5 (646.5)	24 (2.1)	166 (7.3)	116 (5.2)	51 (2.1)	0.468 (0.032)
Tiassale female (n = 6)	1489.6 (352.4)	25 (2.3)	149 (29)	109 (17)	41 (12)	0.361 (0.045)
Ngusso male (n = 3)	46.1 (16.4)	81 (38)	214 (30)	148 (30)	61 (1.7)	0.114 (0.030)
Tiassale male (n = 5)	119.8 (121.9)	57 (9.3)	212 (9.9)	148 (7.0)	59 (3.7)	0.103 (9.8 x10^{-3})

A



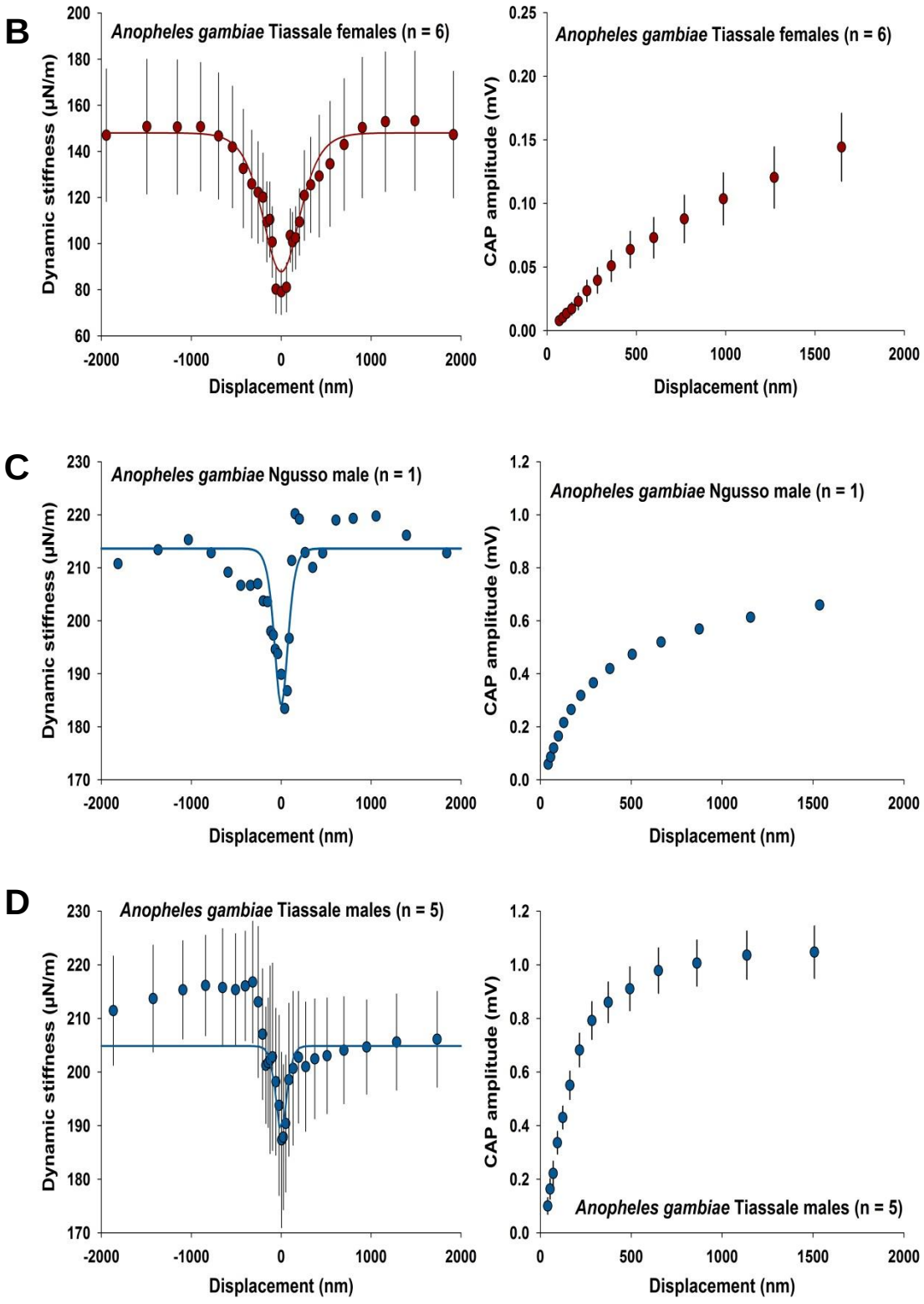
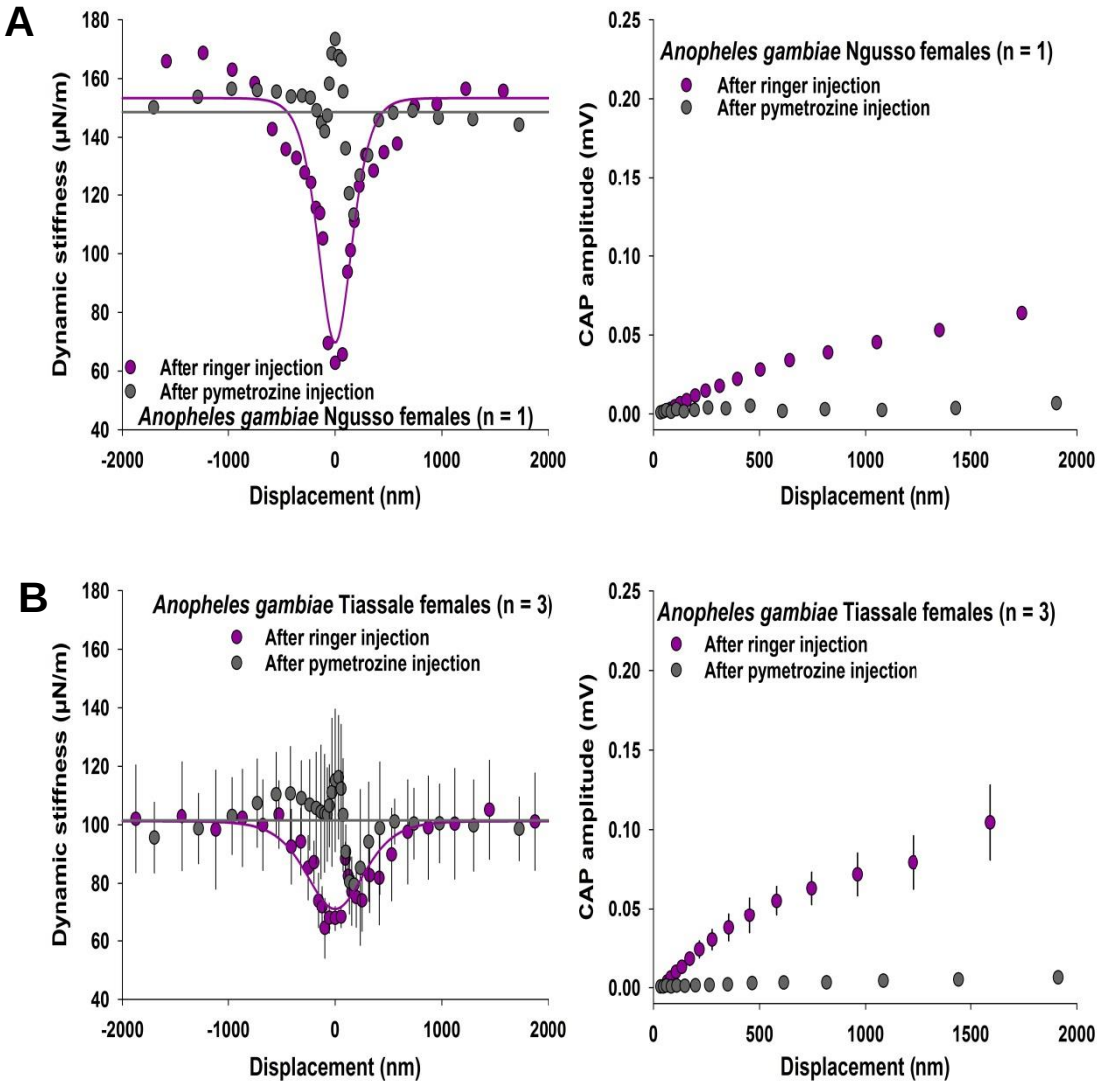


Figure 57. Changes in dynamical stiffness and CAP amplitude in response to changes in antennal displacement to a maximum displacement of ±2000nm for each of the An. gambiae strains and sexes investigated: A) Ngusso females, B) Ngusso males, C) Tiassale females and D) Tiassale males. The bold lines represent two-state gating spring model fits (for a single transducer population) to median data points.

8.3.4.2. Force step electrophysiological recordings for insecticide resistant mosquitoes following pymetrozine exposure

Pymetrozine injection abolished CAP responses to stimulation in both sexes of both insecticide resistant *An. gambiae* strains (as shown in figure 58), although the low sample size used for all groups means that conclusions should be made cautiously. K_{STEADY} remained constant between post-ringer and post-pymetrozine states in females from both strains, whilst only the *An. gambiae* (Ngusso) males seemed to show an increase post-pymetrozine inject (with table 26 providing a full list of stiffness parameter values). Only one such male mosquito was injected with pymetrozine however so no firm conclusions can be drawn from this, except that pymetrozine seemed able to abolish auditory transduction in this specific individual male.



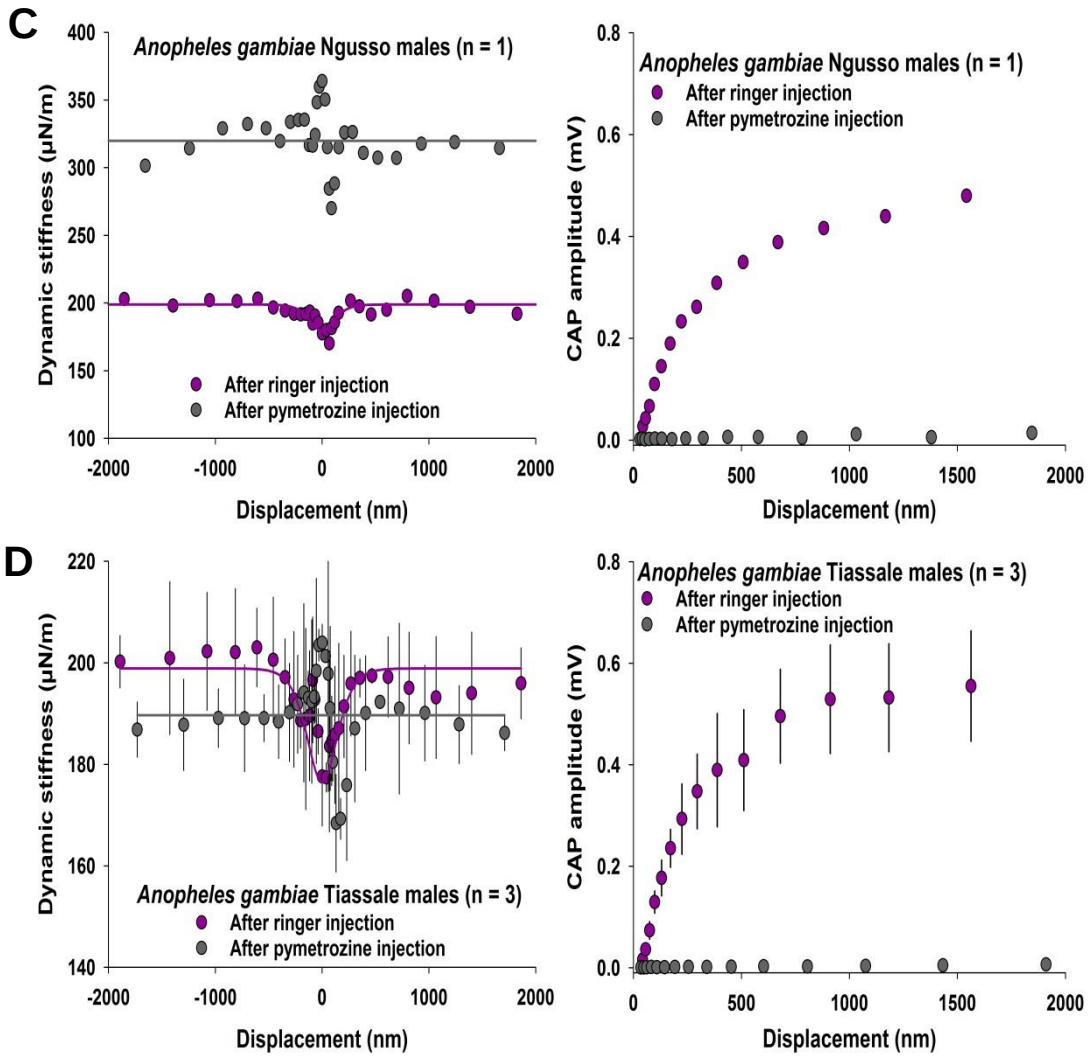


Figure 58. Changes in dynamical stiffness and CAP amplitude in response to changes in antennal displacement to a maximum displacement of ±2000nm for each of the An. gambiae strains and sexes investigated after ringer and after pymetrozine: A) Ngusso females, B) Ngusso males, C) Tiassale females and D) Tiassale males. The bold lines represent two-state model of a single transducer population fits to the median data. Standard error bars (represented by black vertical lines) are included for Tiassale but not Ngusso because for that strain only one injection experiment was recorded for both sexes.

Table 26. Median values for dynamic stiffness parameters before and after pymetrozine injection for An. gambiae (Ngusso and Tiassale) mosquitoes (standard errors are given in brackets were possible, with P-values for comparisons provided for male and female Tiassale mosquitoes).

	Ngusso female (n = 1)	Tiassale female (n = 3)	Ngusso male (n = 1)	Tiassale male (n = 3)
$K_{INFINITY}$ after ringer (µN/m)	153	127 (24.2)	199	203 (22.6)
$K_{INFINITY}$ after pymetrozine (µN/m)	149	121 (17.2)	317	195 (16.7)
Significance value	N/A	P>0.05	N/A	P>0.05
K_{STEADY} after ringer (µN/m)	113	94 (15.4)	121	146 (17.4)
K_{STEADY} after pymetrozine (µN/m)	104	86 (10.5)	224	146 (15.2)
Significance value	N/A	P>0.05	N/A	P>0.05
K_{GS} after ringer (µN/m)	40	33 (8.9)	78	68 (6.8)
K_{GS} after pymetrozine (µN/m)	45	35 (7.1)	93	57 (3.4)
Significance value	N/A	P>0.05	N/A	P>0.05

8.3.5. Mosquito flagellar sensitivity to pure tone stimulation following pymetrozine injection

Figure 59 demonstrates the effect that pymetrozine was able to have not only CAP production in response to pure tone stimulation but also on mosquito flagellar sensitivity; the male *Cx. quinquefasciatus* shown in the figure went from a relative gain of about 10 to approximately 3 following pymetrozine injection.

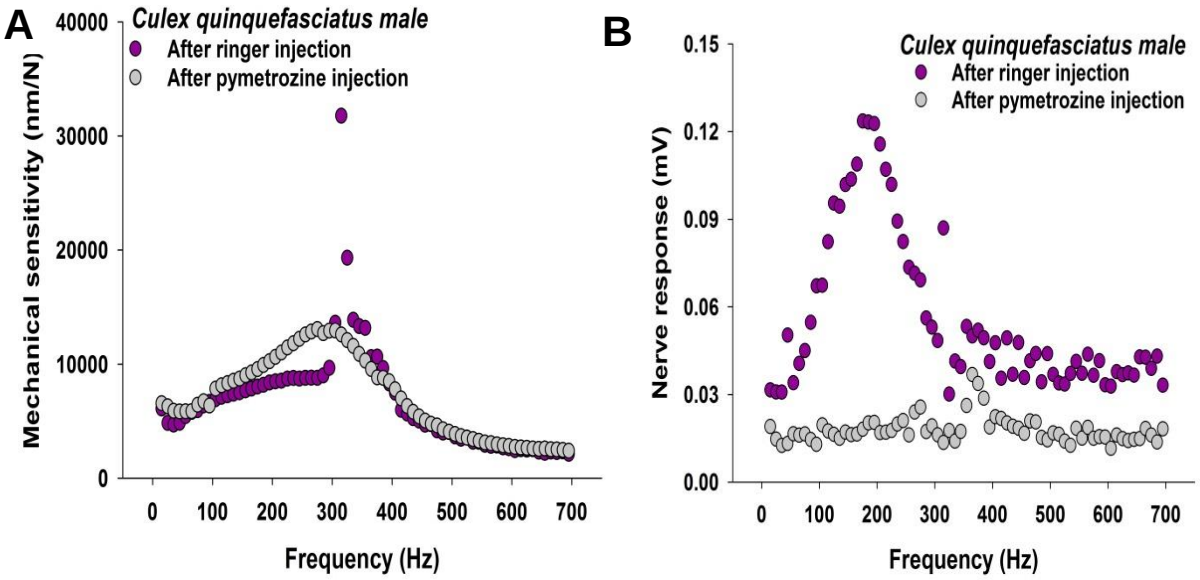


Figure 59. A) Changes in mechanical sensitivity in response to pure tone stimulation both post-ringer and post-pymetrozine injection for a male Cx. quinquefasciatus. B) Nerve response to pure tone stimulation post-ringer and post-pymetrozine injection for a male Cx. quinquefasciatus mosquito.

Pymetrozine injection was also able to ablate the modulatory effect evident at the onset of the pure tone stimulus seen for *Cx. quinquefasciatus* males in figure 60 part A. This flagellar modulation of stimuli, which appears to be male specific, only occurred at frequencies around the peak flagellar frequency and showed the level of immediate increased gain that occurs before the system settled into a steady state. This effect was not seen in post-pymetrozine injected males (as shown in figure 60 part B), and was also absent essentially entirely from females in either a pre- or post-pymetrozine state.

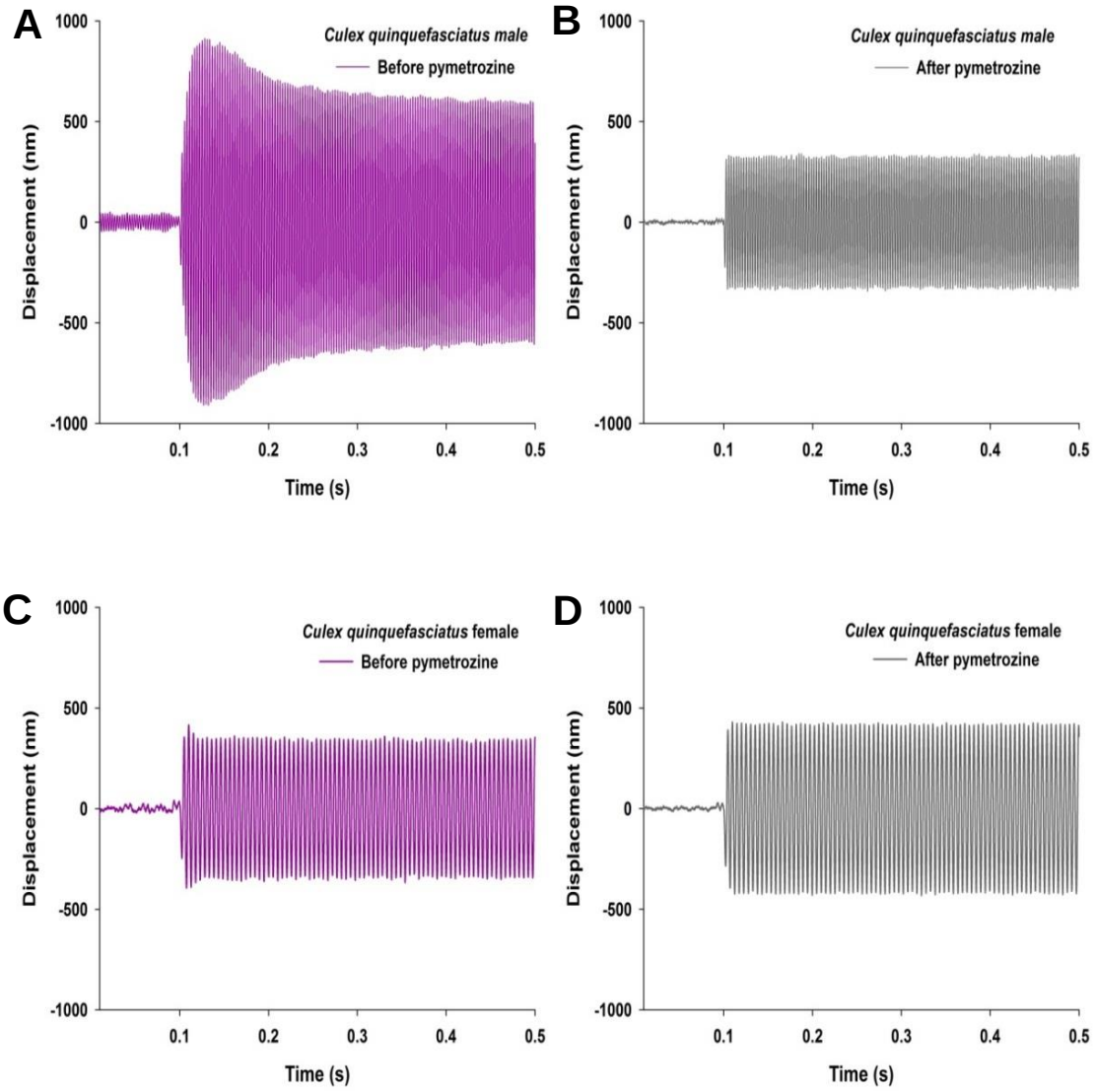


Figure 60. A and B) Flagellar displacement following the onset at 0.1s of a pure tone stimulus (315 Hz) in the pre-pymetrozine (A) and post-pymetrozine (B) states in a Cx. quinquefasciatus male.

C and D) Flagellar displacement following the onset at 0.1s of a pure tone stimulus (215 Hz) in the pre-pymetrozine (C) and post-pymetrozine (D) states in a Cx. quinquefasciatus female.

8.4. Discussion

8.4.1. The effect of pymetrozine ingestion on mosquito auditory systems

Figure 50 demonstrates that all females investigated showed a complete loss of CAP responses to stimulation as well as an abolition of the expected decrease in dynamic stiffness around the resting position shown for *Cx. quinquefasciatus* female mosquitoes in section 7.3.4.3 disappeared. This suggests that pymetrozine has successfully ablated ChO mechanosensory function (as measured by LDV), and therefore implies that ingestion of pymetrozine is sufficient to induce this phenotype.

As discussed in section 6 with regards to *Drosophila melanogaster* injection experiments, experiments utilising pymetrozine ingestion as the exposure method are in some ways preferable to injection experiments because the exposure method used in the field is compound ingestion. Injection experiments therefore could give misleading information as to the extent of pymetrozine's effect on an individual mosquito if insecticide resistance mechanisms only affect compound ingestion (with pymetrozine resistance following ingestion having been documented in several species for example) (321, 322). That similar results were seen for both exposure methods, as observable when comparing the results of sections 8.3.1 and 8.3.3, the *Drosophila melanogaster* results presented in section 6 and previous reports, provides validity to the injection experimental results (27).

Only three female mosquitoes were tested in this section and all were from the same species. In order to confirm that pymetrozine ingestion reliably produces this phenotype across all mosquito species and sexes tested, more replicates must be done involving not only *Ae. aegypti* and *An. gambiae* mosquitoes but also *Cx. quinquefasciatus* males.

The concentration of pymetrozine used in these tests was also at an extremely high, saturating level – exposing mosquitoes to lower concentrations of the compound could therefore provide useful estimates with regards to the minimum exposure concentration necessary for an effect to be observable when the mosquito was tested using the LDV protocol. However, since the compound ingestion protocol does not allow for comparison with a pre-pymetrozine state it would be difficult to estimate an individual baseline for comparative purposes. Proper insertion of the recording electrode would also become more challenging as it could theoretically be possible that the absence of CAP responses is the result of not finding the antennal nerve rather than the effect of pymetrozine.

8.4.2. The effect of pymetrozine injection on mosquito auditory systems – free fluctuations

For all *Drosophila melanogaster* males tested in sections 4 and 6.3.1 (with an example provided in figure 51), pymetrozine exposure resulted in a significant increase in best frequency, from around 200 to 500Hz, in addition to decreases in the maximum antennal velocity amplitude and *Q*. Although decreases in these two parameters were seen for all mosquitoes tested as well, not all mosquitoes showed a significant change in best frequency after exposure – for example, there were no significant changes in best frequency calculated for male *Ae. aegypti* and the significant differences observed for female *Ae. aegypti* and *Cx. quinquefasciatus* involved frequency changes less than 15Hz and as such may only be statistically (rather than biologically) significant (484, 485).

For *Cx. quinquefasciatus* males and both sexes of *An. gambiae*, changes in flagellar best frequency were identical to those seen for the passive, sedated state. This stands in contrast to *Drosophila melanogaster*, for whom pymetrozine exposure results in a significantly different antennal state than that of sedation (as seen when comparing the pymetrozine injected *Drosophila melanogaster* presented in this thesis to previous reports of sedated flies (283, 284)). This strong frequency selectivity and maintenance is indicative of the differences between mosquito and *Drosophila melanogaster* auditory systems.

The difference in velocity amplitude fit function parameters between pymetrozine exposed and sedated states in *Drosophila melanogaster* allows for immediate validation that whilst the antennal system has been affected by pymetrozine, it is in a distinctly different state to that of sedation. For mosquito species however that comparison is much more difficult to make as there were few, if any, significant differences calculated between the sedated and pymetrozine exposed state.

Free fluctuation recordings for mosquitoes exposed to pymetrozine via the ingestion protocol (in section 8.3.1) showed similar peak antennal velocities and best frequency estimates to those injected with the compound, though pre-pymetrozine states could not be measured; thus the possibility remains that changes to the auditory system were the result of some factor other than insecticide ingestion.

Overall therefore, whilst recording free fluctuation measurements of the antennal ear appears to be a reasonable method of confirming the exposure of a mosquito to pymetrozine, comparisons between different auditory states require a greater level of caution for mosquito species than *Drosophila melanogaster*.

8.4.3. The effect of pymetrozine injection on mosquito auditory systems – force step stimulation and compensatory stiffness mechanisms

Compared to the male and female *Ae. aegypti* flagellar responses shown in section 7.3.4.1 (which contains the same force step stimuli for mosquitoes unexposed to pymetrozine) and to the previously reported responses for control *Drosophila melanogaster*, the active processes involved in the recoil of the flagellum after the initial overshoot are far less evident in section 8.3.3 with significantly less swinging of the flagellum taking place following stimulus onset (285). Whereas in a pre-pymetrozine state the receiver displacements in response to the smallest stimuli showed a high level of initial amplification when compared to the largest force steps, this is no longer evident after pymetrozine exposure, as each step response appears identical when adjusted for relative displacement.

The ablation of auditory CAP response to stimulation is evident for all stimuli, in agreement with the pymetrozine injection experiments using insecticide resistant *Drosophila melanogaster* lines in section 6; this indicates a loss of ChO mechanosensory function.

In contrast to the results calculated for *Drosophila melanogaster* in section 6 only one mosquito type studied (*Ae. aegypti* males) showed a statistically significant change in $K_{INFINITY}$ following pymetrozine injection, although female *An. gambiae* and *Cx. quinquefasciatus* mosquitoes did show significant changes to their estimates of K_{STEADY} and K_{GS} following pymetrozine exposure. This could be the result of the systems of compensatory stiffness that were earlier investigated in section 7.3.4, which means that following compound injection changes to stiffness parameters are more difficult to identify.

However, these compensatory systems were also significantly changed following pymetrozine exposure – rather than being almost anti-symmetrical to the values calculated for K_{PEAK} (with the notable exception of *An. gambiae* females), K_{STEADY} now seems almost identical to K_{PEAK} apart from a constant vertical shift. Indeed, calculating K_{PEAK} - K_{STEADY} (as was done in section 7.3.4 for control mosquitoes) now produces an essentially constant estimate of stiffness across all displacements values for male and female mosquitoes from all species tested (with differences in maximum and minimum values of less than 10μN/m), which is indicative of the ablation of ChO mechanosensory function by pymetrozine.

The results shown in figure 56 are highly similar to those presented in section 7.3.4.4 for K_{STEADY} whilst the mosquito was sedated, providing further evidence that K_{STEADY} is the result of some active process (with estimates of K_{PEAK} - K_{STEADY} for sedated mosquitoes are almost identical to those of pymetrozine exposed mosquitoes).

Significant increases in the effective stiffness of the antenna following both sedation and pymetrozine injection were calculated in sections 7.3.2 and 8.3.2 for males and females from all three mosquito species investigated, which is suggestive of the key distinctions between effective stiffness (which includes all stiffnesses present in the entire antennal system) and dynamic stiffnesses (which provide specific information on the changes in stiffness for specific parameters over changes in flagellar displacement).

The significant increases in effective stiffness calculated following either sedation or pymetrozine exposure (as compared to the active, pre-pymetrozine state) were statistically indistinguishable for male and female mosquitoes from all three species tested. The only exception to this was the comparison made for *Ae. aegypti* females, for whom a significantly greater effective stiffness was calculated whilst mosquitoes from this group were in the sedated state than if they had been exposed to pymetrozine.

Given the lack of significant differences observed for other mosquito groups, as well as the absence of significant differences between sedated and pymetrozine exposed states identified in terms of the best frequency (as shown in appendix M), it seems that whilst this difference may be statistically significant it may not be biologically relevant.

8.4.4. Auditory systems of insecticide resistant mosquito species and the effect of pymetrozine injection on these systems

In general the auditory systems (as quantified by the LDV paradigm used throughout this thesis) of male and female *An. gambiae* from the Ngusso and Tiassale lines showed marked similarities to those of the *An. gambiae* Kisumu mosquitoes tested in section 7.3. This suggests that the development of different forms of insecticidal resistance has not led to changes in the auditory system that can be detected using this measurement system – given the low number of mosquitoes tested from the *An. gambiae* Ngusso and Tiassale lines however these comparisons must be made cautiously.

The extremely low CAP amplitudes produced by *An. gambiae* Kisumu females, as shown in section 7.3.4.3, could potentially have been the product of adaptation to cage conditions (although all female *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* mosquitoes tested in section 7 and section 8 had been kept in laboratory conditions for approximately the same length of time) – these adaptations can lead to alterations in mosquito physiology as a result of changes in selective pressure as well as the bottleneck formed by only a handful of female mosquitoes producing the majority of offspring (486). Such adaptations to laboratory conditions have been identified as having an impact on flight performance, as well as associated activities, in both *Drosophila melanogaster* and mosquito species (487, 488).

Both Tiassale and Ngusso strains utilised for experiments however also demonstrated relatively small CAP responses to stimulation in section 8.3.3 despite only been kept in laboratory cages for three generations, making it less likely these lines had fully adapted to cage conditions (for example, these colonies had yet to adapt to blood feeding via the Hemotek system and were still provided blood meals from human volunteers). It also seems implausible given the extremely large number of electrophysiology experiments completed using *An. gambiae* females from all lines throughout this thesis that this is the result of repeated incorrect insertions of the recording electrode.

It is therefore possible that this low level of nerve response to stimulation relative to females from other species is a species-specific feature of *An. gambiae* females, especially given the increased genetic differences between *An. gambiae* and the other two species investigated (as compared to the same differences between *Ae. aegypti* and *Cx. quinquefasciatus*). For example, a decreased focus on audition in female mosquitoes could have resulted in males injecting a greater amount of compensatory focus into their own auditory systems, with the reverse explanation also being plausible.

Female *An. gambiae* mosquitoes clearly retain some sensory capabilities with regards to auditory stimulation however as harmonic convergence is still the primary courtship mechanism in this species (353).

This hypothesis could be tested by repeating the experiments vibrometry experiments conducted in sections 7 and 8 for male and female *An. gambiae* caught in the wild and as such had not had an opportunity to adapt to laboratory conditions. Testing other *Anopheles* species, such as *An. arabiensis*, could also be useful for comparative purposes. In addition to this, more male and female mosquitoes from the insecticidal resistant lines (particularly Ngusso) could be tested to avoid problems associated with drawing conclusions from such a small sample size.

Following pymetrozine injection there was an elimination of CAP response to stimulation and a reduction of the increases in compliance around the resting position seen before exposure in all mosquitoes tested. These changes are in agreement with the results shown in section 8.3.3 for mosquito species that are susceptible to all insecticides. The low sample sizes used for the insecticide resistant lines however prevent concrete conclusions from being drawn – more repeats of the experiment are necessary before firm conclusions can be made.

In addition to this, the method of pymetrozine exposure used in section 8.3.4 was compound injection; as mentioned previously, given that the regular field exposure method of pymetrozine is oral ingestion, experiments investigating the impact of pymetrozine feeding are needed to ensure that both pymetrozine ingestion and injection result in the same effect on the mosquito auditory system. This would also help to guide estimates of suitable concentrations of pymetrozine for use in field situations that would have a significant impact on mosquito populations.

At the very least however, taken together with the earlier results from injecting pymetrozine into insecticide resistant *Drosophila melanogaster* lines, the effectiveness of pymetrozine when used against insects (including both mosquitoes and *Drosophila melanogaster*) that have developed significant levels of insecticide resistance is highly promising and suggests that further experiments be initiated in order to look in more detail at pymetrozine's effectiveness and efficacy against other insecticidal resistant mosquito lines.

8.4.5. The effect of pymetrozine injection on displacement gain as calculated in mosquito auditory systems via pure tone stimulation

The time period over which the potential modulation of pure tone stimuli presented in section 8.3.5 occurs is slightly under 100ms, placing it within the time scale of possible efferent feedback loop control (which can vary extensively according the requirements of the system) (489, 490). As discussed with regards to TTX and TeNT injections in section 7.4.7, efferent modulation has been reported in male *Cx. quinquefasciatus* mosquitoes and it has been suggested that this modulation is linked to identifying flight tones produced by females and is potentially related to mechanical control of sensitivity and the quality factor Q (as described in terms of the free fluctuation velocity fit function in section 6.2.6) (290, 314). This could imply that the modulation of pure tone stimuli is done under the control of an efferent feedback loop.

Female *Cx. quinquefasciatus* have been suggested to also have auditory efferent feedback loops, with the results of TTX/ TeNT injection presented in section 7.3.7 supporting this conclusion (314). In spite of this, flagellar amplitude modulation of pure tone stimuli is at best minor for females from this species, especially when compared to males. This could be indicative once again of the different roles filled by efferent feedback for both sexes.

The elimination of the potential modulation seen in male mosquitoes following pymetrozine exposure is therefore highly interesting, both in terms of the impact that the compound could have on courtship attempts (as males may no longer be able to identify female flight tones) as well as the mechanism by which this modulation is abolished following pymetrozine injection. Pymetrozine's mechanism of action (deterioration of ChO neurons due to a surge in the influx of calcium ions) could directly cause damage to the systems of efferent control for example, or the abolition of ChO mechanosensory function could prevent further mechanisms of control from being initiated (27).

The evidence provided in section 8.4.5 is based on exploratory testing of both this modulation of pure tone stimuli and the impact of pymetrozine on this modulation, and as such would require further testing before statistically valid conclusions could be made. It is also important to test male and female *Ae. aegypti* and *An. gambiae* mosquitoes in order to investigate the potential existence of this modulation in other mosquito species for which efferent feedback loops have not yet been reported (especially in light of the conclusions drawn from section 7.3.7, in which no evidence of efferent feedback could be identified for either female *Ae. aegypti* or *An. gambiae* mosquitoes).

9. Conclusions

9.1. Discussion

9.1.1. Conclusions of behavioural experiments

Pymetrozine has previously been reported to target insect ChOs, with compound exposure leading to an ablation of the organ's mechanosensory function (26, 27). Behavioural assays conducted within this thesis strongly support these previous reports and also explored the effects that a loss of ChO mechanosensory function can have on *Drosophila melanogaster*, with significant reductions in both flight ability and reproductive fitness observed in section 4.

ChOs, and by extension mechanosensation, have been regarded as inessential for the survival of *Drosophila melanogaster* because of the continued viability of ChO mutants (316, 317). This could be seen in the lifespan assay presented in section 4.3.2, with some pymetrozine exposed *Drosophila melanogaster* able to survive as long as control flies. These assumptions should be placed in the context of laboratory conditions however, where there is abundance of available food and no predators. The competitive fitness analysis presented in section 4.3.7 demonstrated that as soon as competition was introduced to the environment then the severe disadvantages associated with a loss of mechanosensation became more apparent.

Such competitive fitness comparisons were not made for mosquito species however, and the experiment described in section 5.3.2 investigating the effect of pymetrozine on *An. gambiae* female fertility and fecundity did not contain sufficiently large group sizes to adequately assess the compound's potential. The issue of small group sizes was also a problem for the larvicidal investigation detailed in section 5.3.1. Experimental repeats are therefore necessary before concrete recommendations regarding pymetrozine can be made.

Drosophila melanogaster are partly able to adapt to a loss of mechanosensation because the mechanism of feeding in this species does not require the insertion of a stylus and as such could be considered not significantly challenging from a mechanosensory control perspective (particularly when compared to mosquito species) (491, 492). Whilst male and female mosquitoes do not need to insert their proboscis into plants in order to feed from sources of glucose, such insertion is necessary for females to acquire a blood meal, which suggests that pymetrozine may have a stronger effect on mosquito species (in terms of female feeding behaviour) than on *Drosophila melanogaster* (493).

The decline in flight ability noted for *Drosophila melanogaster* (in sections 4.3.3 and 4.3.4) following pymetrozine exposure is also highly promising with regards to potential compound effects on mosquito species, with flight forming an essential component of host seeking actions for female mosquitoes (336). If these decreases are also found to be present for mosquito species, which seems likely given the results presented in this thesis and the presumed involvement of the mosquito JO in mosquito flight regulation, pymetrozine could have a highly significant impact on host seeking behaviour (76, 333). These assessments could be made for mosquito species using modifications to the experiments conducted with *Drosophila melanogaster* in the Flycube system described in section 4.2.8, or by utilising other published systems of monitoring mosquito flight behaviour (401, 448).

Overall therefore the behavioural experiments presented in section 4 suggested that pymetrozine exposure had a significant impact on various aspects of the *Drosophila melanogaster* lifecycle and could thus also have a strong negative effect on mosquitoes – these experiments need to be conducted in mosquito species however before firm conclusions can be made with regards to the translation of these effects to other species.

9.1.2. Conclusions of electrophysiological experiments

Sexual dimorphisms in auditory function between male and female mosquitoes have been repeatedly reported - most commonly however, such reports have covered the increased sensitivity of the male flagellum as compared to the female, or have focused on male-specific spontaneous oscillations of the flagellum (34, 289, 363). The analyses provided in this thesis (particularly with regards to the calculated displacement and energy gains discussed in section 7.3) suggest that in a quiescent state, female *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* mosquitoes can demonstrate equivalent (if not greater) levels of mechanical sensitivity to conspecific males.

In general however the level of energy gain calculated for both male and female mosquitoes in section 7.3.1 was of a relatively low level when compared to *Drosophila melanogaster* (283). This may be the result of laser measurements taken in a single plane and thus not accounting for the energy input of other prongs. Once male mosquitoes began to display spontaneous oscillations of their flagellum following TTX or TeNT injection however the calculated energy gains became far greater than those of *Drosophila* species.

That in the unstimulated state male mosquitoes do not make full use of their amplification machinery is in some ways comparable to *Drosophila melanogaster*, who also do not seem to fully exploit the maximum potential of their amplification machinery; energy gains following DMSO injection have been reported as approximately ten times larger than the equivalent gains calculated prior to injection, although it could be the case that DMSO changes the physiology of the fly such that energy gains that could not be attained otherwise become possible (283). Similarly, for nan^{36a} and iav^{1} mutants energy gains have been calculated which are an order of magnitude greater than those of control flies (though still far less than energy gains calculated in this thesis for male *An. gambiae* mosquitoes) (27).

This control and adjustment of energy gain could serve a crucial purpose if its role is to keep the system stable near a critical point of oscillatory instability whilst maximising the antennal sensitivity (494). This critical point (a Hopf bifurcation) has been reported for many different species, including fruit flies, mosquitoes and vertebrates (279, 290, 495). The recent identification of efferent signalling networks in the auditory system of male *Cx. quinquefasciatus* could indicate the potential involvement of efferent control mechanisms with regards to modulation of mechanical sensitivity and energy gain (290, 314).

Maintenance of frequency specificity (in terms of audition) is highly important for both male and female mosquitoes (34, 276). The comparative changes in both K_{PEAK} and K_{STEADY} calculated in section 7.3.3 suggest that for the mosquito species tested in this thesis, the mosquito auditory system is composed of an interlocking system of balancing forces that seek wherever possible to maintain a constant frequency but can also inject a significant amount of energy when required.

A relatively higher degree of similarity (in terms of the measurements of auditory function conducted) was observed between *Ae. aegypti and Cx. quinquefasciatus* females than between either one of these species and *An. gambiae* females; these similarities included the minor (at most) differences in the flagellar best frequency observed between active and sedated or pymetrozine states, results of the single transducer population model fit, displacement gain estimates obtained via pure tone stimulation and comparative sizes of CAP responses to stimulation (shown in sections 7.1 – 7.6).

By comparison *An. gambiae* females seemed distinctly different, underlined by the observed increases in flagellar best frequency observed after pymetrozine injection and the significantly greater extent of nonlinearity calculated. These similarities and/or differences may be the result of *Ae. aegypti and Cx. quinquefasciatus* belonging to the same sub-family (the Culicinae) whilst *An. gambiae* belongs to a different group (the Anophelinae) (56, 76).

However such a level of similarity was not evident for male *Ae. aegypti* and *Cx. quinquefasciatus* with regards to the previously mentioned results – this perhaps suggests that species specific differences in auditory systems have evolved to lie within differences between male mosquitoes rather than females (34).

The uniting factors across both male and female mosquitoes from each of the three species investigated however were the mechanical signatures of transducer gating that were identified in each individual mosquito tested. These signatures included a nonlinear compliance and adaptation to force step stimulation, both of which can be seen 7.3.3 (particularly in the return of the nerve response to baseline following adaptation, rather than engaging in constant neuronal firing or displaying a shift from baseline) (282, 285).

9.2. Critique

9.2.1. Experimental critique

All experiments utilising mosquitoes in sections 5, 7 and 8 used mosquitoes from laboratory colonies. Although the *An. gambiae* Ngusso and Tiassale lines had only been kept in laboratory conditions for several generations, the *Ae. aegypti*, *An. gambiae* (Kisumu) and *Cx. quinquefasciatus* mosquitoes used had been kept as colonies for far longer (and indeed the length of time necessary for adaptation to laboratory conditions to take place is unclear, meaning that several generations may be sufficient for significant changes to have appeared). Whilst this made mosquito rearing in general easier (as blood feeding could be done via the Hemotek system) and allowed for logistical issues to be dealt with quickly, potential adaptations to laboratory conditions can lead to significant differences forming between field mosquitoes and those used for testing (496, 497).

This is particularly important in this case because adaptation to laboratory conditions could entail changes in flight ability or auditory function, two of the major focal points of this thesis. Should there be significant differences between laboratory and field populations with regards to these two important aspects then the results of the behavioural and electrophysiological investigations into hearing should be treated cautiously. Given the conservation of Nanchung and Inactive across species and the initial testing done using insecticidal resistant mosquito lines it seems unlikely that pymetrozine would be completely ineffective on field mosquitoes - the efficacy, or the effect on mechanosensory behaviours, may be significantly altered however (318).

A number of experiments described in section 4 involving *Drosophila melanogaster* utilised CO_2 sedation in order to select virgin flies following eclosion. Whilst CO_2 sedation makes this process relatively straightforward, it can also create experimental problems because of reported after-effects of this type of sedation on *Drosophila melanogaster* behaviour (422, 498, 499). Utilising ice sedation (as was done for many other experiments throughout this thesis, particularly when such experiments involved mosquito species) should minimise the potential effects of sedation and therefore should be utilised for all future experimental procedures.

Testing tethered, solo-flying mosquitoes provided estimations of the WBF for male and female *Ae. aegypti* and *Cx. quinquefasciatus* that were broadly in line with previous reports (as seen in section 5.3.5) (352, 354). Although the results of the same estimations for male and female *An. gambiae* mosquitoes were lower than those given in the literature,

the ratio of male to female WBF remained constant at around 1.5 (353). However, assessing these measurements using individuals does not allow for judgement of a number of variables only assessable when multiple mosquitoes are present; examining WBF modulation and variability for example requires pairs of mosquitoes that interact with one another (34).

In addition to this, the processes involved in preparing tethered mosquitoes (namely the application of glue) could also have led to changes in flight tone characteristics, with experiments using tethered mosquitoes previously being suggested to result in lower WBF estimates than those using free flying mosquitoes (306). This could then have resulted in unreliable estimates of the WBF being made, with corresponding effects on the estimation of distortion products (which were utilised in section 7.3.6).

Interestingly, whilst the frequency of the male flagellar mechanical tuning was consistently at least 40Hz lower across all three species than would have been predicted from the WBF estimates (suggesting that there could indeed have been an issue with this method of measuring the WBF), both the estimated difference tone and the cubic distortion product were broadly in line with the experimental results of the relevant peak mechanical and nerve responses (as discussed in section 7.4.6).

Previous tests exploring harmonic convergence used either the introduction of pairs of tethered mosquitoes to each other or free flying arenas in which the entire courtship procedure could be observed (352, 357, 358). Such courtship interactions provided insight into what fundamental requirements were necessary for copulation to take place, particularly with regards to harmonic convergence, as well as providing information on the heritability of successful copulatory traits (34, 355).

No such experiments were included in this thesis and as such it remains to be seen whether male mosquitoes exposed to pymetrozine are able to harmonically converge with females from the same species (or whether exposed female mosquitoes are able to converge with control males). Without this key information it is difficult to judge exactly how effective pymetrozine could be when targeting mosquito species, though previous reports that deafened mosquitoes were unable to harmonically converge could suggest that pymetrozine exposed mosquitoes would also be unable to harmonically converge (352).

Similarly no trials were conducted to test the ability of pymetrozine exposed female mosquitoes to locate hosts and acquire blood meals; such assays exist and have been published previously (500, 501). These trials would also be essential before any further recommendations could be made regarding pymetrozine's potential as an insecticide targeting mosquito species.

9.2.2. Statistical and modelling critique

A recurring issue noted throughout this thesis was the problem of sample sizes that were insufficiently large enough to guarantee a reasonable level (i.e. at least > 80%) of statistical power. This meant for example that the lack of a significant difference in fertility between pymetrozine exposed *Drosophila melanogaster* and controls discussed in section 4.3.1 may in fact be due to the study being underpowered rather than there being no true difference between the groups (a problem further compounded by the distributions of egg laying habits reported for *Drosophila melanogaster*) (502).

Whilst post-hoc power calculations at least allowed for identification of statistical tests that may be considered underpowered, it would represent a significant improvement if future tests could take advantage of the results presented throughout this thesis and use them to estimate the minimum sample sizes needed to ensure that statistical tests are reasonably powered. The mosquito larvicidal assay (described in section 5.3.1) and the experiment assessing the potential effect of pymetrozine on mosquito fertility and fecundity (in section 5.3.2) for example would both benefit greatly from such an increase in group size, with both of these experiments being crucial for the basis of recommendations regarding pymetrozine's potential involvement in mosquito control programmes.

It is also important to consider the distinctions between statistical and biological significance; for example, female *Ae. aegypti* demonstrated statistically significant changes in flagellar best frequency between active and sedated states, but the absolute frequency difference was less than 10Hz. Distinguishing between the different forms of significance is crucial for accurate sample size calculation and for ensuring that conclusions drawn from the experimental analyses are biologically relevant (484, 485).

The velocity amplitude fit function used to analyse mosquito free fluctuation data in section 7.3 well described female mosquito flagellar free fluctuations (with R^2 values of over 0.95 for all fits included in this thesis) as well as male *Ae. aegypti* data (283). The function described male *An. gambiae* and *Cx. quinquefasciatus* mosquito flagellar fluctuations less well however because of differences in the best frequency of the active and passive system; the passive system was still observable in the free fluctuation recordings taken whilst a mosquito from one of these two groups was in the active state (as shown in section 7.3.1).

As such, modifications to this fit function may be necessary to account for these differences because these fit functions form the basis of energy gain and effective stiffness calculations and therefore it is essential that they describe the data as accurately as possible. In spite of this however, the fit function was still able to perform relatively well for male *An. gambiae* and *Cx. quinquefasciatus* mosquitoes (with all R^2 values calculated being greater than 0.85), suggesting that any necessary modifications may not need to be substantial.

The gating spring model of a single transducer population used in section 7.3.3 to analyse mosquito force-displacement data provided some interesting results as well as a basis for future investigations into mosquito auditory mechanotransduction, but is clearly not sufficient to fully describe such a complex auditory system (especially as it is not sufficient to describe the relatively simple *Drosophila melanogaster* system) (394). Initial attempts to use a more complicated model including multiple populations of transducers were made in section 7.3.4 but were only preliminary because of the lack of knowledge about the biological bases for these transducer populations.

The results of the comparisons of fit quality between the two fit types (i.e. fits including a single transducer population as compared to fits involving two independent populations) also did not produce a comprehensive conclusion as to the number of transducer populations, and future AICc analyses would require greater depth to provide clearer suggestions. This means that whilst more detailed molecular studies are necessary to identify the different populations in the JO, improvements to the gating spring model should also be simultaneously made in order to modify its focus to the more complex auditory systems of mosquitoes.

9.3. Future work

Although the experimental analyses presented in this thesis indicate that pymetrozine has potential for use against mosquito species, because of the effect on mosquito auditory function and *Drosophila melanogaster* flight ability and competitive fitness, much more work is required before the compound could be potentially included in any control programmes.

The first such experiment that must be completed is the aforementioned experiment investigating whether male mosquitoes (from any of the species used in this thesis) exposed to pymetrozine and control females from the same species are able to display the phenomenon of harmonic convergence, with the reverse experiment (i.e. pymetrozine exposed female and control males) being equally important (34).

Given the abolition of auditory function reported for pymetrozine exposed mosquitoes (as shown in section 8.3), and the reported difficulties that deafened – and more generally, ChO impaired - mosquitoes have with flight maintenance, it is unlikely that harmonic convergence could occur but the experimental proof of a possible reduction in harmonic convergence events is vital for the future potential of pymetrozine (352).

Further than this, experiments investigating potential reductions in human biting events following pymetrozine exposure at different concentrations could provide information on the effect that pymetrozine could have if incorporated as part of a mosquito control programme. Cone tests and arm-in-cage assays could thus be utilised to judge any possible changes in biting frequency, after which more extensive trials in experimental huts could be proposed if necessary (501, 503). These tests could then be followed by investigations into tracking female mosquitoes attempting to locate sources of blood meals following pymetrozine exposure (448).

The potential impact of pymetrozine as a larvicide also needs to be examined in greater detail. Low sample sizes used in section 5.3.1 meant that the study was not sufficiently powered to detect the existence of true significant differences reliably and thus could be repeated. The auditory function of adult mosquito exposed to pymetrozine only in the larval stage is also interesting when considering that *Drosophila melanogaster* adults exposed only as larvae demonstrated ablated auditory ability (laboratory data, unpublished). If the same is true for pymetrozine then the compound becomes potentially more useful for application in the field.

Further testing on mosquito lines that have demonstrated insecticidal resistance is also important; should pymetrozine be found to be effective against such strains in field situations (as has been suggested in section 8.3) then the compound would become much more promising in terms of targeting these mosquitoes.

The growing importance of control mechanisms which target females from mosquito species that tend to feed outdoors (such as *An. arabiensis*) alongside the previously mentioned increases in insecticidal resistance prevalence mean that compounds with novel mechanisms of action which can be applied outdoors are of potentially great usefulness (9, 504). The mechanism by which pymetrozine should be administered has still yet to be defined but seems likely to be either as part of an attractive toxic sugar bait programme or as a larvicidal/ oviposition trap toxin.

Pymetrozine has been proven to be effective against insects which feed from plants (26). Whilst mosquitoes do not insert their proboscis into sources of sugar provided by plants in the same manner that aphids insert their stylus into the plant phloem, feeding experiments included in section 8.3.1 utilising *Cx. quinquefasciatus* females (which had no documented resistance to insecticides) showed that merely consuming a source of sugar doped with pymetrozine was sufficient to induce the pymetrozine positive phenotype (76). This suggests that pymetrozine could be sprayed onto vegetation in a similar manner to that previously described (160).

Previous trials of outdoor applications of attractive toxic sugar bait have reduced exposure of non-target species such as bees by spraying the compound onto non-flowering vegetation; pymetrozine could be thus applied in a similar manner (160). It would be necessary to first estimate the minimum concentration of pymetrozine that would be required to have a significant effect on the mosquito population; initial estimates could be gained from using the currently recommended concentrations to target aphids, with field trials which estimate the comparative effect on mosquito population size further refining these estimates.

Finally, it is of the greatest importance that before any new method of mosquito control is introduced into a control programme the potential long term impacts of utilising the control measure are fully assessed. These assessments range from potential toxicity to other non-mosquito species (including humans) to long term environmental effects to the impact that the intervention could have on mosquito population dynamics, all of which are essential in order to avoid repeating previous mistakes made with regards to novel interventions (505, 506).

These preliminary assessments should also take into account not only the selection pressure on the mosquito species targeted, in terms of insecticidal resistance for example, but also on pathogens which are transmitted by this species (because of the influence that pathogens can have on their hosts) (102, 103, 507). For example, should an intervention exert such a strong selective pressure that female mosquitoes begin to take human blood meals earlier in the developmental cycle, or more often, then this could cause a significantly negative outcome on the local human population.

All of the tests described above explore the possible use of pymetrozine in field trials; whilst pymetrozine has the potential for such an application, the evidence presented throughout this thesis also provides support for the use of pymetrozine as a tool to explore mechanosensation. Whilst genetic methods currently exist of ablating ChO function, the specificity of pymetrozine in terms of a pharmacological intervention allows for a greater degree of flexibility in assay design (27).

The possible use of mosquitoes as models of mechanotransduction is also highly promising. The mosquito auditory system in both male and females is highly complex and contains a significant number of interesting properties, such as the manipulation of distortion products for mate seeking, that have yet to be fully investigated (276). The existence of efferent feedback loops in male mosquitoes from species other than *Cx. quinquefasciatus* (or the confirmation of such loops in female *Cx. quinquefasciatus*) for example has not yet been confirmed (314). By modifying the already existing gating spring models, mathematical modelling of mosquito auditory transduction should become more refined which would thus help create higher quality models of potentially multiple transducer populations (in line with previous reports in *Drosophila melanogaster*) (285).

The recent innovations in genetic tools with which to manipulate the mosquito genome also allow for the development of similar assays as those previously published for other insect species (508). The development of a deeper understanding of the mosquito auditory system could therefore not only provide information on potential targets for insecticides but also result in a more complete understanding of the role of auditory (and non-auditory) mechanotransduction in mosquito – and more widely, insect - behaviours.

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Appendices

Appendix A. Selected free fluctuations of *Drosophila melanogaster* CantonS females used in the fertility assay (in section 4.3.1) that were exposed or unexposed to pymetrozine

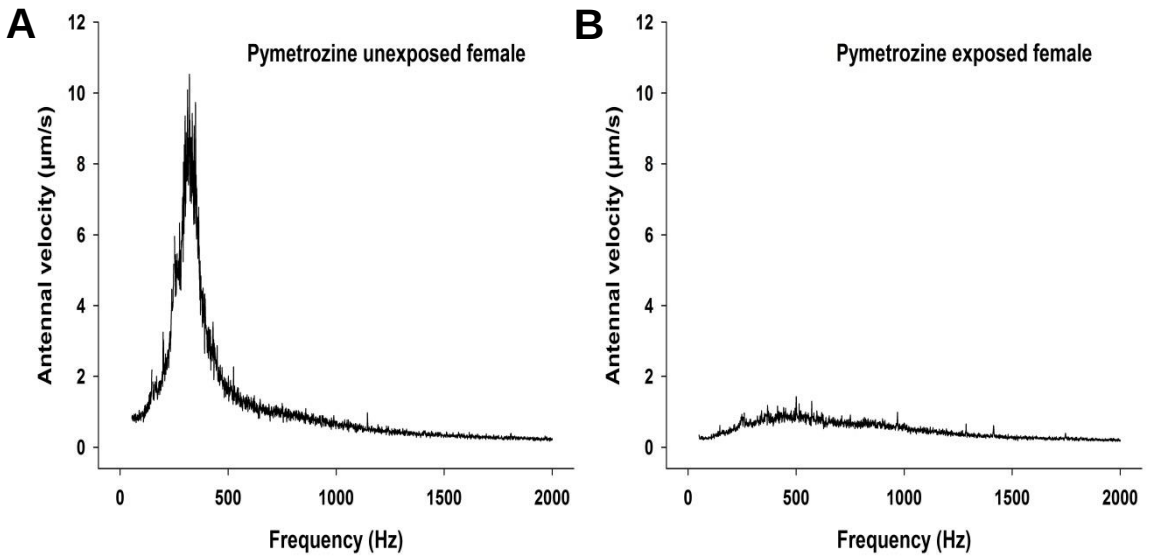

Figure 61. Free, unstimulated fluctuations of female Drosophila melanogaster involved in the fertility assay described in section 4.3.1 for either a pymetrozine unexposed female CantonS (A) or a pymetrozine exposed female (B). The examples shown here are representative of all flies measured from each experimental group.

Appendix B. Selected free fluctuations of *Drosophila melanogaster* CantonS males used in the temperature entrainment assay (in section 4.3.5) that were exposed or unexposed to pymetrozine

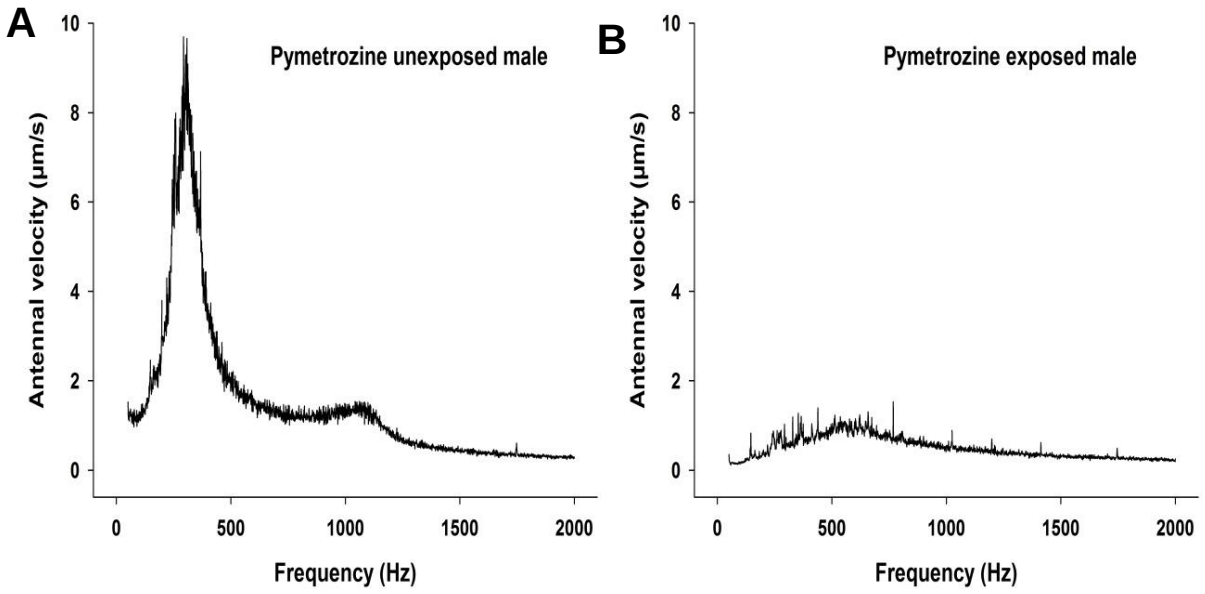

Figure 62. Free, unstimulated fluctuations of male Drosophila melanogaster involved in the temperature entrainment assay described in section 4.3.5 for either a pymetrozine unexposed male CantonS (A) or a pymetrozine exposed male (B). The examples shown here are representative of all flies measured from each experimental group.

Appendix C. Selected free fluctuations of *Drosophila melanogaster* CantonS females and males used in the courtship song assay (in section 4.3.6) that were exposed or unexposed to pymetrozine

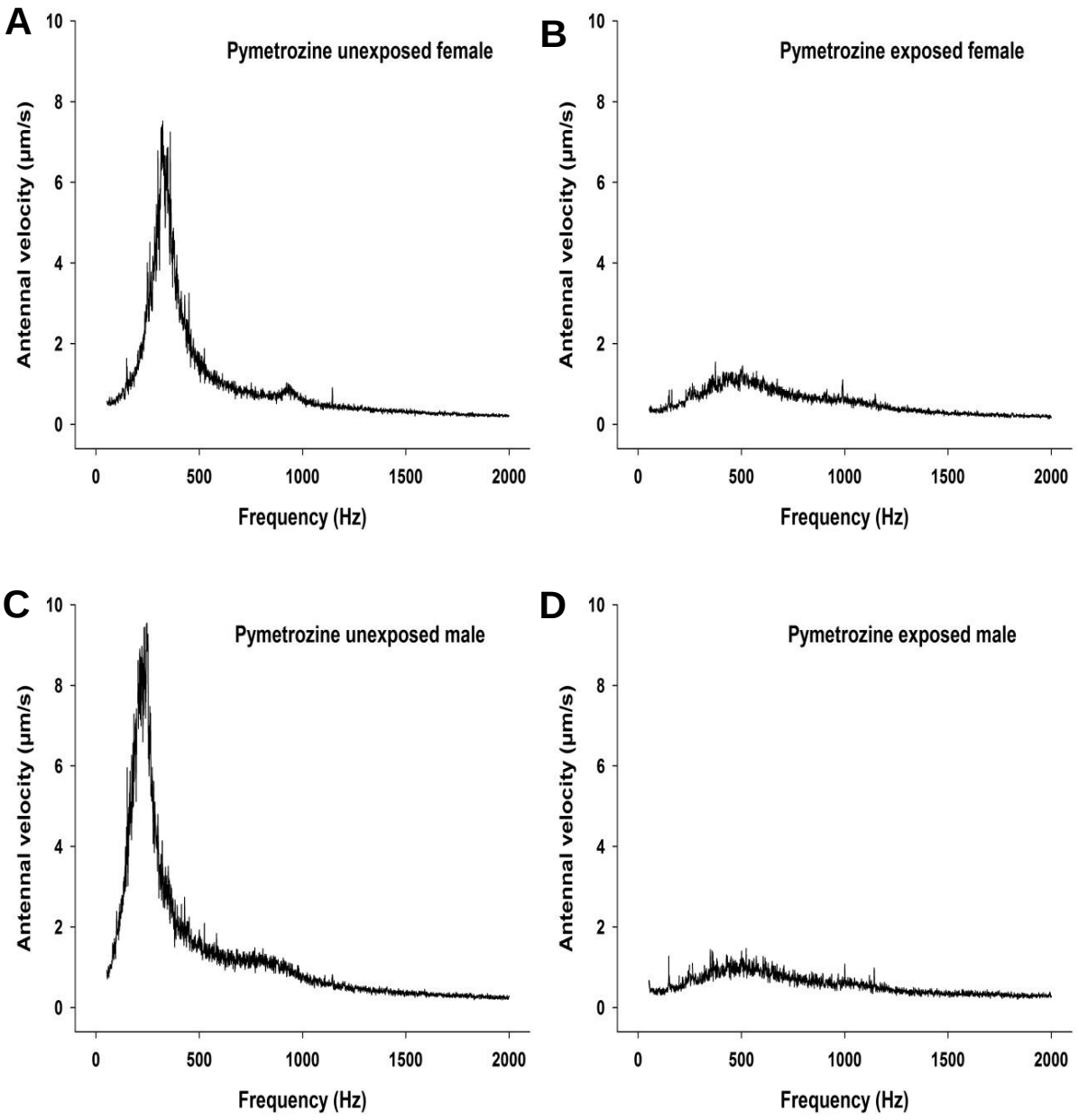

Figure 63. Free, unstimulated fluctuations of a pymetrozine unexposed female CantonS (A), a pymetrozine exposed female CantonS (B), a pymetrozine unexposed male CantonS (C) and a pymetrozine exposed male CantonS (D). Examples shown here were involved in the courtship song assay described in section 4.3.6 and are representative of all flies measured from each experimental group.

Appendix D. Selected free fluctuations of *Drosophila melanogaster* males used in the competitive reproduction assay (in section 4.3.7) that were exposed or unexposed to pymetrozine

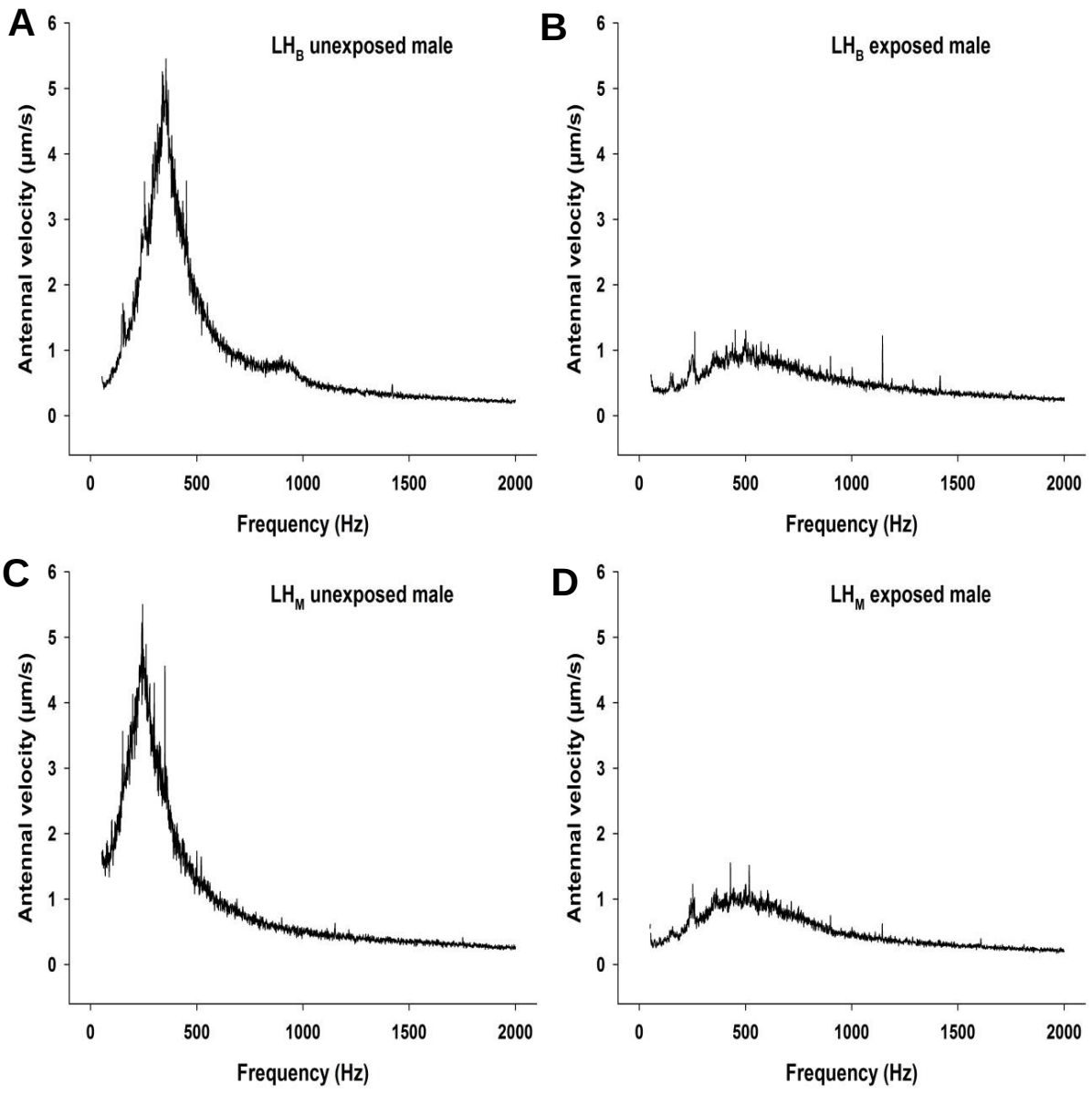

Figure 64. Free, unstimulated fluctuations for a pymetrozine unexposed LH_B –UCL male (A), a pymetrozine exposed LH_B –UCL male (B), a pymetrozine unexposed LH_M –UCL male (C) and a pymetrozine exposed LH_M –UCL male (D). Examples shown here were involved in the competitive reproduction assay described in section 4.3.7 and are representative of all flies measured from each experimental group.

Appendix E. Mosquito flagellomere length measurements

Table 27. Median length values in µm (with standard errors given in brackets) for each of the 13 antennal flagellomeres, the total length of the flagellum from the point of laser focus to the pedicel and the total length of the flagellum in its entirety.

Flagellomere section	*Ae. aegypti* female/µm (n = 8)	*Ae. aegypti* male/µm (n = 10)	*An. gambiae* female/µm (n = 9)	*An. gambiae* male/µm (n = 22)	*Cx. quinque-fasciatus* female/µm (n = 23)	*Cx. quinque-fasciatus* male/µm (n = 16)
1	202.32 (5.45)	381.36 (9.03)	161.58 (2.34)	202.52 (10.12)	184.88 (3.70)	334.42 (6.69)
2	159.38 (4.57)	368.10 (15.99)	134.69 (3.59)	332.77 (15.47)	129.36 (3.90)	271.30 (7.08)
3	151.92 (3.56)	75.84 (1.70)	124.14 (3.52)	114.07 (2.71)	132.00 (3.31)	73.00 (1.12)
4	152.63 (4.24)	76.12 (1.34)	117.59 (3.35)	112.08 (2.00)	140.75 (1.77)	75.82 (1.05)
5	143.78 (4.23)	74.93 (1.73)	117.43 (3.80)	108.96 (2.39)	143.32 (1.58)	74.72 (0.98)
6	141.11 (4.59)	76.12 (1.94)	120.09 (2.43)	111.35 (2.91)	141.77 (1.47)	75.45 (1.09)
7	135.17 (3.53)	77.23 (1.49)	116.50 (1.46)	112.49 (3.20)	142.74 (1.39)	73.36 (0.94)
8	130.36 (4.09)	75.69 (1.39)	109.08 (1.93)	112.26 (3.47)	145.61 (1.19)	70.99 (1.02)
9	123.25 (3.74)	75.46 (1.30)	111.32 (1.81)	113.19 (3.00)	144.60 (1.74)	70.24 (1.34)
10	113.47 (4.41)	73.06 (1.94)	102.12 (1.95)	114.64 (2.75)	144.62 (2.16)	71.83 (1.04)
11	108.49 (3.39)	78.07 (2.04)	98.12 (1.75)	112.70 (2.65)	140.17 (1.70)	71.12 (0.83)
12	104.75 (3.37)	78.07 (3.24)	76.03 (6.01)	107.47 (2.59)	133.51 (2.54)	69.89 (1.30)

13	129.30 (15.4)	113.34 (4.85)	145.81 (8.76)	216.86 (11.90)	147.45 (9.62)	183.09 (14.03)
Length from focus point to base (µm)	1282.30 (18.20)	1244.11 (17.74)	1119.26 (11.72)	1551.68 (18.06)	1447.17 (14.03)	1066.57 (7.96)
Total length (µm)	1795.83 (20.09)	1630.86 (21.52)	1548.03 (12.08)	1736.68 (10.32)	1863.21 (24.24)	1400.99 (9.65)

Appendix F. Comparison of pre- and post-ringer nerve and mechanical responses for *Drosophila melanogaster* and mosquito species

Ringer injection in both *Drosophila melanogaster* and mosquito species was observed to consistently reduce maximum CAP amplitudes produced after stimulation by up to 25% compared to the pre-ringer state. This is almost certainly due to a change in ion concentration following the introduction of the control solution to the insect as well as potential damage caused to the internal system by the injection process itself.

Whilst these reductions were significant, they also seemed stable and CAP amplitude did not appear to be further reduced after the system was given time to recover (after accounting for gradual decreases in CAP amplitude seen if the experiment lasts for a prolonged period of time). The post-ringer maximum CAP amplitudes still remained at such a level as to be statistically greater than in a post-pymetrozine state and allowed for nerve responses to the smallest step stimuli to be identified.

The mechanics of the system remain intact after ringer injection, with both free fluctuations and dynamical stiffness plots suggesting that at worst minimal alterations have occurred. Figure 65 contains demonstrations of typical median reductions in CAP size for *Drosophila melanogaster* and *Ae. aegypti* females as well as the maintenance of the overall changes observable in dynamical stiffness. Table 28 contains comparisons between before and after ringer states for fitting a two state model for either a single transducer population (for *Ae. aegypti* female mosquitoes) or two independent transducer populations (for *Drosophila melanogaster*).

Statistical comparisons were made using paired Wilcoxon signed rank tests with a significance level of 0.05 in Sigmaplot. No statistically significant differences were seen between any of the parameters for either the *Drosophila melanogaster* or *Ae. aegypti* female mosquito model fits, though post-hoc calculations of statistical power gave power estimates below 50% in all cases tested. This suggests that the statistical comparisons may be underpowered and as such had an increase likelihood of type II errors occurring.

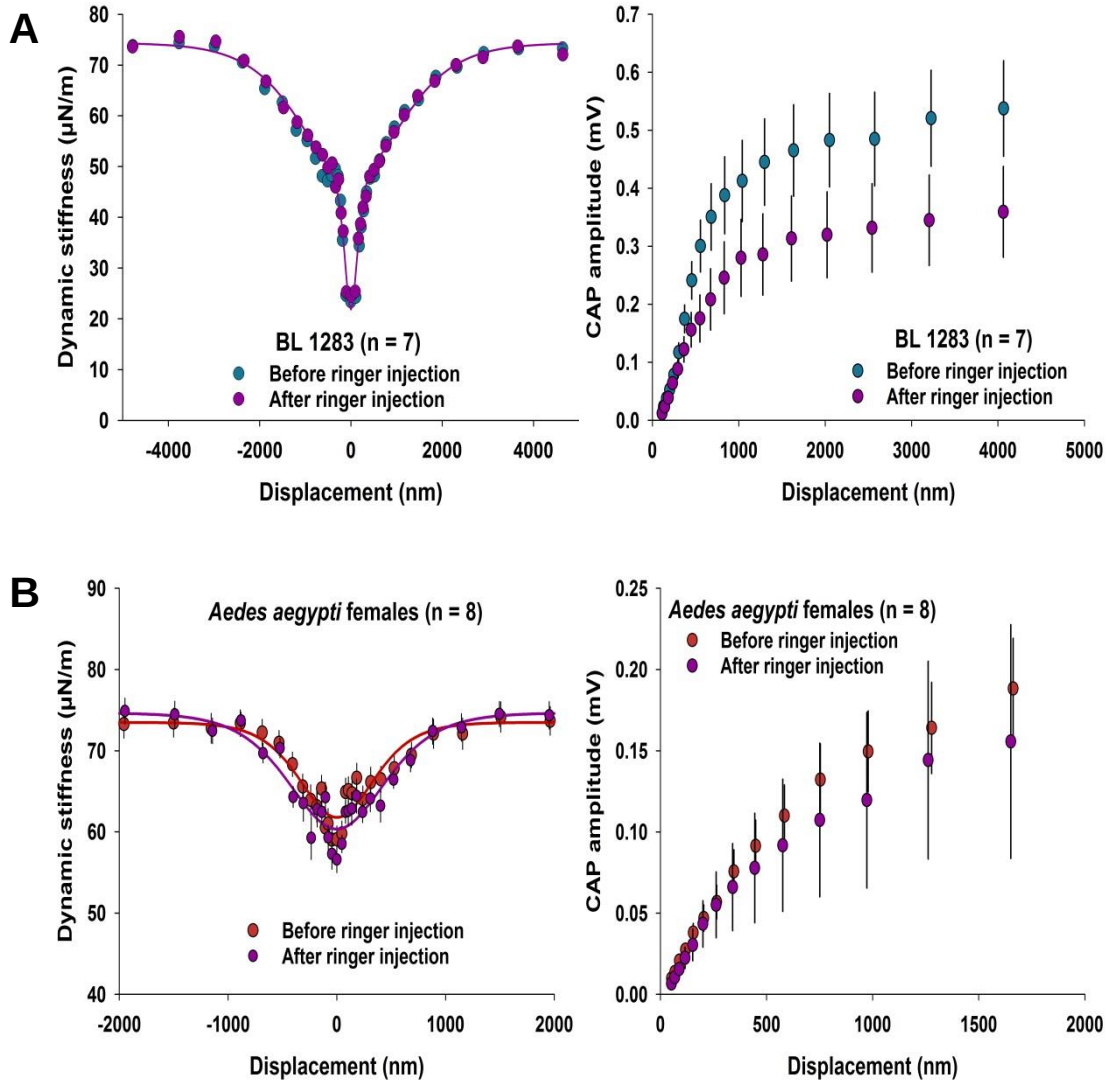

Figure 65. Pre- and post-ringer injection comparisons for changes in dynamical stiffness and CAP amplitude in response to changes in displacement for a) Drosophila melanogaster males from the BL 1283 line and B) female Ae. aegypti mosquitoes. Individual data points represent median values calculated at that displacement, with vertical black bars representing standard errors.

Table 28. Comparisons between before and after ringer injection states of the median values for a Drosophila melanogaster line (BL1283) and Ae. aegypti female mosquitoes using the two state model for either a single population of transducers or two independent populations (no significant differences identifiable between any of the model parameters when comparing before and after ringer states).

	BL1283 before ringer (n = 7)	BL1283 after ringer (n = 7)		*Ae. aegypti* females before ringer (n = 8)	*Ae. aegypti* females after ringer (n = 8)
Number of first population ion channels	206.6 (26.8)	182.9 (38.3)		-	-
Number of second population ion channels	14808.5 (1781.2)	14725.8 (1947.1)		943.9 (178.2)	1492.4 (297.5)
First population gating force (fN)	47 (2.74)	48 (4.22)		-	-
Second population gating force (fN)	5.41 (0.36)	5.34 (0.35)		16 (2.85)	12 (1.07)
$K_{INFINITY}$ (µN/m)	74 (1.9)	75 (2.0)		74 (1.8)	75 (1.5)
K_{STEADY} (µN/m)	49 (1.3)	48 (1.5)		64 (1.3)	66 (1.0)
K_{GS} (µN/m)	27 (0.9)	25 (0.6)		9.0 (0.7)	9.6 (0.7)

Appendix G. Median values obtained from fitting the two-state model for two independent transducer populations to post-ringer injection data for all *Drosophila melanogaster* lines investigated in section 6

Table 29. Median values obtained by fitting the two-state model for two independent transducer populations to post-ringer injection data for the following Drosophila melanogaster lines (numbers in brackets refer to standard errors): 91-S, 91-R, BL 1283 and BL 1675.

	91 – S (n = 7)	91 – R (n = 7)	BL 1283 (n = 7)	BL 1675 (n = 7)
Number sensitive channels	228.5 (72.7)	271.2 (37.0)	206.6 (26.8)	214.5 (20.6)
Number insensitive channels	10544.8 (639.4)	12031.3 (1138.8)	14808.5 (1781.2)	14459.6 (2003.3)
Sensitive channel gating force (fN)	30 (2.77)	30 (1.39)	47 (2.74)	41 (1.05)
Insensitive channel gating force (fN)	5.08 (0.19)	6.44 (0.51)	5.41 (0.36)	5.45 (0.28)
$K_{INFINITY}$ (µN/m)	47 (4.1)	62 (1.1)	74 (1.9)	64 (2.0)
K_{STEADY} (µN/m)	31 (3.3)	37 (1.0)	49 (1.3)	40 (1.3)
K_{GS} (µN/m)	17 (0.9)	24 (0.5)	27 (0.9)	23 (0.8)

Table 30. Median values obtained by fitting the two-state model for two independent transducer populations to post-ringer injection data for the following Drosophila melanogaster lines (numbers in brackets refer to standard errors): w^{1118}xGAL4, J28 7:1xGAL4 and J28 8:1xGAL4.

	w^{1118}xGAL4 (n = 7)	J28 7:1xGAL4 (n = 7)	J28 8:1xGAL4 (n = 7)
Number sensitive channels	323.9 (25.3)	317.4 (22.3)	436.1 (59.4)
Number insensitive channels	15112.7 (1169.1)	17994.5 (884.4)	18508.2 (1150.7)
Sensitive channel gating force (fN)	32 (1.32)	35 (1.65)	31 (1.97)
Insensitive channel gating force (fN)	5.59 (1.57)	5.05 (1.32)	5.13 (0.13)
$K_{INFINITY}$ (µN/m)	60 (0.8)	64 (1.1)	61 (1.7)
K_{STEADY} (µN/m)	38 (0.6)	41 (0.9)	41 (0.7)
K_{GS} (µN/m)	21 (0.4)	22 (0.4)	21 (1.1)

Appendix H. Median velocity fit values for all mosquito species and sexes in all states recorded from

Table 31. Median velocity fit parameters and energy gains for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes before ringer injection (with standard errors provided in brackets).

BEFORE RINGER INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti females	52	6.11×10^{-4} (1.8×10^{-5})	203.06 (2.22)	3.32 (0.21)	3.06 (0.62)
Ae. aegypti males	39	6.87×10^{-4} (3.7×10^{-5})	293.83 (11.72)	1.59 (0.47)	2.81 (1.69)
An. gambiae females	42	7.71×10^{-4} (3.2×10^{-5})	219.70 (3.55)	1.19 (0.24)	1.93 (0.25)
An. gambiae males	35	5.50×10^{-4} (2.6×10^{-5})	336.46 (8.58)	2.87 (1.32)	2.05 (0.58)
Cx. quinquefasciatus females	37	7.12×10^{-4} (3.8×10^{-5})	212.96 (2.41)	5.76 (2.74)	6.26 (2.05)
Cx. quinquefasciatus males	43	5.70×10^{-4} (2.9×10^{-5})	387.89 (6.60)	7.70 (4.34)	1.85 (2.40)

Table 32. Median velocity fit parameters and energy gains for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after ringer injection (with standard errors provided in brackets).

AFTER RINGER INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti females	29	5.58×10^{-4} (2.2×10^{-5})	198.5 (1.42)	3.80 (0.22)	1.72 (0.24)
Ae. aegypti males	24	6.58×10^{-4} (3.1×10^{-5})	290.0 (11.91)	1.50 (3.24)	3.61 (1.68)
An. gambiae females	36	8.63×10^{-4} (3.8×10^{-5})	215.0 (3.95)	1.00 (0.25)	1.84 (0.52)
An. gambiae males	20	5.40×10^{-4} (4.1×10^{-5})	352.0 (13.01)	3.12 (2.79)	3.78 (0.83)
Cx. quinquefasciatus females	29	7.92×10^{-4} (3.6×10^{-5})	221.6 (5.16)	10.6 (3.96)	15.35 (4.88)
Cx. quinquefasciatus males	28	5.29×10^{-4} (4.8×10^{-5})	366.0 (6.76)	19.3 (5.93)	1.46 (1.65)

Table 33. Median velocity fit parameters and energy gains for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after TTX injection (with standard errors provided in brackets).

AFTER TTX INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti females	10	4.81×10^{-4} (3.7×10^{-5})	196.9 (3.09)	2.76 (0.45)	1.56 (0.40)
Ae. aegypti males	9	6.39×10^{-4} (1.2×10^{-3})	445.3 (29.5)	68.13 (58.90)	45.43 (14.62)
An. gambiae females	12	8.73×10^{-4} (4.1×10^{-5})	214.5 (7.22)	1.21 (0.07)	1.70 (0.31)
An. gambiae males	7	4.13×10^{-3} (1.6×10^{-3})	339.6 (6.52)	245.8 (945.4)	19280.68 (14592.2)
Cx. quinquefasciatus females	14	7.70×10^{-4} (8.0×10^{-5})	210.6 (4.54)	16.01 (4.89)	24.63 (4.39)
Cx. quinquefasciatus males	15	1.64×10^{-3} (2.5×10^{-4})	317.1 (7.67)	156.9 (290.01)	722.72 (6004.67)

Table 34. Median velocity fit parameters and energy gains for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after TeNT injection (with standard errors provided in brackets).

AFTER TeNT INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti females	11	6.78×10^{-4} (4.6×10^{-5})	199.27 (2.05)	2.55 (0.24)	1.56 (0.25)
Ae. aegypti males	8	6.16×10^{-3} (7.0×10^{-4})	353.04 (26.76)	94.60 (628.65)	83.43 (37.33)
An. gambiae females	12	8.51×10^{-4} (6.5×10^{-5})	218.70 (3.52)	0.99 (0.16)	1.55 (0.45)
An. gambiae males	8	4.67×10^{-3} (6.6×10^{-4})	327.01 (8.90)	3034.38 (1374.7)	44934.32 (469401)
Cx. quinquefasciatus females	15	7.96×10^{-4} (4.9×10^{-5})	222.44 (6.39)	14.07 (6.98)	21.89 (9.20)
Cx. quinquefasciatus males	14	1.76×10^{-3} (2.3×10^{-4})	301.21 (7.32)	309.65 (162.60)	1078.95 (2055.62)

Table 35. Median velocity fit parameters and energy gains for male Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes whilst their flagella are spontaneously oscillating following either TTX or TeNT injection (with standard errors provided in brackets).

Spontaneously oscillating (male mosquitoes only)

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti males	16	6.39×10^{-4} (6.9×10^{-4})	384.43 (20.45)	93.05 (296.39)	49.70 (19.87)
An. gambiae males	15	4.40×10^{-3} (8.4×10^{-4})	332.04 (5.81)	355.79 (895.09)	40134.86 (254561.08)
Cx. quinquefasciatus males	29	1.64×10^{-3} (1.7×10^{-4})	311.23 (5.52)	239.28 (166.47)	1041.56 (3218.82)

Table 36. Median velocity fit parameters for CO_2 sedated male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (with standard errors provided in brackets).

PASSIVE STATE BEFORE PYMETROZINE INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q
Ae. aegypti females	35	5.16×10^{-4} (1.9×10^{-5})	207.47 (3.99)	1.04 (0.04)
Ae. aegypti males	29	5.78×10^{-4} (4.3×10^{-5})	301.43 (12.32)	0.94 (0.06)
An. gambiae females	33	6.62×10^{-4} (3.2×10^{-5})	325.00 (7.44)	0.67 (0.03)
An. gambiae males	24	5.68×10^{-4} (4.2×10^{-5})	283.55 (5.97)	0.91 (0.07)
Cx. quinquefasciatus females	28	5.41×10^{-4} (2.4×10^{-5})	206.45 (3.32)	1.11 (0.04)
Cx. quinquefasciatus males	31	6.11×10^{-4} (3.0×10^{-5})	263.20 (8.70)	1.00 (0.05)

Table 37. Median velocity fit parameters and energy gains for male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after pymetrozine injection (with standard errors provided in brackets).

AFTER PYMETROZINE INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q	**Energy gain (kBT)**
Ae. aegypti females	36	6.08×10^{-4} (3.3×10^{-5})	210.21 (3.57)	1.31 (0.047)	0.15 (0.08)
Ae. aegypti males	25	6.35×10^{-4} (6.4×10^{-5})	292.67 (11.49)	1.27 (0.08)	0.15 (0.16)
An. gambiae females	26	7.30×10^{-4} (3.0×10^{-5})	305.99 (7.07)	0.94 (0.04)	0.12 (0.12)
An. gambiae males	18	4.52×10^{-4} (4.4×10^{-5})	319.12 (10.65)	1.66 (0.30)	0.15 (0.07)
Cx. quinquefasciatus females	27	4.81×10^{-4} (4.6×10^{-5})	208.93 (1.90)	1.59 (0.10)	0.17 (0.15)
Cx. quinquefasciatus males	35	6.88×10^{-4} (3.5×10^{-5})	258.68 (8.65)	1.16 (0.05)	0.19 (0.09)

Table 38. Median velocity fit parameters for CO_2 sedated male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes after pymetrozine injection (with standard errors provided in brackets).

PASSIVE STATE AFTER PYMETROZINE INJECTION

Species/ sex	Sample size	F_0/m	Best frequency/Hz	Q
Ae. aegypti females	21	6.62×10^{-4} (2.9×10^{-5})	218.38 (4.41)	1.03 (0.05)
Ae. aegypti males	15	6.11×10^{-4} (7.5×10^{-5})	313.92 (13.96)	0.94 (0.08)
An. gambiae females	17	6.04×10^{-4} (4.4×10^{-5})	345.99 (9.97)	0.98 (0.04)
An. gambiae males	8	5.30×10^{-4} (4.8×10^{-5})	317.26 (12.59)	1.04 (0.11)
Cx. quinquefasciatus females	20	5.49×10^{-4} (3.4×10^{-5})	217.83 (2.36)	1.09 (0.06)
Cx. quinquefasciatus males	21	5.84×10^{-4} (3.5×10^{-5})	263.32 (6.34)	0.92 (0.05)

Appendix I. P-values for comparisons between mosquitoes of two state model of a single transducer population parameters

Table 39. P-values for statistical comparisons between male and female mosquitoes from the same species for parameters obtained from the two state model fits of a single transducer population.

Parameter	P-values for *An. gambiae* females vs males	P-values for *Ae. aegypti* females vs males	P-values for *Cx. quinquefasciatus* females vs males
Number of ion channels	P <0.001	P <0.001	P <0.001
Ion channel gating force	P <0.001	P <0.001	P <0.001
$K_{INFINITY}$	P <0.001	P = 0.023	P = 1.000
K_{STEADY}	P <0.001	P <0.001	P = 0.469
K_{GS}	P <0.001	P = 0.388	P = 0.001
Extent of nonlinearity	P <0.001	P <0.001	P <0.001

Table 40. P-values for statistical comparisons between female mosquitoes from different species for parameters obtained from the two state model fits of a single transducer population.

Parameter	P-values for *An. gambiae* vs *Ae. aegypti* females	P-values for *Cx. quinquefasciatus* vs *Ae. aegypti* females	P-values for *Cx. quinquefasciatus* vs *An. gambiae* females
Number of ion channels	P = 0.436	P = 0.436	P = 0.436
Ion channel gating force	P = 0.007	P = 0.738	P <0.001
$K_{INFINITY}$	P <0.001	P = 0.945	P <0.001
K_{STEADY}	P <0.001	P = 0.141	P <0.001
K_{GS}	P <0.001	P = 0.974	P <0.001
Extent of nonlinearity	P <0.001	P = 0.297	P <0.001

Table 41. P-values for statistical comparisons between male mosquitoes from different species for parameters obtained from the two state model fits of a single transducer population.

Parameter	P-values for *An. gambiae* vs *Ae. aegypti* males	P-values for *Cx. quinquefasciatus* vs *Ae. aegypti* males	P-values for *Cx. quinquefasciatus* vs *An. gambiae* males
Number of ion channels	P = 0.230	P <0.001	P = 0.051
Ion channel gating force	P = 0.025	P <0.001	P = 0.539
$K_{INFINITY}$	P = 0.036	P = 0.023	P = 1.000
K_{STEADY}	P = 0.145	P <0.001	P = 0.469
K_{GS}	P = 0.001	P = 0.388	P = 0.001
Extent of nonlinearity	P = 0.552	P = 0.552	P = 0.552

Appendix J. Changes to K_{PEAK} and K_{STEADY} for sedated mosquitoes

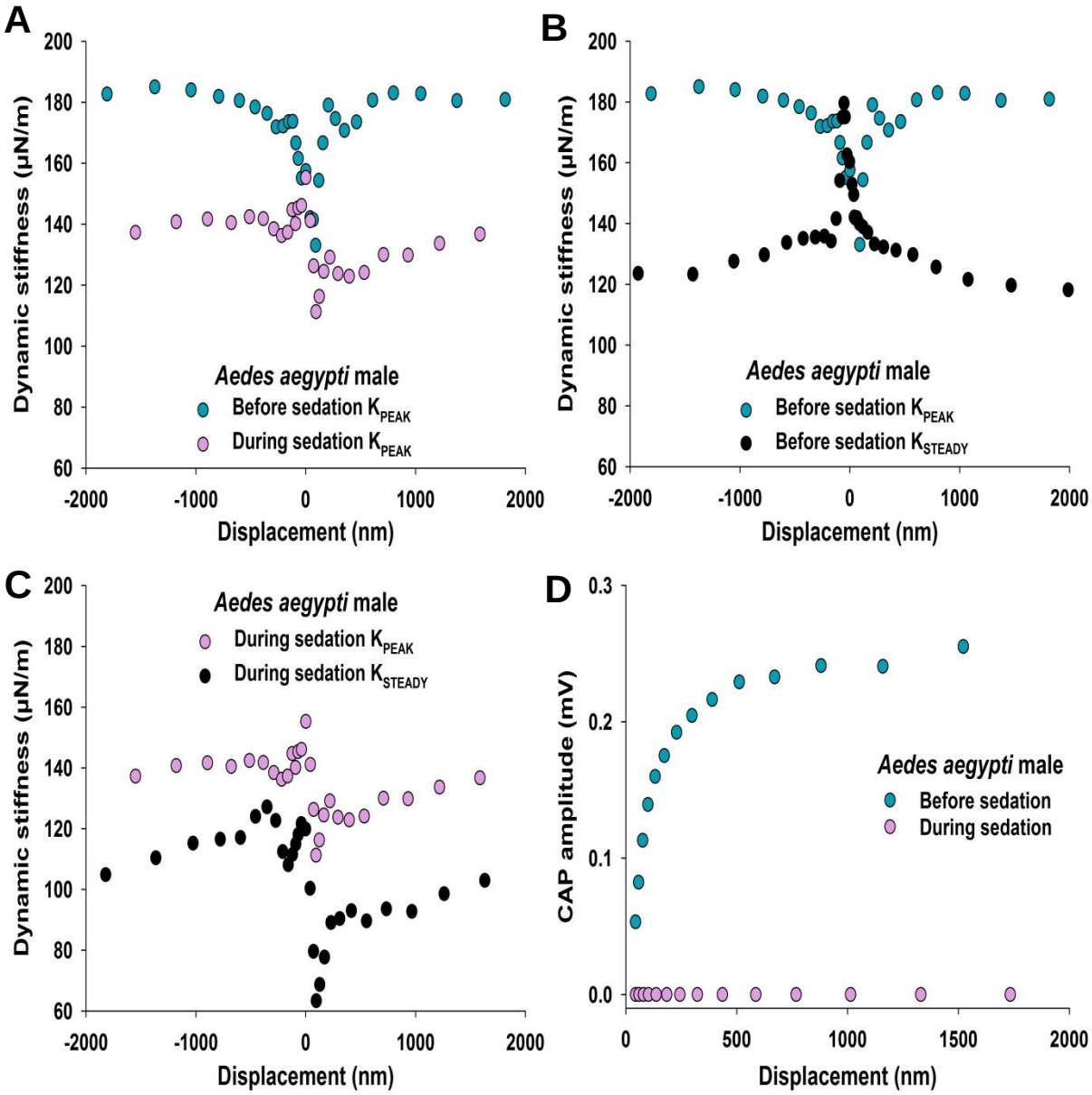

Figure 66. Changes in dynamical stiffness in response to changes to displacement, calculated for an Ae. aegypti male using either values estimated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}); A) K_{PEAK} values for both active and sedated states, B) K_{PEAK} and K_{STEADY} comparison for the active state and C) K_{PEAK} and K_{STEADY} for the sedated state.

D) Changes in CAP amplitude responses in response to changes in displacement calculated for an Ae. aegypti female in both active and sedated states.

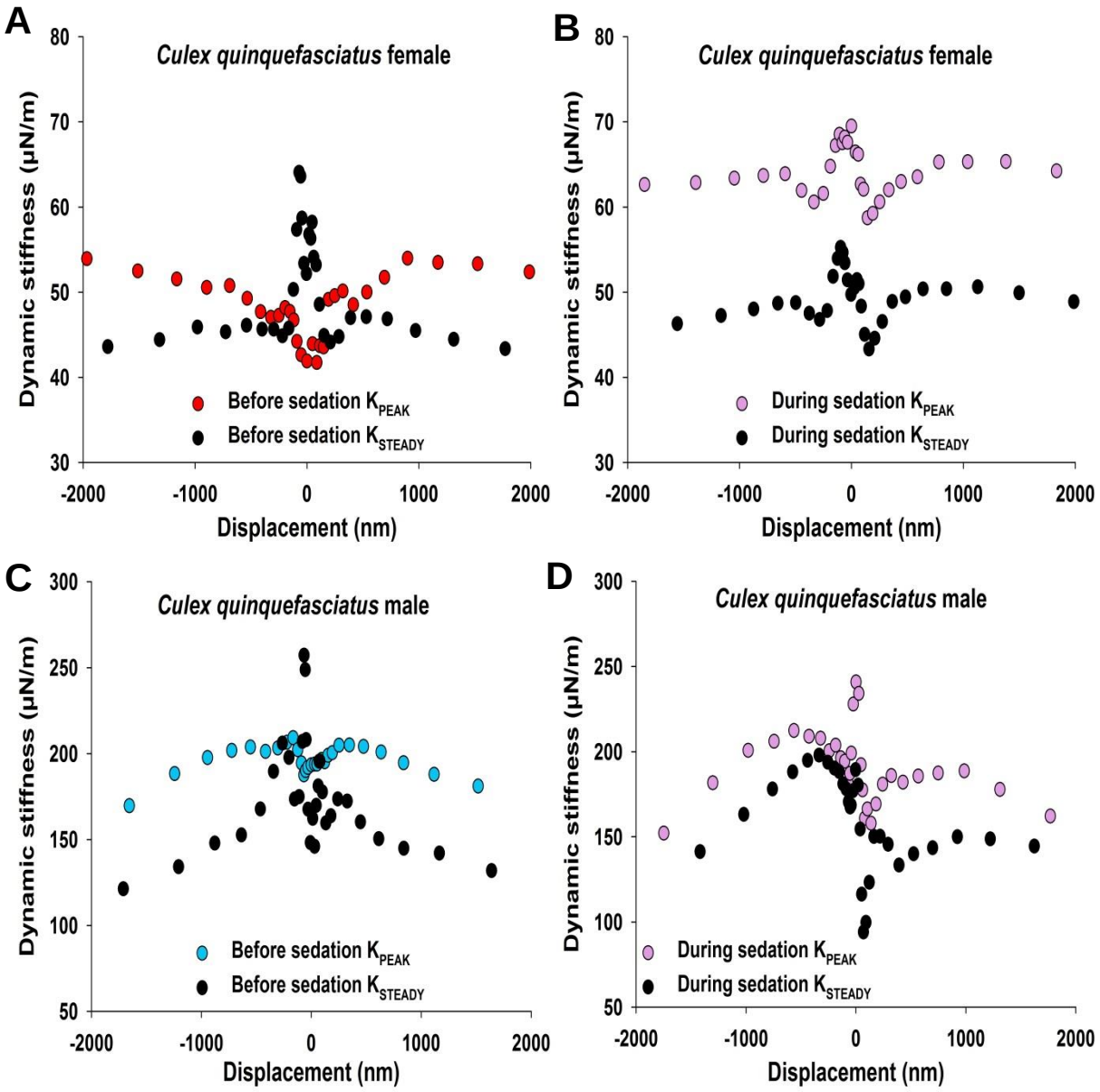

Figure 67. A and B) Changes in dynamical stiffness in response to changes to displacement, calculated using either values estimated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}), for a Cx. quinquefasciatus female before (A) and during (B) sedation.

C and D) Changes in dynamical stiffness in response to changes to displacement, calculated using either values estimated at the peak displacement (K_{PEAK}) or at the steady state (K_{STEADY}), for a Cx. quinquefasciatus male before (A) and during (B) sedation.

Appendix K. Statistical comparisons between single population and two independent population models

Table 42. Number of individuals from each mosquito species and sex which are fit better assuming two independent transducer populations rather than a single population, as determined using the AICc (following previous reports (394)), as well as the AICc for each fit type for female and male mosquitoes from each species.

	Ae. aegypti females (n = 21)	*An. gambiae* females (n = 18)	*Cx. quinq-uefasciatus* females (n = 17)	*Ae. aegypti* males (n = 18)	*An. gambiae* males (n = 15)	*Cx. quinq-uefasciatus* males (n = 15)
Sample size	21	17	18	18	15	15
Number of individuals with better fit for two population model than for one population model	21	17	18	18	1	2
AICc value for one population model fit to median data	-391	-306	-485	-350	-251	-321
AICc value for two population model fit to median data	-398	-320	-490	-354	-250	-315

Appendix L. CAP amplitude and dynamical stiffness changes following TTX and TeNT injection in mosquitoes

From figures 68 and 69 it is clear that for both male and female mosquitoes TTX injection caused an essentially total loss of CAP production, as has been previously reported (353). Following TeNT injection on the other hand female CAP responses persisted although they were somewhat reduced (as shown in figure 68 part D), with this reduction being even more significant in males – the remaining nerve response was still significantly greater than the nerve response after pymetrozine injection however in both sexes.

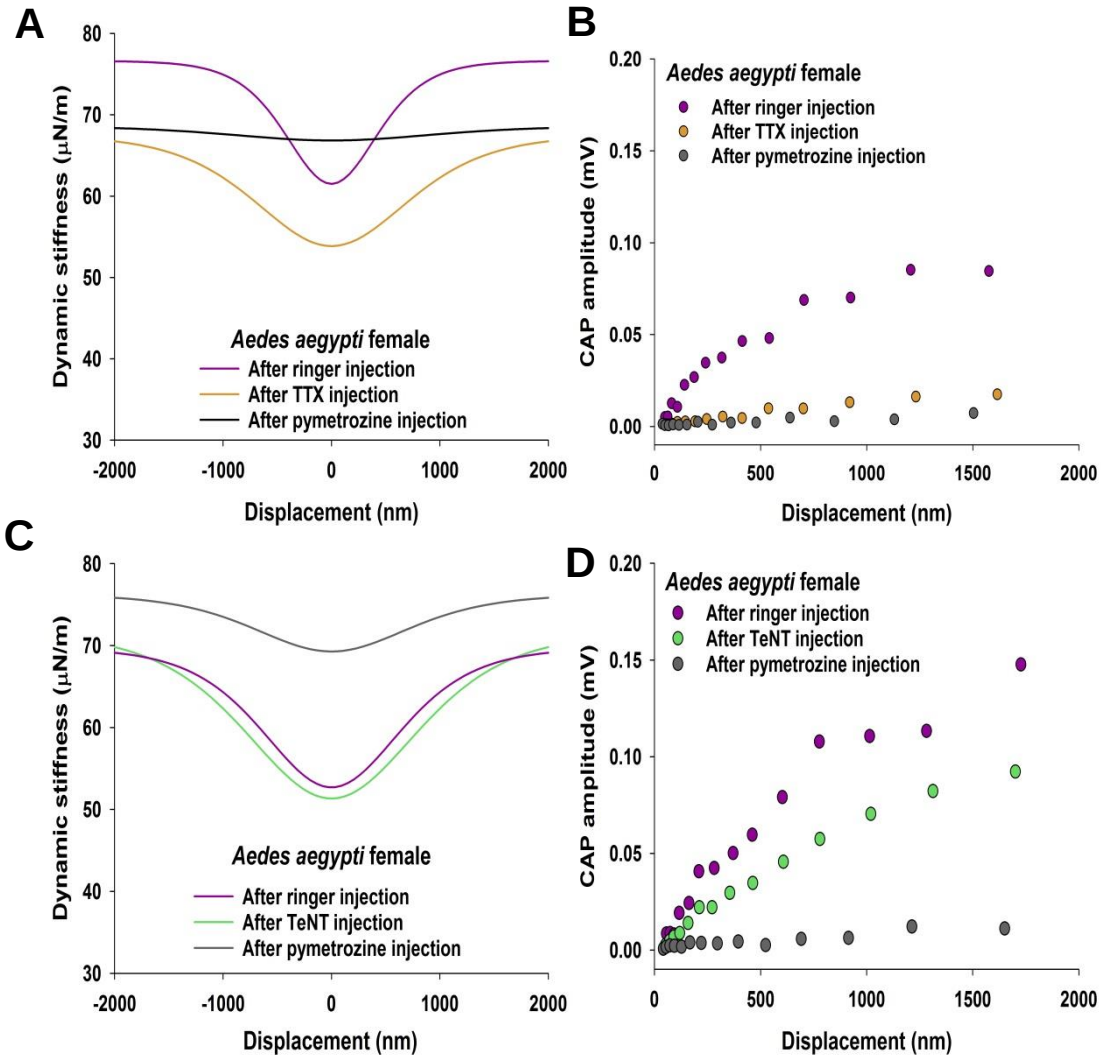

Figure 68. Comparisons of dynamical stiffness and CAP amplitude for an Ae. aegypti female following either TTX (top) or TeNT (bottom) injection – post-ringer and post-pymetrozine states are included as references.

TTX injection in the female *Ae. aegypti* led to a decrease in $K_{INFINITY,}$ though remained some decrease in dynamic stiffness around the resting position, whilst TeNT injection did not significantly change this stiffness decrease and the steady state stiffness remained constant. Estimation of dynamical stiffness was more difficult for *Ae. aegypti* males after TTX and TeNT injection as the system began to spontaneously oscillate which made measuring flagellar responses to force step stimulation challenging (hence no equivalent model fits are presented in figure 69 for male dynamical stiffness data sets). No significant changes in $K_{INFINITY}$ or K_{STEADY} were identified following TTX/ TeNT injection however, with pymetrozine acting as a comparative measure for potential shifts in these two stiffness values.

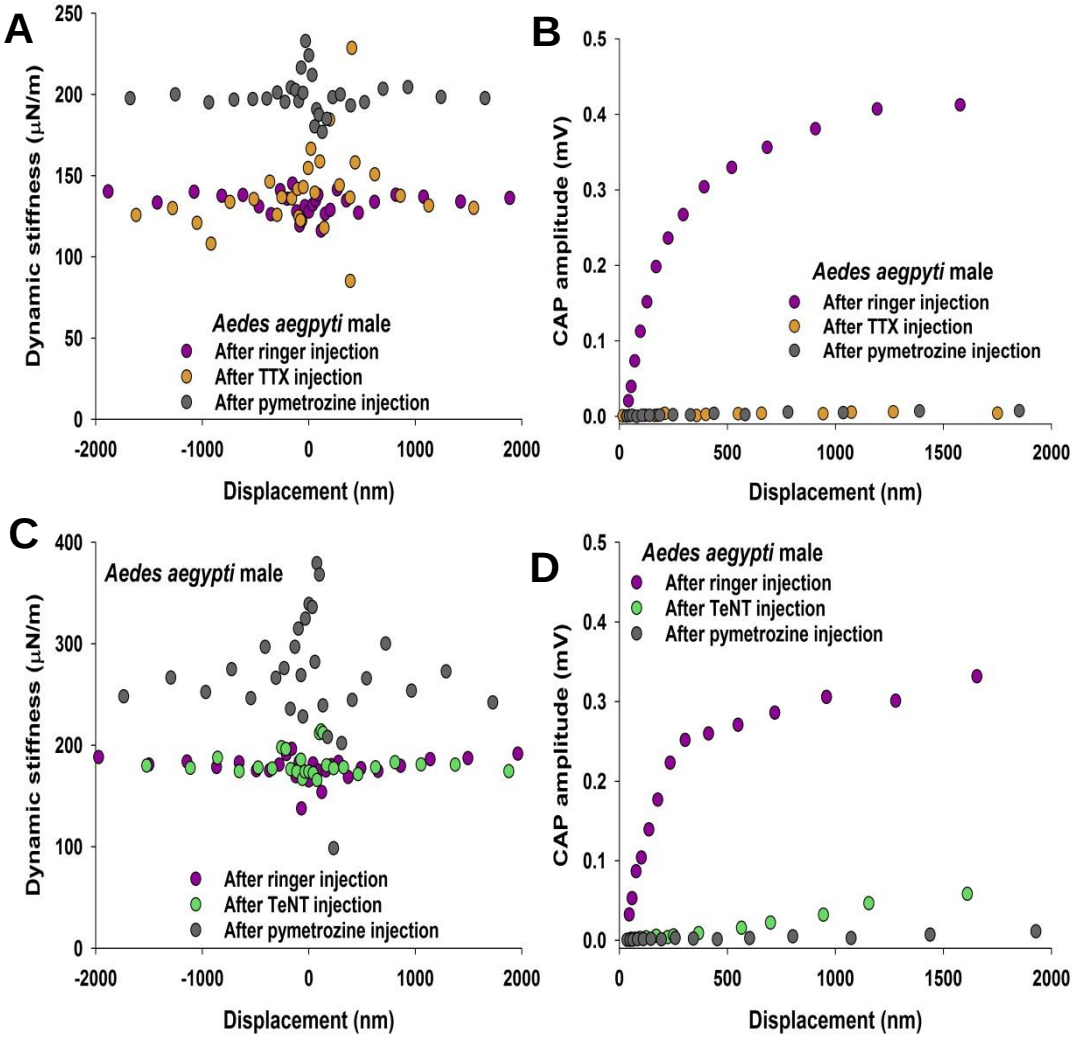

Figure 69. Comparisons of dynamical stiffness and CAP amplitude for an Ae. aegypti male following either TTX (top) or TeNT (bottom) injection – post-ringer and post-pymetrozine states are included as references.

Appendix M. Effective stiffness and best frequency shifts between control, sedated and pymetrozine injected states in mosquitoes, as well significance values for all relevant comparisons

Table 43. Median values of best frequency for sedated or pymetrozine exposed male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (numbers in brackets are standard errors) as well as P-values for all comparisons between the two states.

Mosquito species/ sex	Best frequency (sedated state) (Hz)	Best frequency after pymetrozine (Hz)	P-value
Ae. aegypti females (n = 36/ 35/ 36)	207.5 (3.99)	210.2 (3.57)	P = 1.0
Ae. aegypti males (n = 25/ 30/ 25)	297.99 (11.90)	292.7 (11.49)	P = 0.706
An. gambiae females (n = 29/ 33/ 26)	325 (7.44)	306.0 (7.07)	P = 1.0
An. gambiae males (n = 20/ 22/ 18)	283.7 (5.70)	319.1 (10.65)	P = 0.06
Cx. quinquefasciatus females (n = 29/ 29/ 27)	206.5 (3.26)	208.9 (1.90)	P = 1.0
Cx. quinquefasciatus males (n = 28/ 33/ 35)	274.1 (10.81)	258.7 (8.65)	P = 1.0

Table 44. Median values of effective stiffness for sedated or pymetrozine exposed male and female Ae. aegypti, An. gambiae and Cx. quinquefasciatus mosquitoes (numbers in brackets are standard errors) as well as P-values for all comparisons between the two states.

Mosquito species/ sex	Effective stiffness (sedated state) (µN/m)	Effective stiffness after pymetrozine (µN/m)	P-value
Ae. aegypti females (n = 36/ 35/ 36)	74.0 (7.34)	40.5 (5.04)	P < 0.003
Ae. aegypti males (n = 25/ 30/ 25)	148.1 (24.4)	91.7 (27.1)	P = 0.261
An. gambiae females (n = 29/ 33/ 26)	157.0 (21.2)	125.9 (13.5)	P = 0.399
An. gambiae males (n = 20/ 22/ 18)	162.5 (12.4)	182.6 (22.6)	P = 1.0
Cx. quinquefasciatus females (n = 29/ 29/ 27)	58.7 (2.35)	42.3 (2.63)	P = 0.179
Cx. quinquefasciatus males (n = 28/ 33/ 35)	107.9 (21.6)	86.6 (8.36)	P = 0.160