Evolutionary implications of artificial night-time lighting for the mosquito *Culex pipiens*: insights from genetics, physiology, behaviour and population structure

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“In order for the light to shine so brightly,
darkness must be present”

Francis Bacon (* 1561 – † 1626)

Well before the advent of electrical lighting these words were coined. While it is intended to be understood metaphorically, it nicely summarises the findings of my work, if taken literally. The nearly four years it took, to scientifically prove that Francis Bacon had a point, were among the most intensive and rich in my life. I am indebted to more people for their help and support than I can possibly name here. Therefore, I will limit myself to name only a few and ensure the rest that their support was highly appreciated and will not be forgotten.

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Summary

The term urbanisation describes, on the one hand, the process of outward expansion of cities and on the other hand a changed behaviour of rural populations, i.e. functional urbanisation. Especially the physical expansion of cities creates increasing pressure on the surrounding environments and its plant and animal communities. To date, there are 32 Megacities (>10 million inhabitants) around the globe, 11 of them with more than 20 million inhabitants.

As a habitat, cities are quite challenging as they are highly dynamic, crowded, noisy, polluted and extremely patchy in terms of comparable habitat types or local temperature.

One important feature accompanying the majority of human settlements is artificial light at night. In fact, its presence became as natural to us as the moon and stars. However, light itself is a stimulus to above-ground species that regulates timekeeping, daily activity and seasonal behaviours. Consequently, light could potentially impact a given organism on an array of levels, for example genetically or behaviourally, but also over different time scales, for instance daily or seasonally.

In order to assess the effects of artificial light at night I analysed different levels, i.e. gene expression, activity, fecundity and population structure, in one species, the common house mosquito *Culex pipiens.* In the first chapter of my thesis I examined the transcriptome, which is the entirety of the transcribed genes at a given moment in time, of male and female mosquitoes after exposure to different light regimes (1. “control”: light:16h and dark:8h; 2. “twilight” light:16+3h and dark:5h). I found that gene expression between males and females differed by more than 50%, independent of the light treatment. This underlines the importance of studying both sexes separately, rather than pooling the data, in order to avoid under- or overestimation of the effect of artificial light arising by opposing or additive patterns of sex-specific gene expression. The extended light treatment elicited a reduction in the expression of genes related to gametogenesis, immune response and lipid metabolism in males but not in females.

I then specifically analysed the gene expression of 5 genes (*period, timeless, clock, cycle* and *cryptochrome-2*) comprising most of the central circadian clock using qRT-PCR. The photoreceptor CRYPTOCHROME-1, another clock component, picks up blue light and feeds the information, via a reaction cascade, into the clock, which results in daily fine-tuning. Therefore, information obtained from ambient light levels should lead to a shift in expression levels in the extended light treatment (light:dark: 16+3:8h). I indeed found a down-regulation of the *period* and the *clock* gene expression in the light treatment. When analysing this for each sex separately, I found the same expression pattern in males. However, in females expression of the *period* gene was down-regulated while the *cycle* gene expression was up-

regulated. There was a clear difference in expression, even though I did not find evidence for an overall shift in the clock timing compared to the baseline group.
The circadian clock regulates a number of downstream processes, daily activity being one of them. A change in timekeeping should thus be reflected in daily locomotor activity. This was tested using a Locomotor Activity Monitor, which records activity as the number of crossings of infra red beams in a given time interval. Males and females displayed very different movement patterns, with males being less active than females. The extended light period significantly reduced activity in both sexes throughout the day, not only at the time of application. In constant darkness, the individuals resumed a similar activity pattern, i.e. active phases, indicating that the clock rhythm (~24h) was not shifted, even though the magnitude of activity was reduced.
To understand if less activity in the light treatment had implications for the population I analysed female fecundity as the number and size of eggs produced per population. Less active individuals may have less time for feeding and thus the nutritional status may not be optimal. Optimising reproductive output could then be achieved by producing either fewer eggs of the same size (as in optimal conditions) or the same number but smaller eggs. I found fewer and smaller eggs per raft in the light treatment, indicating that light may have effects on the population level as well.
The next step was to evaluate whether the presence of artificial light leads to a detectable differentiation between natural urban and rural populations. A microsatellite analysis was conducted to estimate the degree of differentiation along an urban-rural gradient. Unexpectedly, there was no differentiation. Instead there was a high connectivity between local populations ensuring gene flow and thus a high genetic variability in a panmictic population across the study area.
In this thesis, I was able to show that artificial light at night does influence mosquitoes on several levels. Light can be a stressor potentially influencing adult individual fitness. Additionally, the fitness of the offspring might be negatively impacted. On the other hand, the high amount of genetic variability and potential exchange of rural and urban local populations ensures a large gene pool and a high adaptive potential. To date, this is the only work addressing the impact of artificial light at night on several levels. It therefore presents important information on potential influences of light pollution. Furthermore, valuable insights on mosquito ecology, especially in males, have been gained, which may be of great interest for estimating the success of control programs.

Zusammenfassung

Urbanisierung beschreibt die physische Expansion von Städten einerseits, zum Anderen aber auch das zunehmend verstädterte Verhalten von Bewohnern kleinerer, ruraler Ortschaften. Dieser Vorgang wird als funktionelle Urbanisierung bezeichnet. Insbesondere die Ausdehnung von Städten sorgt für steigenden Druck auf Flora und Fauna der umgebenden Landschaft. So gibt es bis heute 32 Megastädte (> 10 Millionen Einwohner), 11 davon haben bereits mehr als 20 Millionen Einwohner. Als Habitat sind Städte eine Herausforderung da sie hoch-dynamisch, überfüllt, laut und verschmutzt sind. Vergleichbare Habitat Typen sind sehr ungleichmäßig über Stadt verteilt, vor allem auch in Bezug auf lokal vorherrschende Temperaturen.
Ein weiteres Merkmal urbaner Lebensräume ist künstliches Licht in der Nacht. Tatsächlich ist künstliches Licht für uns so alltäglich geworden wie Mond und Sterne. Nichtsdestotrotz, Licht als solches ist ein wichtiger Stimulus für überirdische Lebewesen, der Zeiterfassung sowie tägliche und saisonale Aktivität reguliert. Dies verdeutlicht, das Licht einen Organismus möglicherweise auf einer ganzen Reihe von Ebenen beeinflussen könnte, zum Beispiel genetisch oder verhaltensbiologisch, aber auch über Zeiträume hinweg, etwa im Tages- oder Jahresverlauf.
Um den Einfluss künstlichen Lichts bewerten zu können, analysierte ich unterschiedliche Ebenen innerhalb einer Stechmückenart, der gemeinen Hausmücke (*Culex pipiens*). Im ersten Teil dieser Arbeit untersuchte ich das Transkriptom, die Gesamtheit der Gentranskripte zu einem bestimmten Zeitpunkt, von männlichen und weiblichen Stechmücken nachdem sie unterschiedlichen Lichtregimes (1. „control“ Licht: 16 Std. und dunkel:8 Std.; 2. „twilight“ Licht: 16+3 Std. und Dunkel: 8 Std.) ausgesetzt waren. Mehr als 50% der Gene wurden allein beim Vergleich von Männchen mit Weibchen unterschiedlich exprimiert. Ein Befund, der die Wichtigkeit unterstreicht, beide Geschlechter getrennt zu untersuchen, anstatt die Genexpression aller Tiere gemeinsam zu betrachten. Nur so lässt sich vermeiden, dass aufgrund von gegensätzlichen oder additiven geschlechtsspezifischen Genexpressionsmustern die Auswirkungen künstlichen Lichtes unter- oder überschätzt werden. Des Weiteren verursachte die verlängerte Lichtperiode eine Reduktion der Expression von Genen, die in der Gametogenese, der Immunreaktion und im Fettstoffwechsel involviert sind in Männchen, jedoch nicht in Weibchen.
Danach untersuchte ich mittels qRT-PCR speziell die Expression von 5 Genen (*period, timeless, clock, cycle* und *cryptochrome-2*) der zentralen, circadianen Uhr. Der Fotorezeptor CRYPTOCHROME-1 nimmt Photonen blauer Wellenlängen auf und setzt so eine Reaktionskaskade in Gang. Auf diesem Weg gelangen Informationen zur Tageszeit zum zentralen Uhr-Mechanismus, was für dessen Feineinstellung sorgt. Eine Veränderung des

Lichtregimes, wie im durchgeführten Versuch, sollte also einer Veränderung der Genexpression führen. Tatsächlich fand eine Herunterregulierung der Genexpression von *period* und *clock* bei verlängerter Hellphase statt. Bei der Untersuchung nach Geschlechtern getrennt fand sich das gleiche Expressionsmuster bei Männchen. Bei den Weibchen wurde das *period*-Gen ebenfalls herunter- das *cycle*-Gen jedoch heraufreguliert. Obwohl es insgesamt klare Unterschiede in der Genexpression zwischen Mücken beider Lichtregimes gab, konnte ich keinen Hinweis auf eine allgemeine Verschiebung des circadianen Rhythmus finden.

Die innere Uhr reguliert die Funktion einiger nachgeschalteter Prozesse, tägliche Aktivität ist einer davon. Unterschiede in der Zeiterfassung sollten daher in divergierenden täglichen Bewegungsmustern resultieren. Dies wurde mit Hilfe von einem Locomotor Activity Monitor getestet. Dieses Gerät zählt die Anzahl der Durchtritte durch Infrarot Schranken in einem gegebenen Zeitintervall. Männchen und Weibchen zeigten sehr unterschiedliche Aktivitätsmuster, die Männchen waren generell weniger aktiv. Bei verlängerter Hellphase war die Aktivität beider Geschlechter über den ganzen Tag hinweg signifikant reduziert, nicht nur in der Phase, in der Licht appliziert wurde. In ständiger Dunkelheit zeigten die Individuen beider Gruppen ähnliche Aktivitätsmuster, d. H. ähnlich lange Phasen der Aktivität. Dies deutet darauf hin, dass der Rhythmus der Uhr (ca. 24h) nicht verändert war, obwohl das Ausmaß der Aktivität reduziert war.

Um zu verstehen, ob reduzierte Aktivität Auswirkungen für die gesamte Population hat untersuchte ich die Fekundität der Weibchen anhand der Anzahl und Größe der Eier pro Population in beiden Lichtregime-Gruppen. Weniger aktive Individuen könnten weniger Zeit zur Nahrungsaufnahme haben und somit einen schlechteren Ernährungszustand. Im Falle einer optimierten Nachkommen-Produktion könnte das wiederum entweder zu weniger aber gleich großen Eiern pro Eischiffchen führen, oder aber zu kleineren Eiern, die aber ähnlich zahlreich sind wie bei gutem Ernährungszustand. Ich fand heraus, dass im Lichtversuch die Anzahl der Eier pro Schiffchen geringer war. Gleichzeitig waren die Eier aber ebenfalls kleiner. Dies deutet darauf hin, dass künstliches Licht auch Auswirkungen auf Populationsebene haben könnte.

Somit war der nächste Schritt zu evaluieren, ob die Präsenz künstlichen Lichts zu detektierbarer Differenzierung zwischen natürlichen urbanen und ruralen Populationen führt. Mittels einer Mikrosatelliten-Analyse wurde der Grad der Differenzierung entlang eines urban-rural Gradienten bestimmt. Entgegen den Erwartungen konnte keine Differenzierung gefunden werden. Stattdessen scheint eine hohe Konnektivität zwischen lokalen Populationen zu bestehen, die dann Genfluss und somit eine hohe genetische Variabilität in einer panmiktischen Population über das ganze Untersuchungsgebiet sichert.

Zusammenfassung

In dieser Arbeit konnte ich zeigen, dass künstliches Licht in der Nacht Stechmücken auf unterschiedlichen Ebenen beeinflusst. Licht kann als Stressor fungieren mit potenziellen Auswirkungen auf die individuelle Fitness der Adulten. Zusätzlich dazu kann auch die Fitness der Nachkommen negativ beeinflusst werden. Auf der anderen Seite besteht eine hohe genetische Variabilität und somit die Möglichkeit des genetischen Austausches von urbanen und ruralen Populationen. Dies bedeutet ein großes Potenzial für Adaptation.
Bisher ist dies die einzige Studie, die versucht, den Einfluss künstlichen Lichts auf unterschiedlichen Ebenen zu erfassen. Eine Reihe der hier gewonnenen Einsichten sind daher von großer Bedeutung bei der Erfassung von Ebenen innerhalb einer Art, die von künstlichem Licht beeinflusst sein können. Des Weiteren wurden wichtige Erkenntnisse zur Ökologie von Stechmücken erlangt, insbesondere für die der Männchen. Dies kann vor allem für den Erfolg von Maßnahmen zur Kontrolle und Verbreitung, von zum Beispiel invasiven Arten, wichtig sein.

Thesis outline

This thesis is composed of two manuscripts that are currently under review and one manuscript in preparation. Each of them constitutes a separate thesis chapter including its own introduction, methodology, results and discussion section and is therefore independently readable from the other chapters. The layout of the individual chapter is following the Journal regulations the manuscripts have been or will be submitted to. The broader context of this thesis is provided by a general introduction and all findings are reviewed in an overall discussion. All manuscripts have been printed with the kind permission of the respective publisher. A list of all tables and figures used is presented. The literature cited is presented at the end of each chapter and a complete list of all references used can be found at the end of the thesis (page 107).

Chapter I

Ann-Christin Honnen[1*‡], Paul R. Johnston[2‡] and Michael T. Monaghan[1,3]: Artificial light at night elicits sex-specific changes in the transcriptome of the mosquito *Culex pipiens* f. molestus.

Manuscript submitted to Philosophical transactions of the Royal Society B, currently under revision, printed with kind permission of Royal Society Publishing.

Author contributions: AH and MTM conceived the study. AH, PRJ and MTM designed the experiment. AH conducted the experiment and the sampling. AH and PRJ analysed the data and drafted the manuscript. AH, PRJ and MTM contributed to the final manuscript.

Chapter II

A.-C. Honnen[1*], J. Kypke[1,2], M. T. Monaghan[1]: Changes in clock-gene expression, activity, and fecundity in the mosquito *Culex pipiens f. molestus* exposed to artificial light at night.

Manuscript submitted to Molecular Ecology currently under revision, printed with kind permission of Molecular Ecology and the publisher John Wiley & Sons Ltd.

Author contributions: AH and MTM conceived and designed the experiments. AH and JK maintained the laboratory colony and conducted the sampling as well as the laboratory work. AH analysed and drafted the manuscript. MTM contributed to writing the manuscript.

Chapter III

A-C Honnen & M.T. Monaghan: City-dwellers versus and country folks: mosquito population differentiation along an urban-rural gradient.

Manuscript in preparation

Author contributions: AH and MTM conceived and designed the study and conducted the field work. AH identified the specimen and conducted the laboratory work. AH analysed the data and drafted the manuscript. MTM contributed to writing the manuscript.

General introduction

Urban environments

A city is a densely built-up area thus creating microclimatic niches, with more or less connected greenery; it is never completely silent or dark, regardless of the time of day (Luniak 2004). Side products of human activity are various types of pollution of water sources or air but also noise. Amongst the ecologically most relevant features of urban environments for animals are noise, temperature and light, since these are all key factors determining habitat suitability. Sound is a means of communication and mate choice, most prominently in birds. High levels of background noise in cities are known to lead to altered timing of singing but also to louder singing in city-dwelling *Turdus merula* (LINNAEUS, 1758) (Nordt & Klenke 2013). Temperature and light are important zeitgebers for the timing of daily or seasonal events (Goto & Denlinger 2002), such as flowering in plants or timing of reproduction in animals. Both, temperature and light (in terms of intensity as well as spectral composition), can occur along gradients in highly structured microhabitats. Those microhabitats can vary greatly even in close proximity (for example cemeteries: Vezzani 2007). The effects of artificial light have been largely neglected in non-human species, even though adverse effects in humans are well documented (Navara & Nelson 2007). Only recently ecological light pollution has become a focus in research again. One of the earlier works analysed the effect of skyglow on natural populations of *Daphnia* (Moore *et al.* 2000) Skyglow describes upward-directed light that undergoes scattering off of molecules or aerosols (Kyba *et al.* 2012). Zooplankton migrates to the water surface at night to feed, thereby reducing predation pressure. In a lake close to a city and influenced by its skyglow, daphnids migrated normally when they were protected from skyglow. When unprotected, vertical migration was significantly reduced (Moore *et al.* 2000). This exemplifies how light in very low quantities can disrupt daily rhythms.

Artificial light at night and light pollution

In order to be able to explain the effects of artificial light it is important to explain natural light, as this is the light environment organisms evolved in and hence shaped their physiology and behaviour.

The revolution of the Earth around its own axis produces daily light-dark cycles, while its elliptical trajectory around the sun creates differences in day length, light intensity and

temperature. The central axis of the earth is tilted by 66.56° from the mean inclination from its trajectory around the sun and day length varies substantially at different latitudes with 24 hour days, or nights, at the poles. This also creates different degrees of seasonality across the globe. Thus organisms have evolved in the presence of varying light conditions on daily and seasonal temporal scales. But the dark period of the day is seldom completely devoid of light. In cloudless nights there is the light of the stars and the moon, with the amount of moonlight varying when it orbits the Earth. Nocturnal animals use these light cues to structure their daily cycle.

Light can be measured in various ways, depending on the portion of interest but also on the instrument used. The most common measure is lux (Longcore & Rich 2004), which is the illuminance in luminous flux (lumen)/m². As the measure of lux has been developed based on human vision it is not representative of insect vision (Longcore & Rich 2004). On the other hand its advantage is that it can be easily measured and has thus been used widely. This leads to some degree of comparability between studies.

Natural light levels vary by many orders of magnitude throughout the day (Table 1). The intensity of artificial light varies according to its purpose and lamp type.

Table 1: Natural light intensities throughout the day. The experimental light refers to the conditions of the laboratory experiment (see methods, chapter II).

light source	illuminance in lux
Direct sunlight	100000-130000
Full daylight (Not direct sunlight)	10000-20000
Overcast day	1000
Very dark day	100
Twilight	10
Deep twilight	1
Fullmoon	0.1
Quartermoon	0.01
Moonless, clear night sky	0.001
Moonless, overcast night sky	0.0001
experimental mid-day	~857
exp. additional light	~275

The invention of electrical light made it possible for humans to disregard the day and night rhythm, as light could be switched on when necessary. Daily life extended into the night and shift work became the norm in some professions (Schernhammer & Thompson 2011). Ever since, technology has been constantly improved and used more and more widely on a global scale.

The term light pollution was first used in the astronomy community, describing the phenomenon that, due to increased light levels in the atmosphere, the stars have become less visible, resulting in moving observatories to remote areas. In 1973 Riegel defined light

pollution as "[…] the unwanted sky light, produced by man […]" (Riegel 1973). In this study the author predicts that light pollution will increase dramatically and he estimated a yearly growth of 20% for the USA alone (Riegel 1973). Even now, more than forty years after Riegel's (1973) plea for light policy changes, the amount radiating into the sky is increasing, in some areas still by 20% per year, and by an average of 6% on a global scale (Hölker *et al.* 2010).

Light can influence any given organism in a multitude of ways and on various levels. First, there is the direct application of light. It must be distinguished between illumination, which is the entirety of light perceived in a given moment; and luminance, which describes the intensity of light emitted from a surface per unit area in a given direction. Due to the surface reflection light also polarises. Additionally, very different spectra might be covered. Spectral sensitivity can vary among species and usually depends on the habitats in which a species, more specifically their light-sensitive pigments, evolved in (Warrant & Johnsen 2013). Thus each lamp type and each illuminant may influence different species in very different ways (c.f. Fig. 1).
The term 'ecological light pollution' was proposed to " […] describe artificial light that alters the natural patterns of light and dark in ecosystems [...]" (Longcore & Rich 2004).

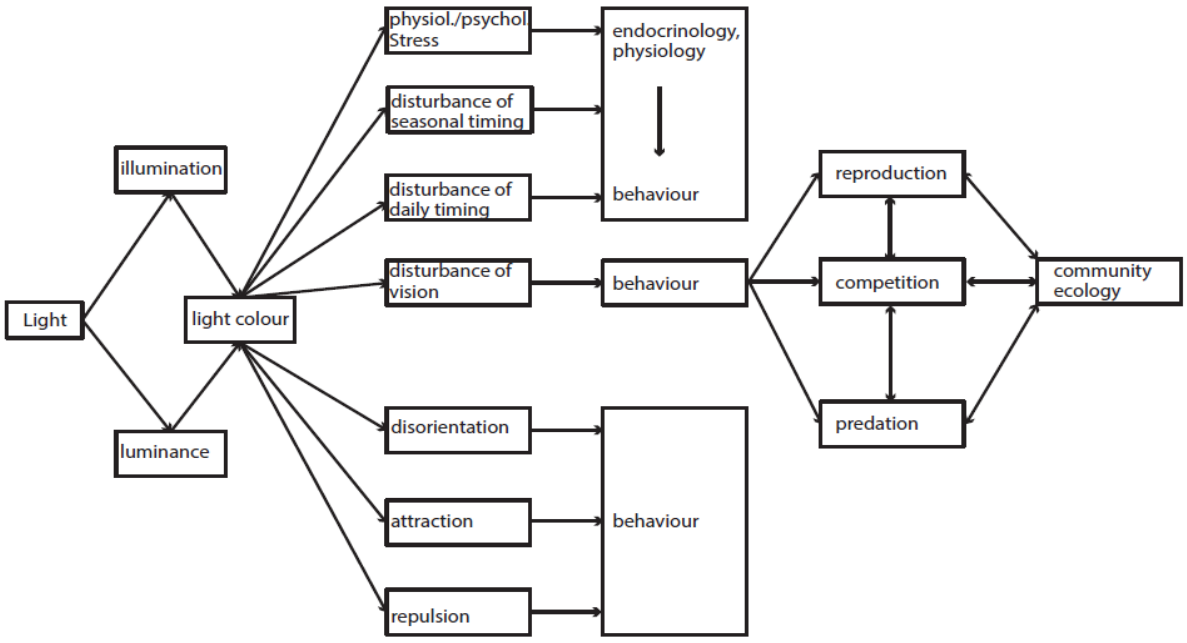

Fig. 1: Schematic drawing of ways in which artificial light might affect an organism and the potential implication on inter- and intrapopulation level. Redrawn after Health Council Of The Netherlands (2000).

I will provide an example to underline a possible set of influences of ecological light pollution. Lightning bugs use light or flashing light as a means to recognize conspecifics, communicate danger or disturbance but also to attract mates (Lloyd in Rich & Longcore 2006). Skyglow and streetlights can create a high ambient light intensity thereby influencing the visibility of the light flashes of the beetles (Lloyd in Rich & Longcore 2006). This effect is enhanced with cloud cover, as the clouds reflect the skyglow back to earth. With the communication of the individuals being disturbed or even completely disrupted, fewer pairs might form and reproductive success, and with it future generations, are influenced. This may lead to differences in local population sizes, depending on the proximity to the light source (i.e. close to settlements versus pristine areas). The overall different numbers of individuals will then also influence the predators of fireflies, as one prey item decreases and needs to be replaced by another. This way light might impose a potential change in the whole community at one location. Additionally, light may not influence all members of a community to the same degree.
This exemplifies an ecological approach to the effects of light pollution. It remains to be tested which physiological and underlying genetic processes, if any, are influenced by light and what the implications for the organism are. As can be seen from Fig. 1 seasonal and daily timekeeping may be influenced. If this impacts the individual this should result in a measurable effect in behaviour. A change in behaviour might not be ecologically relevant, if all members of a population shift their behaviour in the same direction. But if discrepancies arise this might have implications for the population as a whole.

Individual time keeping

Natural light is one of the most important zeitgebers for most organisms, regulating daily activity and behaviour, and also timing physiological processes (Longcore & Rich 2004; Navara & Nelson 2007). Diurnal species, i.e. active during the day and resting during the night, are physiologically adapted to a rhythm following the timing provided by day and night cycling. Seasonal timing is provided by the day length. This holds true, in reverse order, for nocturnal species (i.e. night length). For any kind of organism it is therefore vital to recognise whether it is dark or light or how long a day is, in order to prepare for the coming season. Examples include physiological preparations for winter, e.g. diapause in *Daphnia* (Stross & Hill 1968) and mosquitoes (Goto & Denlinger 2002).
It is therefore beneficial to have an internal clock to serve as a counter and also as a driver for regulating the timing of physiological processes. There exist a multitude of clocks,

depending on different habitats, such as intertidal, lunar or circadian clocks (Schwassmann 1980).
The internal clock underlying such circadian (about a day) cycles keeps its rhythm, independent of changing day length or constant (light or dark) conditions (Panda *et al.* 2002). Yet, in order to synchronise the internal timekeeping with the environment, light cues are used. Bünning and Moser (1969) suggested that the time of synchronisation is probably in the morning or evening when light intensities are low but the rate of change in intensity is high, so that variability caused by cloud cover is lowest. Many insects and mammals indeed use a blue-light receptor (CRYPTOCHROME) to set their circadian clocks in the early morning (Kyriacou *et al.* 2008; Sandrelli *et al.* 2008; Kyriacou 2009; Tomioka & Matsumoto 2010). This makes them especially vulnerable to artificial light. Many of the lamp types used in outdoor lighting possess a large emission peak in the blue part of the spectrum (Eisenbeis & Hassel 2000; Eisenbeis & Eick 2011). An important question is therefore whether artificial light could interfere with the timing of the gene expression of the genes involved in the circadian clock (see Chapter II) and thus disturb the clockwork. If timekeeping is disrupted it is very likely that the regulation of downstream processes is affected as well.

Study species

Among the animals commonly found in cities are mosquitoes (Diptera, Culicidae). There are about 46 mosquito species in Germany, but the species inventory is currently under evaluation since invasive species have established stable populations in the recent past (Becker *et al.* 2011). The species-specific habitat requirements vary greatly (Becker *et al.* 2012) and only few species are able to populate cities (Rudolf *et al.* 2013; Townroe & Callaghan 2014, chapter III). One of these species is *Culex pipiens* (LINNAEUS, 1758) the Northern house mosquito with a distribution range spanning the entire Northern hemisphere (for a recent map see: Smith & Fonseca 2004).
Females are known and much disliked for their bloodsucking behaviour bringing about the annoying buzz of flight activity. A blood meal is needed to facilitate egg development. It is less well known that male mosquito diet consists solely of nectar and also females rely on this food source when they are not inseminated (Robich & Denlinger 2005; Gary Jr & Foster 2006; Foster 2008). This calls for a different set of behaviours in males and females in search for food and a different timing of searching activity. Since timing has to coincide with resting periods of hosts or with flowering periods of plants, as some nectar sources provide better nutrition than others and are thus preferred. One example of a very strong link between mosquitoes and a plant is *Silene otites* (LINNAEUS, 1758), the Spanish catchfly. This

plant is pollinated by mosquitoes, mainly *Culex pipiens* and *Culiseta annulata* (SCHRANK, 1776), relying heavily on their regular visits (Jhumur *et al.* 2008).

Culex pipiens can be subdivided into two ecotypes: *pipiens* f. pipiens (LINNAEUS, 1758), the nominate form, and *pipiens* f. molestus (FORSKÅL, 1775). These two types differ in their physiology, behaviour, habitat requirements, and, to some extent, also genetically (Becker *et al.* 2012). The most striking difference is the feeding behaviour of the females. *Culex pipiens* f. pipiens females are obligate bloodfeeders with a strong preference for birds whereas *pipiens* f. molestusis a facultative bloodfeeder, meaning it is capable of producing offspring autogenously. But it will readily take blood, preferably from mammals. Moreover, f. molestus is stenogamous (mates in confined spaces) and does not diapause in winter. It is thus not too surprising that there are stable populations in man-made structures, such as underground tunnels (Kothera *et al.* 2010). In sympatric areas the two forms can hybridise (Fonseca *et al.* 2004; Kothera *et al.* 2010; Becker *et al.* 2012; Gomes *et al.* 2013). As blood feeders both types are capable of transmitting diseases from one host to the next, which is of increasing importance in a world of global trade and travel. In fact, the steadily increasing globalisation constitutes one of the main long-distance dispersal ways for mosquitoes (Service 1997), the Asian tiger mosquito (*Aedes albopictus;* SKUSE, 1894) being one of the most prominent examples (Paupy *et al.* 2009). Additionally, the global increase in mean annual temperatures is believed to facilitate the establishment of new pathogen populations to be carried along with mosquito species (Githeko *et al.* 2000; Becker *et al.* 2011).

As mentioned above, hitchhiking on human-made devices represents an effective way of dispersal for mosquitoes, with the destination likely being a city of some sort. The habitat suitability at this place then determines, whether a stable population can be established or not. Here light comes into play as an urban habitat characteristic. Like other organisms, mosquitoes perceive light and use it to adjust their internal timekeeping, which is the basis for the timing of physiological processes and daily behaviours (Gentile *et al.* 2006, 2009; Meireles-Filho & Kyriacou 2013). Given the differences in male and female biology, the effects may differ between sexes. A prominent factor that potentially renders male mosquitoes especially vulnerable to disturbance by artificial light is the mating biology. Male mosquitoes build swarms at particular times of day over landmarks and structures. Females then fly into these swarms and mating follows (Anophelines: Howell & Knols 2009, *Culex*: Reisen *et al.* 1985; Becker *et al.* 2012). It has been shown for Anopheline males that swarming is triggered by certain, species-specific wavelengths (Howell & Knols 2009). Interestingly, this has not yet been investigated in *Culex* mosquitoes, but since this species swarms at dusk a timer of some sort must be involved, and it is likely to be connected to the perception of light at particular wavelengths. If the effects of artificial light are indeed different

between sexes, this could lead to less overlap in daily rhythms and we may then expect implications for the population as a whole.
If the influence of artificial light at night has implications on population level, i.e. selecting for adaptive genotypes, it depends on the connectivity of adjacent (urban and rural) populations whether this influence can be passed on. Human-assisted dispersal is not the only means of long distance transportation. Mosquitoes can also disperse by wind-drift (Service 1997). In order to do so, they fly vertically upwards and pass through the boundary layer where they get carried away by the prevailing winds (Service 1997). It is not yet clear, if this crossing of the boundary layer can be considered as motivated flight, i.e. controlled by the individual, or if it occurs by chance and what might be the cause. High density in the larval habitat has been suggested to serve as a trigger (Service 1997). The most important mosquito movements are the motivated ones. These are the only flights that are under full control of the individuals (Service 1997). One motivation for flight activity is mate search, which bears the potential of connecting populations. *Culex pipiens* has been classified as strong flyer with an average maximum distance of 9695m (Verdonschot & Besse-Lototskaya 2014). Although the majority of all flights will be restricted to much shorter distances (Service 1997), this species is capable of covering larger ranges. Hence, if artificial light induces permanent changes in the population, propagation of the effects is possible.

Aim of the thesis

Artificial light is a feature that is so commonplace, we seem to barely notice that it is there at all. Although light is such a strong environmental cue underlying very basic functional traits, such as the circadian clock, we must acknowledge, that there is no clear picture of how it might interact or interfere with plants and animals. The purpose of this thesis is therefore to determine whether artificial light at night elicits a response on the genome level and determine the functional processes that are influenced, even though they are not equipped with or connected to a light receptor. Furthermore, the effects of artificial light on the circadian clock, behaviour and egg production are evaluated. In a third approach, I investigated, whether living in artificially lit habitats can give rise to different genetic architectures in natural city populations. I used the common mosquito *Culex pipiens* as a model for a flying insect species occurring in urban as well as rural and pristine habitats. Studies on mosquitoes generally focus on females, because of their disease vector potential. There is a scarcity of information about male mosquitoes. In order to fully understand if and how populations might be influenced by light and what the implications might be, for example

concerning vector control measurements, it is essential to consider both sexes. Therefore, males and females are analysed separately in this work.

Chapter I

This chapter is devoted to determining functional processes that might be influenced by light. To do so mosquitoes from a laboratory colony, raised under the same light conditions, were tested in two light conditions (with and without additional light at night) and the transcriptome was sequenced using the Illumina HiSeq 2000 platform. Profiles of differentially expressed genes have been determined for males and females as well as for the two light regimes.

Chapter II

The influence of artificial light at night on the individual on several levels was investigated in this chapter. First, the expression of the genes comprising the central circadian clock feedback loop was measured under two different light conditions and compared between males and females. In a second step it was evaluated whether the behaviour was influenced. This was done recording daily movement patterns under the same light conditions as used for the gene expression study. Again the data was tested for differences between males and females. Next, I examined the reproductive output of the population, as eggs produced, under different light conditions. All of these approaches necessitate the use of a viable mosquito population kept under controllable environmental conditions. I therefore established a laboratory colony of *Culex pipiens* f. molestus.

Chapter III

In this chapter the goal was to investigate whether urban habitats promote a population differentiation from surrounding rural populations. In a first step, the numbers of different species occurring in the city of Berlin and its surroundings have been screened. Second, I used microsatellite markers to analyse population substructuring and gene flow along an urban-rural gradient in *Culex pipiens* f. pipiens.

Chapter I

Artificial light at night elicits sex-specific changes in the transcriptome of the mosquito *Culex pipiens* f. molestus

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Abstract

Artificial light at night is a typical feature of urban areas across the globe, but few studies of its effects on nocturnal organisms have examined their response to artificial light at the molecular level. Mosquitoes in the *Culex pipiens* complex (Diptera, Culicidae) commonly occur in urban habitats and can act as vectors of vertebrate disease. We used RNAseq to evaluate the transcriptome response in a laboratory population of *Cx. pipiens* f. molestus exposed to near-natural conditions (light:dark 16:8 hours, “control”) and light-at-night conditions with 3 additional hours of light (“twilight”, 16+3:5 hours). Females showed no expression changes when exposed to the light treatment. In males, 151 genes with a range of important functions including gametogenesis, immune response and lipid metabolism had reduced expression in the twilight treatment (119 genes). Many of the genes more highly expressed in twilight (32) had unknown functions. There were 11660 genes differentially expressed in males and females (51% of studied genes) regardless of treatment or timepoint. Eleven genes of the circadian clock were female-biased and three were male-biased. The results indicate a strong response to artificial light in males of *Cx. pipiens*, with potential implications at the population level.

Introduction

Artificial illumination at night is a typical characteristic of human settlements and thus constitutes part of the habitat for organisms living in urban and suburban environments. Its global use is estimated to increase by up to 20% per year [1]. The result is that many habitat types are increasingly influenced by artificial light either directly by illumination, or indirectly by sky glow. Light is, along with temperature, one of the most important environmental cues for organisms. Natural light is involved in triggering activity on a daily (foraging) or seasonal (migration) basis [2], thereby affecting the ecology and physiology of many organisms. The occurrence of artificial light during scotophase can affect the metabolism [2, 3], behaviour [4] and circadian clock regulation [5–8] in an organism.

Mosquitoes (Diptera, Culicidae) are an ecologically important group of insects and many species occur in close proximity to humans [9]. The *Culex pipiens* complex is a widespread and abundant group of mosquitoes in Europe [10]. They occur commonly in urban and suburban areas [11], [12] and are thus widely exposed to artificial light. Blood-feeding females act as vectors of a number of vertebrate diseases (e.g., west nile virus, avian malaria) [13]. There are pronounced differences in the behaviour and the physiology of male and female mosquitoes, e.g. in food preference and daily activity [8]. The majority of research is focused on females because, as blood-feeders, they play an important role in the transmission of diseases. However, both sexes should be considered in a study of the consequences of artificial light at the population level. A recent study of the malaria mosquito *Anopheles gambiae* reported a large proportion of genes to show sex-biased expression [14]. To our knowledge this is the only study addressing this issue in mosquitoes.

The circadian clock is expectedly the main process affected by light as it is equipped with a blue-light receptor CHRYPTOCHROME -1, [15, 16] which directly picks up environmental light information. But, Rund et al. [8] suggest that there are also a number of non-clock genes that are expressed in diel patterns in response to the surrounding light environment. These genes relate to metabolic detoxification, immunity and nutrient sensing (e.g. glutathione-S-transferase, serine protease inhibitor and *takeout genes*, respectively) [8]. This implies that also processes not under direct circadian control would be influenced differently under different light conditions increasing the number of downstream processes affected. The effect on the whole organism could potentially be large. Therefore we aimed to obtain an overview of changes in transcript abundance as a response to different light regimes.

In the absence of genomic data for *Cx. pipiens*, the *Cx. quinquefasciatus* genome provides a basis for our analysis. Both species belong to the *Culex pipiens* complex and they are known to hybridise [10]. There is still some debate on the species status concerning *Cx. quinquefasciatus* [10, 17, 18]. The *Cx. pipiens* complex includes two forms (or ecotypes) with partially sympatric distributions, pipiens and molestus. Females of the pipiens form depend on blood meals (anautogeny), and males build mating swarms. The molestus form is autogenous and can mate in confined spaces. These characteristics make the molestus form highly suited to rearing in the laboratory, allowing for testing of different light regimes under otherwise constant conditions.

Here we tested whether exposure to artificial light at night altered gene expression in *Cx. pipiens* by combining laboratory experiments in controlled conditions with RNAseq. We expected sex-biased gene expression, with potentially different responses in males and females because of the potentially different role of light as an environmental cue. Our results indicate a more pronounced response to artificial light by males across a wide range of gene functions including gametogenesis, immune response, and lipid metabolism, with potentially important implications at the population level.

Materials and Methods

All mosquitoes came from a laboratory colony established in 2012 and were reared in a light:dark (L:D) regime of 16h:8h ("control" hereafter). Light was provided by cool-white LEDs (LED flex SMD, 24VDC, 24W, 1A, 60 LEDs/m, 500cm, cool-white single chip, Barthelme GmbH & Co. KG, Nürnberg, Germany). Light levels in the colony were controlled with custom-made software based on the LabView runtime environment (National Instruments v 8.5.1). We specified voltage at 15 time points over a 24-hour period (19 time points in twilight, see below) time points to which the software fit a hermite spline curve. The result was a smooth change of voltage, and thereby light intensity, over each 24-hour period. This allowed us to produce a near-natural light regime with gradually changing light intensities throughout the day (dark: 0 lux, mid-day: 300 lux; supplementary figure S1).

Pupae were placed in rearing cages and allowed to emerge. Adult individuals were removed from the cages with an aspirator. Adults were between 1 and 8 days old prior to removal from the cages. We therefore assume that all females were mated. Individuals were placed in continual darkness for 48h where a 10% saccharose-solution was available *ad libitum* for the first 36 h. This was done to uncouple the individuals from light cues and thus prevent the onset of processes arising from light-induced anticipation of the time of day. Feeding was

stopped 12h prior to sampling in order to avoid inflated expression of genes related to digestion relative to other physiological processes. Adult individuals were then exposed to two different light regimes: control (16h:8h Light:Dark) and twilight (16+3h:5h). The first 16h were lit as in the rearing cage (described above) followed by a sudden increase to 112 lux for 3 additional hours (S1). Exposure started at 04:00 and seven adults of each gender were sampled after 3 days at two timepoints (19:40 and 22:30) for each treatment resulting in eight samples. In the control group light intensities were 25 lux (19:40) and 0 lux (22:30), while intensity was 112 lux at both timepoints in the twilight group. Expression levels related to specific metabolic processes can differ among tissues (e.g. clock genes) [8, 14]. Because no data were available for *Cx. pipiens* f. molestus transcriptome (as defined by Wang *et al.* [19]) expression, we chose to examine whole bodies in order to investigate whether additional light at night would impose effects prevalent in the whole organism. A disadvantage of our approach was that tissue-specific differences in expression might not be detected. Nevertheless, the advantage of using whole bodies is that it provides an overview of the main processes affected without running the risk of overestimating the results based on tissue-specificity.

For RNA extraction, first TRIzol® Reagent (Ambion®, Invitrogen, Carlsbad, USA) was added to the sample. The tissue was then homogenised with an ULTRA-TURRAX® disperser (IKA®, Staufen, Germany) on ice. All following steps were done according to the manufacturers' protocol (Ambion®, Invitrogen, Carlsbad, USA) and the RNA-pellet was dissolved in 50µl RNase-free water (Carl Roth GmbH und Co. KG, Karlsruhe, Germany). The construction of 8 TruSeq cDNA libraries and sequencing on the Illumina HiSeq2000 platform for 207 cycles was performed by LGC Genomics GmbH (Berlin, Germany). The resulting 100-bp paired end reads are available from the NCBI SRA under BioSample accession PRJNA257052.

Read pairs were mapped to the *Culex quinquefasciatus* reference genome version CpipJ1.21 from ensembl metazoa using RSEM v1.2.12 [20] and Bowtie 1.0.0 [21]. For ordination analysis only, count data were transformed using the variance stabilizing transformation available in the DESeq2 v 1.4.5 package for R from Bioconductor [22] which evaluates the integral of each count value by numerically integrating the reciprocal of the square root of the variance from the fitted dispersions. This yields transformed values that become asymptotically equal to log base 2 for large counts.

Principal component analysis (PCA) was performed on the transformed data using the R function prcomp without scaling. Differential gene expression was determined in R using DESeq2 and raw counts. Genes with at least a 2-fold change in expression and an adjusted

p-value of 0.05 or lower were considered to be differentially expressed. Over-representation of Gene Ontology (GO) terms in groups of differentially expressed genes was determined using the GOstats v. 2.14 package [23] for R, using a hypergeometric test with a *p*-value of 0.05 and the GO terms from the CpipJ1.21 proteome annotation as the gene universe. Gene orthology data were obtained from OrthoDB v. 7 [24] and sex-biased *Anopheles gambiae* gene expression [14] were downloaded from the Sebida database [25].

The experimental design allowed testing for the effects of treatment and time but not for interaction effects. Our analysis is conservative, because in order to detect an effect of treatment, the direction of differential expression must be the same across time points (and vice versa). Sex-specific gene expression has been observed in a range of organisms [26, 27], therefore we tested for sex-specific differences across all samples using DESeq2 as above. We specified the design formula as “design = ~ sex + treatment + time”. This allowed for a comparison of levels for each factor. Gene expression differed markedly between males and females (see Results). As a result, all subsequent comparisons were made for each sex separately using “design = ~ treatment + time”.

Results

Principal component analysis of expression patterns showed a strong separation by gender along the first component (PC1), accounting for more than 90% of the total variation in gene expression observed (figure 1*a*,*b*). Separation between timepoints occurred in males along PC2 (ca. 2% of variation, figure 1*a*). Separation between control and twilight treatments occurred along PC3 in males (ca. 2% of variation, figure 1*b*). There was no clear separation of female samples by either timepoint or treatment in any of the eight principal components (supplement S2).

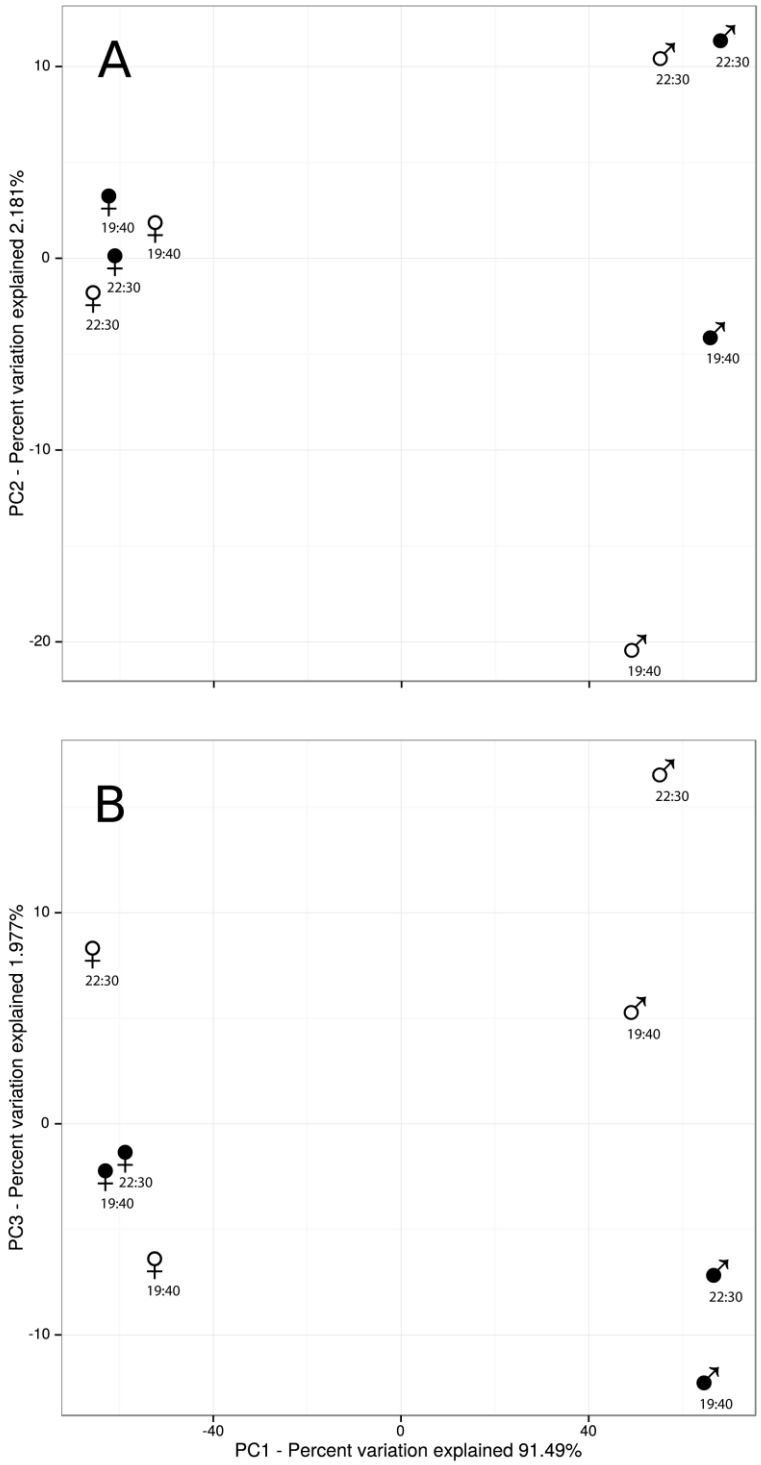

Figure 1: PCA plots of differentially expressed genes showing the percentage of variation explained by sex (PC1) and (A) time (PC2) and (B) sex and treatment (PC3). The colours correspond to the treatment: black is control (L:D 16:8h) and white is twilight condition (16+3:8h). Sampling timepoint is given for each sample.

The majority of genes showed relatively low eigenvector coefficients within PC1 (S3) with only a single gene showing a coefficient greater than 0.05 (S5). This was a gene encoding

an LWamide neuropeptide precursor with strongly female-biased expression (S4). In contrast, there were 20 genes loading on PC2 and 24 genes loading on PC3 (S5, S6). Genes along PC2 included 7 genes involved in metabolic processes such as protein catabolism and gluconeogenesis, and 3 genes encoding components of the opsonophagocytosis machinery (S7). The majority of genes loading on PC3 had no functional annotation (S8), but included several genes involved in gametogenesis, including *oskar* and *exuperantia*, as well as a gene encoding long-wavelength sensitive opsin.

Effects of artificial light treatment

In males, 151 genes showed differential gene expression in response to the light treatment. Thirty-two genes were more highly expressed in the twilight condition, while 119 genes were more highly expressed in the control (figure 2A).

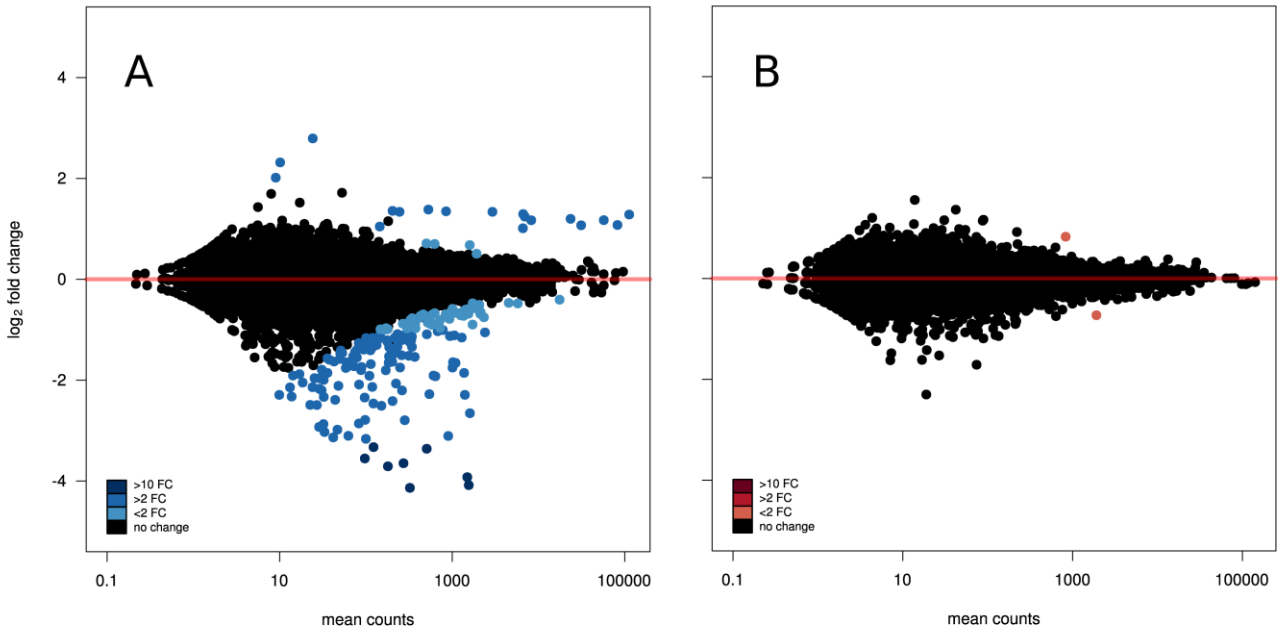

Figure 2: MA plots of differentially expressed genes between treatments for (A) males and (B) females. The colours refer to the differences in fold changes (FC). Positive and negative fold changes indicate genes with treatment- and control-biased expression, respectively.

A striking signature of gametogenesis-related gene expression was identified in the male response to altered light conditions, where expression of genes involved in DNA replication, mitosis, meiosis, spermatogenesis and germ cell proliferation was elevated under near-natural light conditions (S9). This included a substantial portion of the DNA replication machinery including all six DNA replication licensing factors (*mcm2-7*), and the components

of the origin recognition complex, *geminin*, *orc1* and *cdt1*, as well DNA primase and DNA polymerase and an ortholog of *fizzy* which regulates transition to meiosis. An identical pattern of expression was observed for orthologs of 10 genes that have shown strong germ-line stem cell bias in *Drosophila* [28], including the major regulator of zygotic genome activation *smaug*, and the cognate activating kinase *pan gu* (S9). Furthermore, there was clear coordinate expression of several genes with crucial roles in spermatogenesis, such as *ance* [29] and *importin alpha* [30]. Gene ontology (GO) term enrichment analysis also highlighted the signature of gametogenesis with enrichment of numerous related GO terms (S10) including “reproductive process” (GO:0022414), “gamete generation” (GO:0007276), “regulation of cell cycle” (GO:0051726).

Differential expression of immune genes was also observed with elevated expression of genes encoding DUOX, which mediates epithelial immunity [31], two c-type lectins which function as opsonins, and *dorsal*, which encodes the Toll-activated NF-kappaB transcription factor responsible for induction of immune gene expression [32] in the control treatment. A similar pattern was observed for the orthologs of the *Drosphila* genes *yellow-f* and *yellow-h* which encode dopachrome conversion enzymes that participate in the melanization response [32]. Several genes encoding proteins with functions in lipid metabolism and transport were also differentially expressed in response to artificial light including a triacylglycerol lipase, lipoprotein receptor, apolipoprotein and a cluster of three genes encoding sterol acyltransferases. In contrast to males, females exhibited differential expression between light regimes in only two genes. These encode a transferrin and phospholipase A1. The magnitude of induction, however, was marginal in both cases (figure 2B).

Expression changes over time

In males, 63 genes were differentially expressed over time; 25 exhibited higher expression at the earlier timepoint and 38 showed higher expression at the later time point. Genes with higher expression in the earlier timepoint were enriched for GO-terms "gluconeogenesis" (GO:0006094) "regulation of endopeptidase activity" (GO:0052548) and "mitochondrial ATP synthesis" (GO:0042775) (supplement S11). These also included several genes with predicted functions in digestion and the midgut such as chymotrypsins 1 and 2, peritrophin, and phosphoenolpyruvate carboxykinase [33]. Genes which exhibited higher expression at the later timepoint included many hypothetical proteins, an ortholog of *Drosophila rdl* which acts in neurons to promote sleep [34], and 5 genes encoding additional gated ion channels. In females, seven genes differed in their expression over time, four of which were down-

regulated in samples taken at 22:30h when compared to the 19:40h sample. These four genes encoded *amino acid transporter, trypsin 4, myosin III* and a hypothetical protein.

We compared the differentially expressed genes of treatment and time in order to assess whether there was an overlap, i.e. genes that are differentially expressed in response to treatment and over time. This could only be performed for male data because we did not detect differential gene expression in females in response to light treatment. A total of 13 genes overlapped between time and treatment in males, including *gaba receptor*, *chymotrypsin 2*, and 2 hypothetical proteins.

Sex-biased gene expression

Independent of treatment or timepoint, 11660 genes were differentially expressed in males and females (figure 3). This amounts to ca. 50% of all genes studied. Of these, 8704 genes exhibited at least a 2-fold sex bias. Previous work in *An. gambiae* reported 1487 female-biased and 1226 male-biased genes [14]. These belonged to 1351 and 1030 ortholog groups, respectively. By comparing the expression of genes belonging to ortholog groups that are conserved between *An. gambiae* and *Cx. pipiens* f. molestus data here, we found that 46% and 79% of these groups also possess sex-biased genes in *Cx pipiens*. Analysis of gene ontology terms that were specifically enriched in each sex showed a remarkable overlap with *An. Gambiae*, including "cellular iron homeostasis" (GO: 0006879) and "ion transport" (GO: 0006811) in males and "ligase activity" (GO: 0016874) in females.

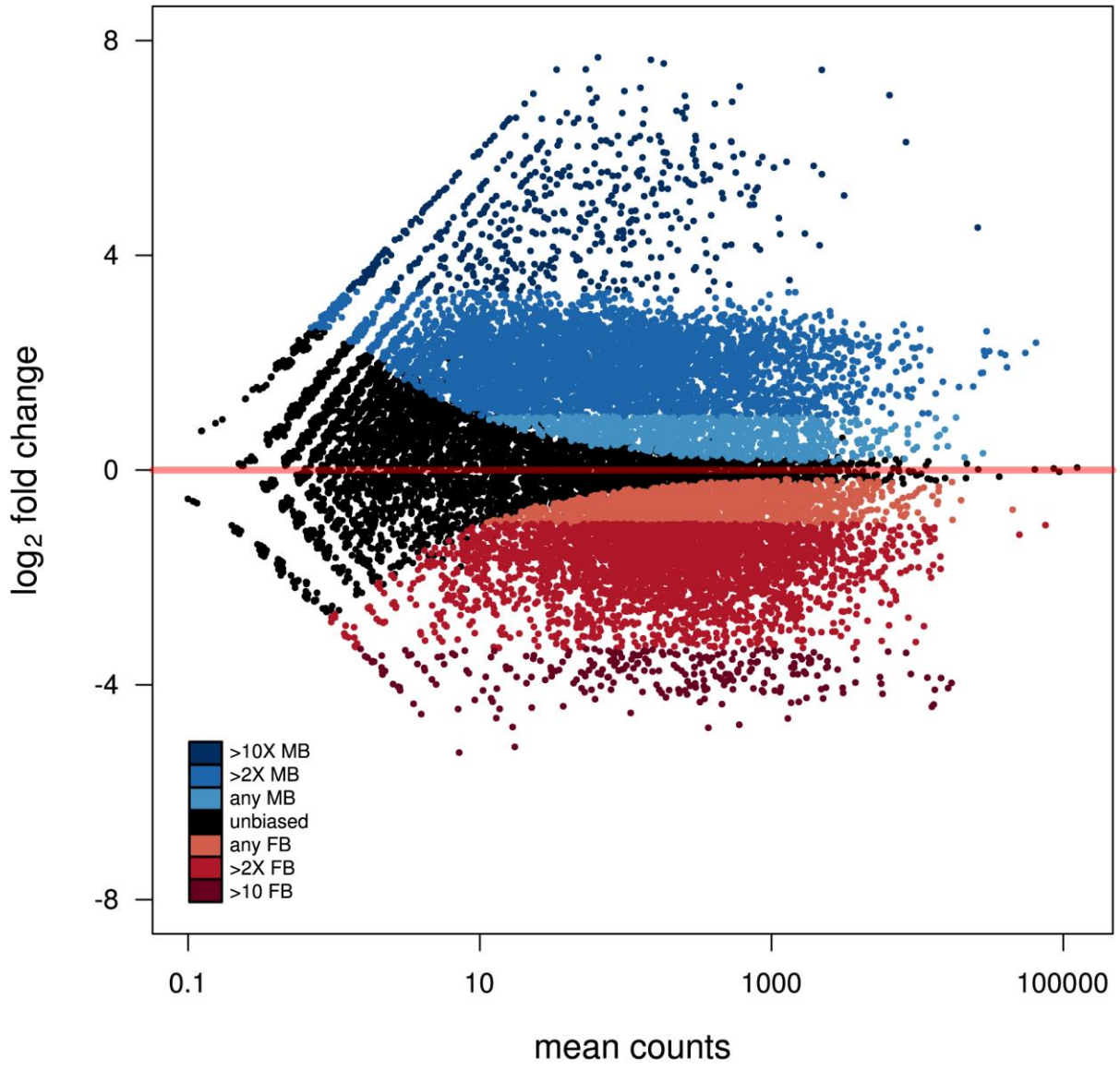

Figure 3: MA plot of differential expression between males (blue) and females (red). The colours refer to the differences in fold change (FC).

clock genes

An important link to environmental light are the clock genes, via the photoreceptor CRYPTOCHROME-1 [15, 16]. Consequently, we searched for clock genes among all differentially expressed genes. Fourteen genes related to circadian clock function were differentially expressed between males and females (table 1). The genes *photolyase, disc overgrown protein kinase* and *timeout/timeless-2* were male-biased while the rest were female-biased.

Table 1: circadian clock and related genes differentially expressed in females compared to males.

	gene name	gene id	fold change	SE
canonical clock genes	Clock[1]	CPIJ002146	- 0.284	0.165
	Clock[1] - continued	CPIJ002147	- 0.272	0.122
	cryptochrome-1	CPIJ009455	- 0.398	0.141
	photolyase[2]	CPIJ017734	+ 3.906	0.186
	Duplication of cryptochrome-2[3]	CPIJ015481	- 0.297	0.164
	cryptochrome-2	CPIJ018859	- 0.347	0.110
	cycle[4]	CPIJ014938	- 0.350	0.184
	period	CPIJ007193	- 0.338	0.162
	timeless protein	CPIJ007082	- 0.314	0.112
genes related to circadian rhythm	timeout/timeless-2[5]	CPIJ000660	+ 5.914	0.194
	par domain protein[6]	CPIJ014920	- 0.301	0.136
	discs overgrown protein kinase[7]	CPIJ003503	+ 2.785	0.100
	hypothetical protein	CPIJ016941	- 0.411	0.090
	mck1	CPIJ006114	- 0.763	0.126

[1] circadian locomotor output cycles kaput protein (contains artificial break point)

[2] annotated in reference as cryptochrome 1

[3] annotated in reference as cryptochrome 1

[4] circadian protein clock/arnt/bmal/pas

[5] paralog of timeless

[6] homolog of par domain protein 1 (pdp1) in *Drosophila melanogaster*

[7] alternative name: doubletime

Discussion

We assessed the effects of artificial light, time, and gender on gene expression in the entire body using RNASeq. Half of all genes were differentially expressed between males and females, with the majority being more highly expressed in males. Because of the strong sex-specific expression, the effect of light treatment was evaluated within sex. Comparing twilight and control treatments, we detected changes to gene expression in males but not in females.

Given our experimental design it is possible that an interaction effect between timepoint and light treatment confounded our ability to detect differential expression leading to an underestimation of the number of differentially expressed genes. Although this constitutes a conservative approach in determining the gene expression changes we find a clear effect of treatment in males.

The treatment effect in males

The majority of differentially expressed genes in males showed higher expression in control. These mainly related to gametogenesis, immune response and lipid metabolism. There is a scarcity of knowledge about the effects of artificial light on mosquitoes, in particular about males. In the control treatment the individuals experienced decreasing light and darkness. Under natural conditions, decreasing light triggers activity in nocturnal mosquitoes. At this time individuals start to search for food as well as for mates, and swarm formation starts in males. Hence, it is tempting to speculate that the expression of genes involved in gametogenesis should also be initiated, possibly as a downstream process of circadian clock expression. No literature is available so this remains an idea to be tested in the future. One aspect to consider are activity levels. If they are low with light at night, which personal observation suggests, that would be in accordance with the idea sketched above. Again this is subject to future studies.

Genes involved in lipid metabolism comprise the second group of differentially expressed genes. This might be related to increased metabolic rates in males that were generally more active in control than in twilight. The last food uptake was 12 hours prior to sampling which could mean that the carbohydrate reserves had been used up. Additionally, we found genes involved in immune response to be differentially expressed in the control condition in males. Some immune genes have previously been detected as rhythmically expressed in *An. gambiae* [8, 35], and we might observe rhythmicity here as well. This could mean that immune gene expression might potentially be negatively influenced under artificial light conditions, which remains to be tested in future studies.

Of the genes differentially expressed in the twilight condition, the majority encode conserved hypothetical proteins, rRNA and a gene encoding for a guanine nucleotide binding protein. In mammals the guanine nucleotide binding proteins couple transmembrane receptors to effector components of a variety of signal transduction pathways [36]. These genes are seemingly conserved across taxa. To date, it is not known whether they carry out the same function in mosquitoes.

Thirteen genes were differentially expressed between treatments and also between timepoints in males. Such a pattern might arise when (i) a given gene is rhythmically expressed but with treatment-specific amplitude or (ii) when expression changes in response to the presence or absence of a stimulus (e.g. food). In order to distinguish between these two scenarios time series sampling would be required, which was not the objective of the present study. Of the 13 overlapping genes only 2 have a functional annotation: gaba receptor, an ortholog of Drosophila *rdl* which acts in peptidergic neurons to promote sleep [34]; and the digestive protease chymotrypsin 2. Neither gene is known to be expressed cyclically.

Time effect in females

In females differential gene expression was detected only in response to time with four genes expressed at elevated levels at the early timepoint. One is a transmembrane *amino acid transporter* that is expressed in brain neurons but also in gut epithelia in mammals [37]. This function seems to be conserved across taxa [38]. *trypsin 4* has a function in digestion, especially of blood meals, in *Aedes* [39]. The expression decreases over time suggesting that down regulation of digestive processes occurs as a function of time since the last blood meal. The third gene that was found to be down-regulated over time is *myosin III*, an ortholog of Drosophila *ninaC* [8]. This gene is part of the *inaD*-signalling complex responsible for organising the phototransduction cascade. *ninaC* is rhythmically expressed in *Aedes aegypti* and *Anopheles gambiae* [8]. It is striking that the differences in light regime did not seem to induce changes in its gene expression. Partly, this is due to expression changes over time, as they may obscure possible more subtle responses to treatment. Another explanation is that in our experiment the females are likely to be inseminated. Male accessory gland secretion is a powerful modulator of female behaviour and activity [40]. It might be that their presence renders females insensitive to light at night. This could potentially have implications for biting propensity as accessory gland secretion can trigger ovulation and oviposition behaviour.

Sex-biased expression

In *Drosophila*, approximately 50% of genes are sex-biased [40] which is comparable to the present findings. In the mosquito *An. gambiae* 72% of genes show sex-biased differential gene expression when measured using whole bodies [14]. By determining orthologue relationships between the sex-biased genes from our study and those in *An. gambiae* [14]

we found the majority of sex-biased genes to overlap in males but not females. This pattern could be explained by greater conservation of male-biased gene expression between *Anopheles* and *Culex*. Alternatively, this could reflect differences in ecology of females from these two species. *An. gambiae* is an obligate blood feeder whereas *Cx pipiens* f. molestus displays facultative autogeny (a blood meal is not essential). Furthermore, the present study sampled individuals reared on a sugar diet whereas *An. gambiae* data were derived from blood-engorged females and sugar-fed males [14], potentially contributing to differences in female-biased gene expression.
Sex-biased differences in gene expression are known to occur in a number of species and sexual dimorphism (in morphology, behaviour and physiology) is believed to be a main driver of this [26]. In *Drosophila melanogaster* as much as 50% of genes can be differentially expressed in gonadal tissue alone [41].

Among the genes that are DE between females and males are the clock genes. Apart from the sex-bias in their expression it is noteworthy that the degree of expression was different, male-biased genes being generally much more highly expressed compared to females, whereas female-biased genes were only slightly (but significantly) upregulated. This suggests that the internal circadian clock system is influenced differently between males and females. The highly expressed male-biased genes included *photolyase*, which is a domain of the CRYPTOCHROME-1 protein. It contains two light-harvesting cofactors and is mainly responsible for DNA repair after UV- and blue-light exposure [42]. *timeout/timeless-2* is a paralogue of circadian clock gene t*imeless* and is involved in chromosome stability and light entrainment [43]. *disc overgrown protein kinase,* in *Drosophila* also referred to as *doubletime,* phosphorylates the PERIOD protein and thus contributes to circadian rythmicity [44]. All of these genes are involved in the perception of light and relate directly to circadian clock function suggesting a greater influence of the twilight treatment on male timing than on females.

The light regime we employed in this study was designed to mimic the natural, gradual increase and decrease of light intensity throughout the day. Work by Veronesi *et al.* [45] suggests that certain light levels (below 5 lux for *Culex pipiens*) function as threshold for commencing or ceasing activity. Our light regime thus provides a cue for anticipation of the onset of darkness but no cue is given that allows anticipation of the artificial “twilight” (112 lux in our experiment). Therefore it is reasonable to believe that we are measuring a response to artificial light rather than to a longer day. However, our choice to deprive individuals of light cues and thus not allow for synchronisation to the ambient light environment constitutes an acute change relative to the light regime experienced before. The objective of the study was

to determine physiological processes that are potentially affected by light at night. Our results then allow for comparison of, for example, different lamp types or natural and artificial light based on target processes identified in this study.

Gonotrophic status can strongly modify female behaviour [40]. An evaluation of the effect of light at night at different stages (e.g., virgin, inseminated, or after oviposition) arguably provides important insights on its ecological implications. However, since females are mated soon after emergence [46] it is reasonable to assume that the vast majority of females at a given point in time are inseminated. Our study thus addresses an important portion of the population. Inseminated females of most species actively search for a blood meal, therefore understanding the effects of light at night on this group may be of importance for disease transmission.

As a nocturnal species *Culex pipiens* is naturally exposed to varying, albeit low, light intensities produced by moonlight. It has been suggested that lunar cycles have some influence on the activity and biting propensity of mosquitoes [47, 48]. In our study we use an artificial light source with a distinct spectral signature. Additionally, there were no cues regarding the lunar cycle provided in the laboratory setup. Our results are thus likely to be the response to light at night. However, the presence of moonlight in combination with artificial light may produce a different gene expression profile, which remains to be addressed in future studies.

Our work raises questions that require additional research in a range of fields before it will be possible to fully understand the implications of light at night for mosquito ecology. Nonetheless, the results suggest that additional light at night does have an influence on *Culex pipiens* f. molestus, males and females being affected to a different extent. This suggests possible implications for reproduction biology and, consequently, population-level impacts.

Data accessibility

The datasets supporting this article are freely available on the website of the research group http://monaghanlab.org/data/cxpip_rnaseq/

Competing Interests

The authors do not have competing interests.

Authors' contributions

AH and MTM conceived the study. AH, PRJ and MTM designed the experiment. AH conducted the experiment and the sampling. AH and PJ analysed the data and drafted the manuscript. AH, PRJ and MTM contributed to the final manuscript. All authors gave final approval for publication.

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Chapter II

Changes in clock-gene expression, activity, and fecundity in the mosquito *Culex pipiens molestus* exposed to artificial light at night

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Running title: Effects of artificial light on mosquitoes

Abstract

Artificial light at night is a global phenomenon. As the human population grows and becomes increasingly urbanized the influence of artificial light is spreading. The implications are potentially far-reaching since light is an important environmental cue for many organisms. We quantified the effects of artificial light at night on the expression of six circadian clock genes, activity and fecundity in the widespread mosquito *Culex pipiens molestus* (Diptera, Culicidae). This species regularly occurs in close proximity to humans and potentially acts as disease vector. Laboratory populations were exposed to16h:8h daily light:dark cycles followed by darkness (control) or by 3h of additional light at a constant, lower intensity (twilight, 16+3:5), a scenario that mimics many urban lighting schemes. Individuals that experienced twilight exhibited significant changes in the expression of *period* and *Clock* genes. Notable sex-specific differences were found in the *cycle* gene (up-regulated in females) and the *Clock* gene (down-regulated in males). Daily activity was reduced by nearly half in twilight-exposed individuals, with changes differing markedly between genders. Female fecundity was impacted resulting in fewer eggs per egg raft and smaller eggs with additional light. Our results indicate that artificial light regimes prevalent in urban settings may influence linkages among the circadian clock, daily activity, and fitness with far-reaching implications for their life history.

Introduction

Artificial light at night is a prominent feature of most areas of human habitation. Despite its global extent and continued growth (up to 20% p.a. in urban areas, Hölker *et al.* 2010) little is known about how artificial light in otherwise dark periods affects the physiology, behaviour and, ultimately, the fitness of organisms (Moore *et al.* 2000; Emerson *et al.* 2008). A number of studies of artificial light have focused on behavioral changes. This includes how insects are attracted to light (Perkin *et al.* 2011, 2014) and how other organisms may benefit from this, e.g. bats that feed on insects attracted to light (Blake *et al.* 1994). Other studies documented negative consequences such as loss of orientation and exhaustion (Eisenbeis & Hassel 2000). Less studied are the effects on diel rhythms of organisms, where a prominent role of light is to act as environmental cue to set and synchronise circadian timekeeping. The circadian clock regulates entire metabolic pathways (Longcore & Rich 2004; Navara & Nelson 2007) and controls locomotor activity in a broad range of organisms (Panda *et al.* 2002). Disruption can lead to changes in the timing of key behaviours like foraging and mating activities (Navara & Nelson 2007) or navigation (Froy *et al.* 2003).

Mosquitoes (Diptera, Culicidae) are a globally distributed group of insects with aquatic immature (egg, larva, pupa) stages. Many species thrive in areas of human habitation (Vezzani 2007) and are therefore very likely to be exposed to artificial light at night. Additionally, mosquitoes play an important role in the functioning of many aquatic and terrestrial ecosystems. The aquatic larvae consume organic carbon (Kraus & Vonesh 2012) and are a major food source for aquatic organisms. Aerial adults are fed on by other insects or birds and act as pollinators (Jhumur *et al.* 2008). Adult females of most species are blood-feeding and thus act as important vectors of disease in vertebrates (Githeko *et al.* 2000). Hence, understanding the potential effects of artificial light on physiology, behaviour and fecundity of mosquitoes may have important implications for both natural and anthropologically influenced ecosystems.

Studies of the circadian clock in mosquitoes have shown expression to be cyclical (Gentile *et al.* 2006, 2009) and altered light regimes (i.e. day lengths) have led to altered circadian rhythmicity in flesh flies (Goto & Denlinger, 2002). But we are not aware of any studies that investigated artificial light at night.
The genetic basis of the mosquito circadian clock (Fig. 1) includes the genes *Clock, cycle, period, timeless, cryptochrome-1* and *cryptochrome-2* (Mathias *et al.* 2005; Gentile *et al.* 2006, 2009; Rund *et al.* 2011, 2013). These genes and their products comprise a central feedback-loop that rhythmically regulates transcription and repression. The mosquito clock

differs from the well-studied *Drosophila* clock most notably by the possession of *cryptochrome-2*, which is thought to represent the ancestral clock state (Zhu *et al.* 2005, 2008; Yuan *et al.* 2007; Meireles-Filho & Kyriacou 2013).

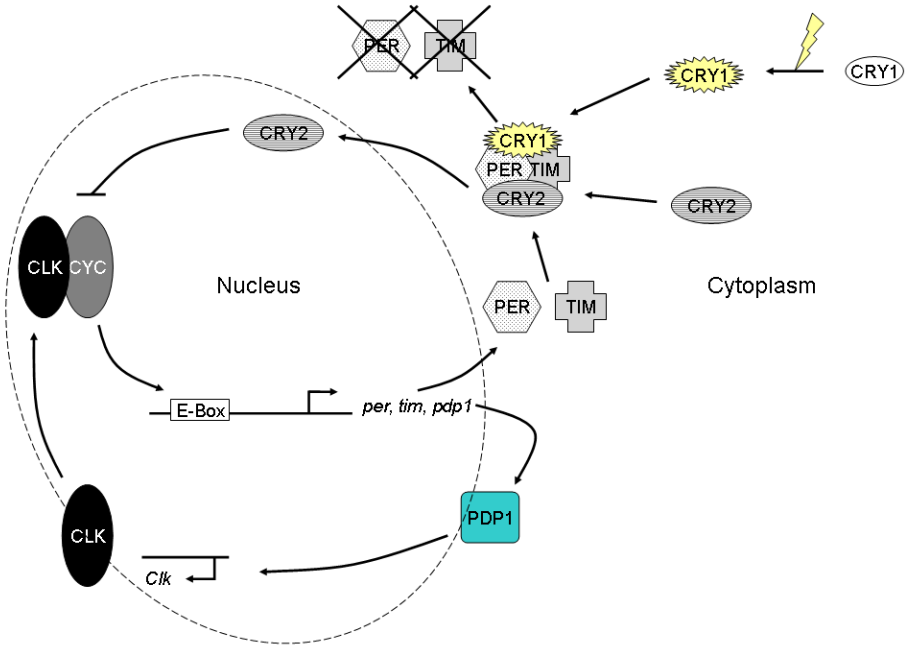

Fig. 1: Model of the mosquito central circadian clock feedback loop, redrawn from Rund et al. (2011) and Tomioka and Matsumoto (2010). Gene loci are indicated with italics and proteins are indicated by capital letters.

Environmental light cues are transported to the clock via the blue-light receptor CRYPTOCHROME-1 (excitation maxima in Drosophila: 445 and 375 nm, Okano *et al.* 1999; Yuan *et al.* 2007) which binds to the PERIOD-TIMELESS-CRYPTOCHROME-2 protein complex, causing it to break up via degradation and phosphorylation of PERIOD and TIMELESS (Stanewsky *et al.* 1998; Ceriani 1999; Emery *et al.* 2000). Free CRYPTOCHROME-2 molecules repress the CLOCK-CYCLE-complex which is regulating the transcription of *period, timeless*, and *PAR-domain protein1 (pdp1)* (Fig. 1; Zhu *et al.* 2005, 2008; Yuan *et al.* 2007; Tomioka & Matsumoto 2010; Rund *et al.* 2011). PDP1 binds to the promoter of *Clock*, activating its transcription initiating the cycle anew (*Anopheles gambiae*: Rund *et al.* 2011; *Culex quinquefasciatus*: Gentile *et al.* 2009; Meireles-Filho & Kyriacou 2013).

The circadian clock affects a number of downstream physiological processes; consequently, changes to the expression of one or more clock genes may have substantial effects on their behaviour. Mosquitoes exhibit regular activity patterns related to the activity of their preferred

hosts (Lee *et al.* 2002, Jhumur *et al.* 2006, 2008). Light-mediated changes of the clock rhythm may lead to changes in activity patterns, which could then affect host search, feeding, and mating. For example, experimental manipulation with light pulses led to changes in blood-feeding patterns in *Anopheles gambiae* (Das & Dimopoulos 2008). A largely neglected aspect is that light may affect males and females differently. Anopheline males form mating swarms that are triggered by certain wavelengths, in a species-specific manner (Howell & Knols 2009). This has not been studied in *Cx. pipiens* although males swarm (Becker *et al.* 2012). Moreover, male mosquitoes feed exclusively on sap (Jhumur *et al.* 2006; Foster 2008) which likely leads to different activity patterns compared to females. Altered activity patterns may either reduce (avoiding light) or expand (using light) feeding time. In females changes in feeding time may influence the nutritional status thus possibly leading to altered energy allocation to egg production.

We used laboratory experiments to quantify the physiological, behavioural and reproductive effects of artificial light at night in the mosquito *Cx. pipiens molestus*. Specifically, we quantified changes in clock-gene expression, daily activity, and egg size and number. *Cx. pipiens molestus* is a biotype of *Cx. pipiens* that can mate in confined spaces and does not require a blood meal, making it particularly suitable for controlled laboratory experiments (Becker *et al.* 2012; Gomes *et al.* 2013). Although artificial light at night is a common attribute of urban mosquito habitats, we are not aware of any studies that manipulated the light environment of this species.

Material and Methods

Laboratory colony and light treatment

A laboratory colony of *Culex pipens molestus* was established in April 2012. Founder individuals (eggs) originated from an existing colony at Bernhard-Nocht-Institute for Tropical Medicine in Hamburg, Germany, which was established from larvae in July 2011 collected near Karlsruhe, Germany. We reared our mosquitoes in a climate chamber maintained at 26 ± 1 °C with a relative humidity of ca. 60-90 %. Adults were kept in mesh cages (60 x 30 x 30 cm) and fed with ~ 10 % saccharose solution offered on cotton pads *ad libitum*. Males and females were mixed to allow mating. Petri dishes were provided for oviposition. These were filled with filtered, sterilised (autoclaved) lake water that was collected weekly from nearby Lake Müggelsee. Egg rafts were removed from the Petri dishes daily and placed in open trays (16 x 29 cm) filled with the same water. After hatching larvae were fed with ground algal

flakes (JBL Spirulina, JBL, Neuhofen, Germany). Pupae were transferred to water-filled beakers (50 ml) in the cages and allowed to emerge.

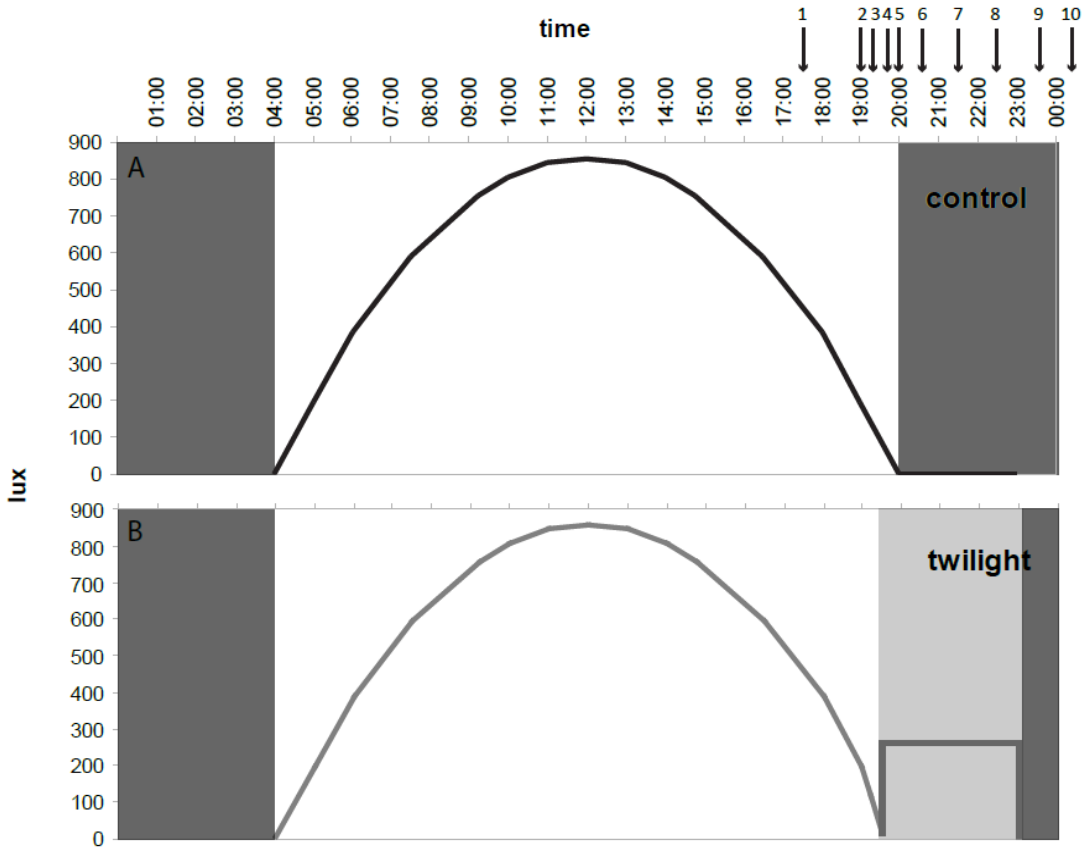

Fig. 2: Light (cool-white LEDs) intensity throughout the day for the control (A) and twilight (B) treatments. Dark grey shading indicates periods of darkness and light grey shading indicates the period of added light (30% of mid-day lux) in the twilight treatment. Arrows indicate sampling times for the gene expression study (17:30, 19:00, 19:20, 19:40, 20:00, 20:30, 21:30, 22:30, 23:30, 00:30).

The climate chamber was divided into two parts, in which the mosquitoes fulfilled their entire life cycle. Each compartment had its own independent light treatment; one (“control” hereafter) had a light:dark (L:D) cycle of 16h:8h and the second had a L:D cycle of 16+3h:5h. The 3 additional hours were at a constant and lower light intensity (“twilight” hereafter, Fig. 2) described below. The control treatment (L:D 16:8) mimicked early summer daylight hours at study latitude (N52° 26' 51.986"). Illumination levels in both treatments were the same throughout the day, the three additional hours of low light intensity (ca. 257 lux, i.e. 30% of the mid-day light level) in the treatment chamber mimicking a suburban environment where street lighting occurs only during the early part of the night. Light was produced using LED illuminants (LED flex SMD, 24VDC, 24W, 1A, 60 LEDs/m, 500cm, cool-white single

chip, Barthelme GmbH & Co. KG, Nürnberg, Germany) arranged in seven strips of 48 LEDs each. These were attached to a board (88 x 34 cm) and suspended horizontally over the cages. Light levels were controlled with custom-made software based on the LabView runtime environment (National Instruments v 8.5.1, cf. Fig. 2). We specified voltage at 15 (19 in twilight) time points to which the software fit a hermite spline curve. The result was a smooth change of voltage, and light intensity, over each 24-hour period with the exception of the three hour constant, low-level additional light in the twilight treatment (Fig. 2; supplementary S1). Light intensity was measured 75cm below the illuminant board inside a cage using a light meter (ILT1700, International Light Technologies, Peabody, MA, USA).

Expression of circadian clock genes

A subset of adult mosquitoes was removed from the control population and sorted by gender prior to gene expression experiments. Ten individuals were placed in transparent plastic sample containers (50 ml) for each sampling time point and sex. Containers were covered with mesh to allow for feeding and placed in continual darkness for 48 hours. Gentile *et al.* (2006) reported that 48 hours efficiently entrain to a given light/dark cycle. A subset of individuals for each treatment and gender was removed at each of ten time points starting at 17:30 and ending at 00:30 (Fig. 2). Individuals were not fed for the twelve hours prior to sampling and containers were placed into their respective treatments by 04:00 am. In order to evaluate whether expression changed with length of exposure and whether expression was cyclic, sampling was repeated on day two (i.e. beginning after individuals were exposed to the respective lighting regime for 41.5 h) and day four (beginning after 89.5 h). All sampled individuals were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Heads from three individuals of each gender and time point were removed on ice and total RNA was extracted using TRIzol® Reagent (Ambion®, Invitrogen, Carlsbad, USA). We used only heads because the expression of clock genes differs between tissues (Gentile *et al.* 2009; Rund *et al.* 2011, 2013). Due to the small amount of starting material, the manufacturer's protocol was adjusted as follows: heads were disrupted and homogenized in 500 µl TRIzol Reagent on ice with an ULTRA-TURRAX® disperser (IKA®, Staufen, Germany) for 20 sec. Chloroform (100 µL) was added, the sample was thoroughly mixed (15 sec), incubated at room temperature (ten min), and centrifuged (15 min, 12,000 g at 4°C). A volume of 200 µL from the aqueous phase was transferred to a new reaction tube and precipitated with 200 µL of 100% isopropanol and incubated (ten min at room temperature, followed by incubation overnight at – 20°C). After centrifugation (twelve min, 12,000 g, 4°C) the supernatant was discarded and the pellet was washed with 200 µL of ice-cold 75% ethanol followed by another centrifugation step (six min). The supernatant was discarded and

the RNA pellet was re-suspended in 20 µL RNase-free water (Carl Roth GmbH und Co. KG, Karlsruhe, Germany) and RNA content was quantified using a NanoDrop 1000 (peqlab-Biotechnology GmbH, Erlangen, Germany).

cDNA synthesis was carried out using AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol with 14 ng cDNA /µL in a final volume of 12.7 µL. A negative control (RNase-free water instead of RNA) was included with every reverse transcription. We used a primer mix consisting of 0.25µl oligo-dT 15 (100 pmol/µl), 0.25µl oligo-dT 20 (100 pmol/µl) and 1 µl of random hexamer primer (100 pmol/µl) per sample (Mr. Martin, Agilent Technologies, personal communication). All primers were obtained from Gentile *et al.* (2006, 2009). qPCR reactions used Brilliant III Ultra Fast SYBR® Green QPCR Master Mix (Agilent Technologies) following the manufacturer's protocol and were analysed on a Stratagene MxPro3000P or MxPro3005P (Agilent Technologies). Samples and calibrators (consisting of pooled samples used for all subsequent runs) were diluted 1:4. In each run we added three calibrators, the reverse transcription negative control, and a negative control for the qPCR. For each sample (and all genes) a double determination was done at the following conditions: initial denaturation at 95°C for three minutes, then 40 cycles of 95°C (17 sec), annealing at 60°C (25 sec) and elongation at 72°C (25 sec). In an additional cycle, a melting curve was established in three steps: 95°C (40 sec), 55°C (30 sec) and 95°C (30 sec; run settings: supplementary S2). Changes in expression were determined using the ΔΔCT method (Pfaffl 2001).

Diel activity

Individual pupae from each treatment population (control, twilight) were transferred to separate mesh-covered containers for emergence. This ensured that individuals remained unmated, and activity patterns reflected only the search for food or mates and not oviposition sites. Adults were collected within three days after emergence and 26 haphazardly selected individuals were transferred to a locomotor activity monitor (LAM, TriKintecs Inc., Waltham, USA). The LAM consists of individually monitored tubes (12.5 x 2.5 cm) and records the number of times any one of the nine infrared beams is crossed within two-minute time windows. Data acquisition began approximately seven hours after individuals were placed in the tubes. Each treatment was run for five days of light:dark cycles (hereafter "LD"), where light treatments were the same as above (Fig. 2). This was followed by five days of complete darkness (hereafter "DD"). LD and DD periods were separated by 24 hours without measuring in order to allow time for adaptation. Survival of the mosquitoes throughout the

experiment was high. Only three individuals died during the measurements in each treatment. These individuals were considered dead when there was no movement recorded for at least twelve hours, in which case only data up to one hour after the last recorded movement were used. Conditions were identical in the two treatments, except that for the twilight experiment adults were collected up to four days post-emergence (n=23).

Fecundity

Within each treatment, a subset of pupae was transferred to a new cage where they were allowed to emerge and reproduce. Densities in these cages were monitored so that control and twilight cages were equal. Dead individuals were counted and sex was determined (males:females control: 1.03:1; twilight: 1.19:1). Throughout the study period (205 days) the density never exceeded 210 individuals per cage. The number of egg rafts and the number of eggs per raft were recorded daily. Egg diameter was measured in a subset of rafts during three time periods over the course of the experiment: March (i.e. 11 months after the colony was established), August, and October 2013. Diameter was measured as viewed from above using a microscope-attached camera (Nikon SMZ1500 and Nikon Digital Sight DS-Fi1, magnification: 100x) and the line-measuring function implemented in the NIS Elements D 3.10 software.

Data analysis

We designed the *gene expression* study to test for changes with respect to treatment, gender, length of exposure, and sampling time. In a first step we calculated a Spearman rank correlation for the relative mRNA expression levels between all pairs of genes using all available data per gene and time point. To reduce the influence of outliers we used medians calculated per gender and time point. This reduced data set was then paired by time point. A multi-level analysis was carried out using Wilcoxon Signed Rank tests (WSR), testing for changes in overall expression levels between the two treatments and was carried out for each gene by pooling all time points and sexes. Each gender was then analysed separately. Changes in gene expression levels over time were examined by testing for differences between time points within treatments using a Kruskal Wallis test (KW). When gene expression differed significantly over time we used Mann Whitney U tests (MWU) for pairwise comparisons per gene (suppl. S3a, S3b). To correct for multiple testing we adjusted the p-value using Bonferroni correction ($p = 0.001$).

Because *activity* patterns can be highly individual, we translated the count data into a binary (1/0) activity matrix (active/inactive), calculated a sum per 2-minute time-window across individuals, and used this value for data analyses. All subsequent tests were carried out for LD and DD separately. We used MWU (n=7200) to compare the two treatments. *Cx. pipiens molestus* have been reported to exhibit bimodal circadian acivity, peaking at the beginning and end of the dark period (Veronesi *et al.* 2012). We therefore divided each day into five activity phases; one: night (23:30 to 04:00), two: increasing light (04:00 – 09:00), three: day (09:00 – 15:00), four: decreasing light (15:00 – 20:00) and five: additional light (20:00-23:00). Only phase five differed between treatments. Differences among phases were tested using KW (n=3600), followed by pairwise MWUs when significant. Within each phase, treatment effects were tested with MWU. Sex-specific differences in activity within treatment and phase were compared using MWU for LD and DD separately.

Fecundity was examined using counts of eggs, rafts and diameter measurements as proxy for egg size. Differences in the number of egg rafts and number of eggs per raft were calculated with MWU. Spearman rank correlation was used to examine relationships between raft and egg counts over time and within each treatment. Because correlations between date and number of eggs were significant for the control (but not twilight) condition, we used KW to evaluate whether this pattern existed for the diameter as well. Diameter, measured in a subset of eggs during three sampling periods (March, August, October), was compared using pairwise MWUs.

Results

Expression of circadian clock genes

We used ΔCT values for the evaluation of the constitutive control rp49. ΔCT is the relative expression corrected for inter-run differences (see Methods). There was no difference between treatments (MWU all available data, $p > 0.05$) or sampling times (KW, $p > 0.05$), although we observed significantly higher ΔCT values in females compared to males in the twilight treatment.

However, when we tested for differences between exposure times within each treatment we found significant (KW, control (n=170): asymp. $p<0.0001$, twilight (n=187): $p=0.006$) responses of the constitutive gene. This pattern was driven by the 1-d-exposure, which likely represents a response to the new light regime (after 48h constant darkness) that triggers a change in the individuals' metabolic rates. Mosquitoes show reduced metabolic rates in resting periods (Gray & Bradley 2003), and our data clearly shows reduced activity

(increased resting) in constant darkness. Since this pattern was not detectable in the comparison of treatments the phenomenon must have occurred in both treatments to the same degree. Because we used ΔΔCT values calculated for each individual, relative expression of target genes was obtained with respect to individual rp49 baselines and thus reflects a true difference in expression.

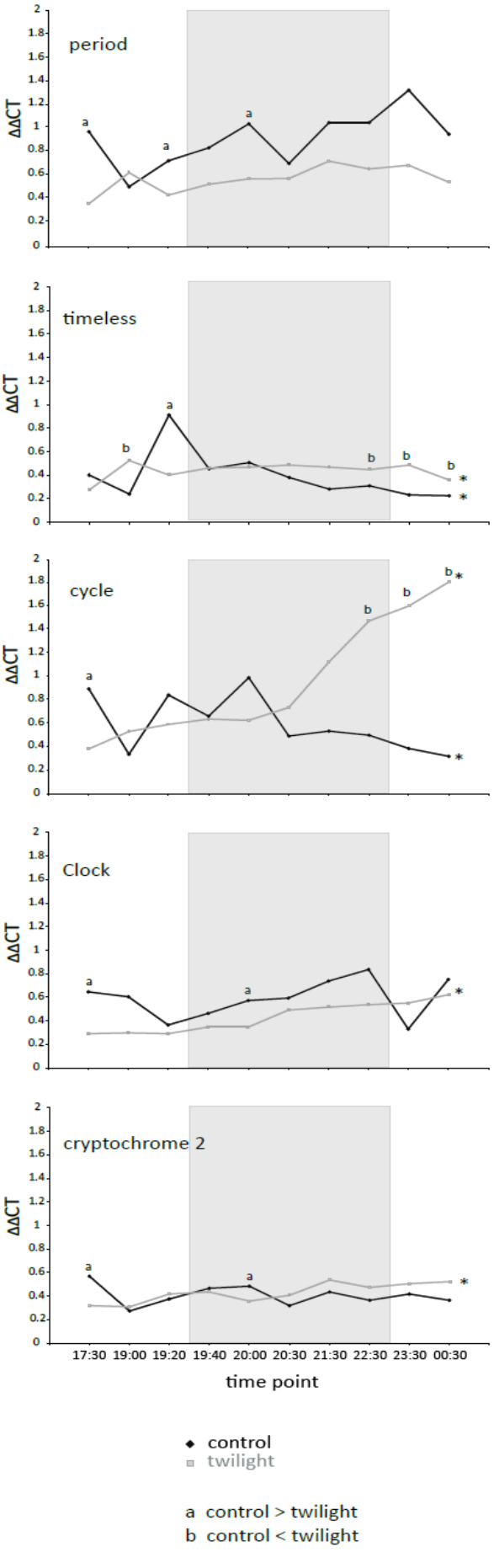

Fig. 3: Median relative gene expression of *Cx. pipiens* f. molestus over the ten sampling time points. Expression levels measured after three different exposure periods (1d, 2d, 4d) were not significantly different (see Results) and all three periods are therefore combined. Grey shading indicates the times with different lighting in the control (0 lux) and twilight (*ca*. 300 lux) treatments. Asterisks indicate overall significant differences within treatment. Letters indicate significant difference between treatments at a given time point.

We first tested for overall differences in expression resulting from length of exposure (1, 2, or 4 d) and found no significant difference except that cycle expression in the control varied with exposure length (KW, df = 2, asymp. p = 0.029, medians per gender, time point and gene). So we pooled data from the different exposure lengths for subsequent analyses. Because the studied genes comprise most of the central circadian feedback loop, the expression of some genes is expected to be correlated. We observed significant correlations between all genes, but a few relationships were particularly strong: cycle was correlated positively with *cryptochrome-2* (ρ = 0.772) and with *Clock* (ρ = 0.531). *Clock* was, in turn, correlated with *cryptochrome-2* (ρ = 0.626). It is noteworthy that the expression of these three components is regulated in different feedback-loops.
Period and *Clock* expression was significantly down-regulated in individuals exposed to twilight conditions, pooling all 10 time-points and genders (WSR, n = 60; Table 1).

Table 1: Median expression difference ($\Delta\Delta C_T$) per gene and treatment for all individuals ("all") and for females and males separately. Arrows give the direction of regulation in twilight compared to control in cases of significant differences. Significance level for comparison of treatments (WSR) over all and within gender is indicated as: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

	period	*timeless*	*cryptochrome2*	*cycle*	*Clock*
all - control (n=60)	0.94	0.36	0.43	0.54	0.55
all - twilight (n=60)	0.62***↓	0.44	0.43	0.74	0.42** ↓
p_a(control vs twilight)	0.000	0.104	0.918	0.062	0.009
females - control (n=30)	1.00	0.32	0.35	0.47	0.52
females - twilight (n=30)	0.61***↓	0.42	0.42	0.78* ↑	0.40
p_f(control vs twilight)	0.000	0.106	0.237	0.030	0.131
males - control (n=30)	0.84	0.38	0.46	0.66	0.60
males - twilight (n=30)	0.66** ↓	0.46	0.43	0.67	0.44* ↓
p_m(control vs twilight)	0.001	0.478	0.371	0.530	0.028

Separating the data by gender revealed that *period* was down-regulated in both sexes and *Clock* was down-regulated in males but *cycle* was up-regulated in females (Table 1). Examining individual time points, all five genes exhibited significant differences between control and twilight conditions at one or more time points (Fig. 3). Four genes differed at 17:30, when light conditions were identical in both treatments, and three genes differed at 20:00, with contrasting light levels (control = dark; twilight = 30 % of mid-day; Fig. 2). Expression at 19:40 (low light in both treatments), 20:30, and 21:30 (dark in control, light in twilight) were never significantly different between treatments. Testing for differences across time points within treatments showed that two genes exhibited temporal changes in the control (*cycle* and *timeless*; KW, df=9, asymp. p < 0.05; Fig. 3) whereas four genes varied in twilight (*cycle, timeless, cryptochrome2*, and *Clock*; p <0.05; Fig. 3). The fact that exposure

time did not differ significantly indicates that this observed temporal variation was cyclical over a period of four days of exposure in each condition.

Diel activity patterns

In both treatments, mosquitoes were more active when experiencing light-dark cycles (LD, Table 2) compared to constant darkness (DD, suppl. Table S4, p asymp. < 0.0001, both). When analysed for temporal differences, activity levels varied among the five phases (pairwise comparisons, MWU; LD asymp. p < 0.0001 – 0.003; DD: p < 0.0001-0.005, Bonferroni-adjusted significance =0.005).

Table 2: Mean activity (sum of all active windows per 2-minute interval divided by n individuals) in each phase of the LD (light:dark) experiment. Significance level (SL) for the comparison of treatments within phase is indicated as: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$),.ns = not significant.

	phase				
LD	1	2	3	4	5
all (SL)	***	***	ns	***	***
control	0.251	0.098	0.010	0.105	0.419
twilight	0.213	0.053	0.008	0.038	0.051
females (SL)	***	**	***	***	***
control	0.334	0.132	0.017	0.148	0.538
twilight	0.425	0.103	0.029	0.102	0.141
males (SL)	ns	***	*	***	***
control	0.166	0.063	0.003	0.061	0.307
twilight	0.198	0.051	0.001	0.021	0.026

The only exception was in the control population under constant darkness (DD) where there were no differences between phases one and four (p = 0.217) and between phases two and five (p = 0.853). Activity was nearly halved in most phases in twilight when compared to the control (Fig.4). Control treatment activity peaked during the two dark phases (one and five), as expected for a nocturnal insect with bimodal activity. Three additional hours of light led to reduced activity in phase five in the twilight treatment (Fig. 4). In LD, activity corresponded closely and inversely with light intensity. The pattern was similar in DD but the evening peak occurred earlier (phase four). Compared to LD this might reflect earlier anticipation of darkness, indicating that changing (i.e. in- or decreasing) light intensity is an important trigger for activity. The individuals of the twilight treatment markedly increased activity in the phases four and five in DD. When analysed separately, females were almost always more active than males (Fig. 4) with the exception of phases one and five in complete darkness in the control condition. The locomotor activity of males and females differed significantly within

treatment, across phases (MWU, asympt. p. < 0.0001 for all comparisons and LD/DD; Fig. 4) and within phases (LD, Table 2; DD, suppl. Table S4).

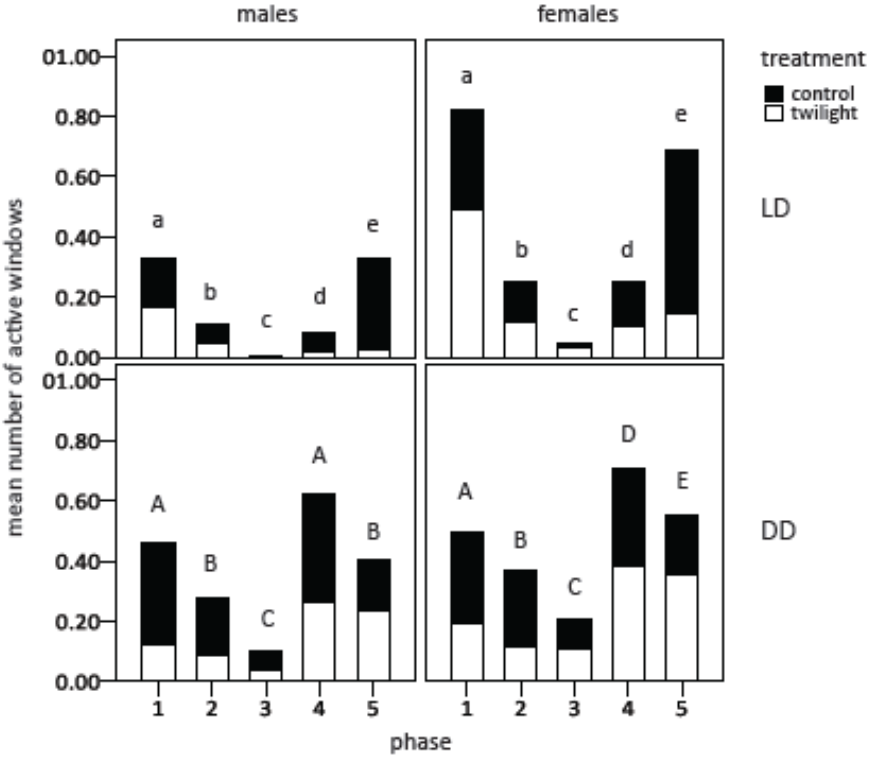

Fig. 4: Activity of *Cx. pipiens* f. molestus as the mean number of active windows in 2-minute time intervals across individuals, separated into 5 phases, and by gender. The upper panels report activity of individuals during the LD phase of the experiment (control L:D = 16:8; twilight L:D = 16+3:5; see Fig. 2). The lower panels report activity during the DD phase (no light in any phase). Letters indicate significant differences between phases (pairwise MWU, Bonferroni-adjusted *p* = 0.005; see Table 2).

Fecundity

The number of eggs per raft was significantly higher in the control treatment (MWU, asymptotic $p_{2\text{-tailed}} = 0.005$), although the number of egg rafts did not differ significantly between treatments (MWU, asymptotic $p_{2\text{-tailed}} = 0.195$). There was a negative correlation between the number of eggs and collection date in the control ($\rho = -0.231$, $p_{2\text{-tailed}} = 0.001$) but not in the twilight treatment ($\rho = 0.077$, $p_{2\text{-tailed}} = 0.275$). There was no correlation between date and number of rafts (control: $\rho = -0.112$, $p_{2\text{-tailed}} = 0.460$, twilight: $\rho = -0.002$, $p_{2\text{-tailed}} = 0.985$). Egg diameter was greater in the control condition than in twilight (MWU, asymptotic p = 0.031, absolute numbers in Table 3).

Table 3: Mean egg diameter at each sampling term for each treatment. Significance level for the pair wise MWU tests of terms within treatment is given when all comparisons were significant indicated as: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

	term	n	mean [mm]	SD
control	march	93	0.105***	0.015
	august	110	0.134**	0.025
	october	271	0.125**	0.015
twilight	march	88	0.118	0.017
	august	125	0.128**	0.024
	october	224	0.119	0.014

Since there was a correlation between number of eggs and date in control, we subdivided diameter measurements into three periods. Within treatments there was a significant effect of period (KW, df = 2, control asymp. $p < 0.000$; twilight: $p = 0.004$). The diameter differed between all samplings and treatments except between March and October in twilight ($p = 0.282$). Testing for the effect of light within sampling campaigns showed significant differences in all periods (Table 3). Egg diameter was not significantly different in March (11 months after the colony was founded) but was larger in the control population in August and October (asymptotic $p_{2tailed} < 0.000$; Table 3).

Discussion

Artificial light at night has been a common feature of urban and suburban environments for over a century. Despite its potential to affect the physiology, behaviour, ecology, and fitness of a wide range of organisms, most studies have been limited to single factors (e.g., migration: Moore *et al.,* 2000; attraction: Eisenbeis & Hassel, 2000). A key mechanism by which artificial light may influence organisms is through its interaction with the circadian clock, which can have subsequent impact on physiology, metabolism, behaviour (Meirleles-Filho & Kyriacou 2013), and ultimately fitness (Emerson *et al.* 2008). Here we report clear evidence that artificial light at night simultaneously influenced clock-gene expression, altered activity patterns, and effects reproductive output in the mosquito *Cx. pipiens molestus*, a widespread species of the temperate zone often occurring in urban or suburban areas (Townroe & Callaghan, 2014). Some of the observed effects were different in male and female mosquitoes, which may have important consequences because of their differing roles in ecosystems.

Expression

The genes of the circadian clock have been fairly well studied in several mosquito (Culicidae) species, including the closely related *Cx. quinquefasciatus* (Gentile *et al.* 2009). Most research on clock interaction with light cues is concerned with changes in day length (i.e. changes in L:D ratios) and interactions with other factors (e.g., temperature) (Goto & Denlinger, 2002, Mathias et al. 2005). We were specifically interested in artificial light at night of the kind generated by street and other lighting, which in contrast to different L:D ratios consists of an abrupt switching on and constant brightness for some period of time that would otherwise be dark. An important overall finding was that the patterns we observed were consistent across exposure days (1-, 2-, 4-d exposure to the conditions), indicating cyclical expression and consistent treatment-induced changes over time.
Expression of the circadian clock-genes depends on the abundance of other gene products, both within and outside the feedback loop, thus it was not surprising to find significant correlations among all studied genes. The strongest correlations were observed among *Clock*, *cryptochrome2* and *cycle*, their expression being regulated in different feedback loops (Fig. 1). When considering overall changes in expression, we found that in the presence of artificial light at night the regulation of expression was gene- and also gender-specific, in the sense that one (or both for *period*) sex altered its expression much stronger than the other. There is no general regulation pattern in twilight compared to control. In fact, three out of five genes differ (*period, Clock* and *cycle*) between treatments. Studies of mosquito ecology largely focussed on females as they are potential disease vectors. Here we show that males and females differ in gene expression in different light conditions which might result in sex-specific changes of internal timekeeping.

Based on previous studies of other species, we anticipated *Clock* and *cycle* to peak when the levels of *period*, *timeless* and *cryptochrome2* were low (Gentile *et al.* 2009; Tomioka & Matsumoto 2010; Meireles-Filho & Kyriacou 2013). In contrast, overall temporal changes were minimal in the control individuals, with *Clock* and *period* increasing as *timeless* and *cycle* decreased. This might result in protein levels that impede CLOCK-CYCLE-complex formation possibly disrupting the feedback loop. But our data yields no hint of circadian disruption in either treatment (stability across exposure). In the twilight condition *Clock* and *cycle* increased while *timeless* remained stable and *period* also increased slightly albeit not significantly. This indicates low levels of the respective proteins which is likely due to the light dependant cryptochrome1-mediated degradation of the PERIOD-TIMELESS-CRYPTOCHROME2-complex (Fig. 1, Ceriani 1999; Gentile *et al.* 2009; Meireles-Filho & Kyriacou 2013).
The differences we observed in gene expression are not easy to compare with existing literature, because there are no similar studies on *Cx. pipiens* available, only on its sister

species *Cx. quinquefasciatus.* Gentile *et al.* (2009) sampled every two hours over a 24-h period in 12:12 h L:D in a study of *Cx. quinquefasciatus* and *Aedes aegypti*, as opposed to the higher frequency over 7 hr sampled here. Although closely related, *Cx. quinquefasciatus* has a tropical distribution and may thus differ in its clock-gene expression patterns because of inherent differences in light regime (daily and seasonally), temperature, and other factors relevant to the functioning of the circadian clock (reviewed in: Meireles-Filho & Kyriacou 2013).

Activity

Activity was strongly influenced by light and whether it is in-or decreasing. Mosquitoes were significantly less active in the twilight across all phases except for mid-day, when individuals of both treatments were least active. Once this cue disappeared (i.e. constant darkness) activity continued to vary over the course of each 24-h period, suggesting a role of internal timekeeping.
We found that increasing light led to decreased activity and vice versa (phases 2 and 4), and activity was highest during the dark periods. Accordingly, activity in the twilight treatment was lower and remained lower in constant darkness. In most cases, females were more active than males. Because females are often the only gender studied, it is noteworthy that significant differences in gender activity resulting from changes in light levels may have consequences for populations and ecosystems that have yet to be considered.
Our findings are in accordance with the literature. Veronesi *et al.* (2012) found that host-seeking (i.e. female) flight activity in *Cx. pipiens* was induced when light intensity dropped below 5 lux, resulting in a strong peak two to three hours after sunset with low activity until sunrise. They also reported that changing light intensities led to changing activity patterns. For example, there was a shift in the timing of activity after autumnal equinox to before sunset. Veronesi *et al.* (2012) suggested that this was light-induced because temperatures were still favourable at the time.

Due to a female life span of 20 to 30 days of *Cx. pipiens* it is possible for an individual to encounter seasonal changes, especially emerging late in the year (Veronesi *et al.* 2012). Therefore it is essential to adapt to the light regime as it is important in anticipating seasonal temperature changes and related behaviours such as diapause (Veronesi *et al.* 2012, Robich *et al.* 2007). In diurnal *Aedes aegypti* in urban Trinidad (night time brightness ca. 53-107 lux, converted from foot-candles (5-10fc)) 10% of human landing captures occurred at night, but virtually no captures were made at the same time in dark rural areas (Chadee & Martinez 2000). This exemplifies how artificial light at night influences host-seeking activity of a diurnal

mosquito such that it uses extra light. In contrast, a nocturnal species' foraging time might then be reduced.
We found very different activity patterns in all phases except for mid-day, indicating that artificial light may alter activity patterns throughout the day, not only the timing of onset and end. The reduced activity in twilight condition (i.e. prolonged resting periods) may be followed by a reduction in foraging time and thus decreased nutrient uptake. Additionally, it may lead to fewer mating encounters. We investigated the population-level effect on reproductive output below.

Fecundity

Females produced fewer eggs of smaller diameter in the twilight conditions. Because densities were maintained at similar levels and the number of rafts did not differ significantly between treatments, we presume that females laid eggs at a similar rate and the amount of energy used for finding oviposition sites was comparable. These findings are interesting on their own because of potential effects on fitness resulting from reduced fecundity and lower egg mass. Sota & Mogi (1992) found that in *Aedes* mosquitoes egg size is a predictor of desiccation resistance which is especially important for non-permanent larval habitats that predominate in cities. Larger eggs may have a fitness advantage surviving unfavourable conditions and hence a greater probability to hatch. We found smaller eggs in twilight. Smaller eggs may be produced by i) smaller females in the population or ii) reduced energy allocation to egg production and provisioning. The fact that we find a significant difference of diameter over time in both treatments suggests that adult female size distribution was not skewed. This probably led to increased variation in the data set, which likely caused an underestimation rather than overestimation of the differences. Eggs were smallest in March, largest in August, and again smaller in October. This was found in both treatments but more pronounced in the control, implying that reduced female allocation is a consistent behaviour over time. Our experimental set-up lacks cues of seasonal change (e.g. day length, temperature) so we did not expect such pronounced differences over time. The only cue was the (autoclaved) water used for oviposition and rearing larvae and pupae. This or innate seasonality might have given rise to this pattern. We are unaware of any studies evaluating the egg size of *Culex* over the course of a year. Also, it is long known that there is a complex genetic basis for seasonal rhythms (Tauber & Tauber 1981) but it is not clear how reliable seasonality will be expressed in a constant environment.
We also found fewer eggs per raft in the twilight. This could have resulted from fewer females laying eggs in the twilight treatment. We believe this is not the case, because the number of rafts did not differ between the two treatments. Also, ovary competence is

stimulated through food intake (Wheeler 1996) and sugar meals were available *ad libitum*, we therefore suppose that all females were capable of producing eggs.
These findings are interesting within the study as a whole, because they may be directly linked to the changes in activity we observed (above). Individuals in twilight conditions were less active, perhaps indicating that these individuals feed less frequently. Although we did not measure key variables (e.g., total feeding time, sugar consumption, growth rate) it suggests a link between the two sets of findings.

Limitations

The laboratory setup allowed us to control critical environmental parameters (temperature, humidity) and simulate daily light cycles with artificial twilight. It was especially important to control temperature, as it can play a key role in the circadian clock in insects (Goto & Denlinger 2002, Meireles-Filho & Kyriacou 2013). Nonetheless, an important next step is to carry out research on natural populations. Our control condition mimics an early summer day with 16 hours of light. While this absence of seasonal cues may have influenced our results, we wanted to minimize confounding factors such as shifts in peak expression of clock genes depending on day length (Goto & Denlinger 2002). Regularly attending the colony to monitor densities, supply of sugar solution, change the water and to collect egg rafts, may have introduced an olfactory cue to the females. This may lead to different results than would occur in a host-free environment, but this potential cue was introduced to both experimental groups. Another limitation of our setup was that allowing individuals to complete the entire life cycle within each treatment required the separation of the climate chamber into two compartments, which might have induced divergent processes in the two groups. For the gene expression experiments, individuals were taken from the same source population (control), and largely support the differences obtained in the other two experiments. Activity in LD was lowest in phase three for both groups but differed in DD which depicts the response to light as well as a different behaviour in continual darkness. Therefore it can be assumed that both behaviours are triggered by the light environment rather than being a kind of adaptive response based on larval experience of the system. We chose not to translocate individuals from the control group for the activity experiment because we wanted to exclude the possibility of aberrant behaviour caused by the new environment and the differences of the two treatments.

Conclusion

Our results show that a combined approach of multiple experimental methods can provide insight into a complex interaction of organismal responses to this important environmental cue. A well-running clock is known to have positive effects on individual fitness. Emerson *et al.* (2008) showed that of all fitness components measured (pupal survivorship, embryonic viability, adult longevity and fecundity as mean number of eggs per eclosing female) the number of eggs was significantly reduced in the non-resonant (L:D 10:25) environment in *Wyeomyia smithii*. The mechanisms behind these changes may not be the same because we studied a nocturnal species, different L:D ratios, artificial light at night, and population fecundity rather than individual female fecundity. Nonetheless, the consequence of reduced reproductive output was the same.

Hence a disrupted circadian clock has a large impact on future mosquito generations. The light conditions we tested influenced but did not completely disrupt clock functioning. Note that we only analysed three hours of 'lit night' whereas in many urban areas artificial light persists all night. The clock not only affects fitness but also regulates activity. Gentile *et al.* (2009) speculated *cryptochrome-2* might be involved in controlling activity patterns as there was a remarkable difference in expression patterns between a diurnal (*Ae. aegypti*) and a nocturnal (*Cx. quinquefasciatus*) species. In *Cx. pipiens molestus* mRNA abundance of *cryptochrome-2* was not found to cycle over time and across treatments suggesting that, at least in this species, activity is controlled by other means, f.e. a different gene or via post-translational regulation (Gallego & Virshup 2007). On the other hand activity was clearly reduced under artificial light at night. This suggests enhanced resting which in turn might have influenced reproductive output. Since we only measured the output (i.e. eggs) but no other parameters influencing fecundity we cannot make this link for certain, but it seems unlikely that that the altered clock gene expression, activity and reduced reproductive output observed are a product of chance alone. The sex-specific differences found in the expression as well as in activity underlines the importance of studying both sexes to fully appreciate the potential ecological implications of artificial light at night. We would like to highlight, that this study does not aim to test for methods of mosquito control. In fact, this study suggests a rather large adaptive potential of *Cx. pipiens molestus.*

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Data accessablitity

Data obtained in the study will be made available by the authors upon request.

Appendix

S1: Light regulation in the two treatments in % of maximum voltage and translated into light intensity (lux).

time of day	%	control lx	twilight lx
04:00:00	0	0	0
05:00:00	22.5	192.78	192.78
06:00:00	45	385.56	385.56
07:30:00	69	591.192	591.192
09:15:00	88	753.984	753.984
10:00:00	94	805.392	805.392
11:00:00	98.5	843.948	843.948
12:00:00	100	856.8	856.8
13:00:00	98.5	843.948	843.948
14:00:00	94	805.392	805.392
14:45:00	88	753.984	753.984
16:30:00	69	591.192	591.192
18:00:00	45	385.56	385.56
19:00:00	22.5	192.78	192.78
19:13:00	13		111.384
19:30:00	1		8.568
19:30:10	30		257.04
20:00:00		0	
23:00:00	30	0	257.04
23:00:10	0	0	0

S2: Run settings for Stratagene MxPro3000/MxPro3005p

Thermal profile as reported in manuscript. ROX was used as a reference dye.

<u>Analysis term settings:</u>

Baseline correction: adaptive baseline

Graphical temperature resolution Dissociation point separation: 0.5°

Threshold fluorescence: amplification-based threshold fluorescence lower range 5%, upper range 60%

Threshold has been set manually for each gene when exponential amplification was stable for all genes and used for all subsequent runs

<u>Efficiency settings and threshold:</u>

cryptochrome2: R^2: 0.981, slope: -3.323, Efficiency: 100.0%, threshold: 0.090

period: R^2: 0.994, slope: -3.557, Efficiency: 91.0%, threshold: 0.080

timeless: R^2: 0.993, slope: -3.323, Efficiency: 100.0%, threshold: 0.080

Clock: R^2: 0.992, slope: -3.834, Efficiency: 82.3%, threshold: 0.190

cycle: R^2: 0.998, slope: -3.276, Efficiency: 102.0%, threshold: 0.190

ribosomal proteine 49: R^2: 0.992, slope: -3.438, Efficiency: 95.4%, threshold: 0.100

Table S3a: control treatment: p-values for pairwise comparison between time points (Mann-Whitney-U Test) for all genes. Bonferroni-adjusted significance threshold: p=0.00111

period	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.077	0.519	0.203	0.957	0.239	0.909	0.636	0.67	0.464
19:00		~	0.092	0.335	0.001	0.287	0.17	0.085	0.11	0.628
19:20			~	0.757	0.682	0.636	0.786	0.518	0.345	0.433
19:40				~	0.361	0.815	0.351	0.258	0.224	0.986
20:00					~	0.307	0.882	0.576	0.626	0.325
20:30						~	0.424	0.351	0.224	0.762
21:30							~	0.708	0.709	0.39
22:30								~	0.901	0.176
23:30									~	0.358
00:30										~

timeless	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.035	0.019	0.424	0.109	0.815	0.335	0.126	0.017	0.011
19:00		~	**0.0001**	0.007	**0.0007**	0.032	0.131	0.205	0.763	0.737
19:20			~	0.089	0.444	0.089	0.007	**0.00055**	**0.00012**	**0.000091**
19:40				~	0.443	0.839	0.118	0.027	0.0063	0.0058
20:00					~	0.297	0.031	0.003	**0.00046**	**0.0003**
20:30						~	0.503	0.184	0.117	0.044
21:30							~	1	0.423	0.153
22:30								~	0.276	0.202
23:30									~	0.626
00:30										~

cryptochrome2	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.219	0.318	0.621	0.959	0.239	0.503	0.909	0.621	0.29
19:00		~	0.413	0.157	0.053	0.463	0.079	0.29	0.26	0.39
19:20			~	0.345	0.081	0.568	0.322	0.658	0.401	0.911
19:40				~	0.423	0.281	0.606	0.873	0.669	0.232
20:00					~	0.081	0.901	0.631	0.21	0.045
20:30						~	0.143	0.483	0.237	0.58
21:30							~	0.394	0.736	0.411
22:30								~	0.736	0.71
23:30									~	0.47
00:30										~

Clock	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.525	0.062	0.068	0.646	0.628	0.443	0.684	0.126	0.817
19:00		~	0.413	0.369	0.763	0.858	0.182	0.357	0.465	0.71
19:20			~	0.684	0.074	0.232	0.018	0.14	0.985	0.261
19:40				~	0.164	0.239	0.02	0.083	0.646	0.145
20:00					~	0.932	0.231	0.382	0.128	0.984
20:30						~	0.195	0.525	0.347	0.845
21:30							~	0.734	0.034	0.331
22:30								~	0.326	0.37
23:30									~	0.423
00:30										~

cycle	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.219	0.708	0.303	0.957	0.232	0.59	0.525	0.154	0.025
19:00		~	0.259	0.259	0.132	0.474	0.067	0.29	0.581	0.911
19:20			~	0.61	0.682	0.322	0.496	0.563	0.146	0.02
19:40				~	0.39	0.322	0.563	0.586	0.087	0.002
20:00					~	0.076	0.71	0.23	0.017	**0.000478**
20:30						~	0.122	0.786	0.763	0.069
21:30							~	0.322	0.034	0.003
22:30								~	0.533	0.04
23:30									~	0.093
00:30										~

Table S3b: twilight treatment: p-values for pairwise comparison between time points (Mann-Whitney-U Test) for all genes. Bonferroni-adjusted significance threshold: p=0.00111

period	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.158	0.882	0.319	0.307	0.259	0.044	0.033	0.06	0.294
19:00		~	0.047	0.215	0.398	0.442	0.953	0.854	0.815	0.325
19:20			~	0.326	0.206	0.186	0.03	0.015	0.035	0.262
19:40				~	0.737	0.563	0.092	0.073	0.174	0.923
20:00					~	0.938	0.318	0.198	0.48	0.791
20:30						~	0.258	0.187	0.518	0.646
21:30							~	1	0.573	0.209
22:30								~	0.659	0.206
23:30									~	0.361
00:30										~

timeless	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.004	0.148	0.007	0.025	0.005	0.002	0.009	0.022	0.143
19:00		~	0.107	0.9	0.586	0.59	0.79	0.163	0.318	0.025
19:20			~	0.253	0.416	0.192	0.175	0.606	0.364	0.666
19:40				~	0.769	0.968	0.972	0.492	0.798	0.132
20:00					~	0.832	0.763	0.708	0.961	0.153
20:30						~	0.878	0.279	0.767	0.079
21:30							~	0.266	0.695	0.03
22:30								~	0.521	0.279
23:30									~	0.126
00:30										~

cryptochrome2	17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
17:30	~	0.858	0.065	0.089	0.226	0.031	0.004	0.074	0.011	0.02
19:00		~	0.121	0.154	0.546	0.207	0.026	0.118	0.029	0.045
19:20			~	0.884	0.234	0.945	0.281	0.626	0.308	0.308
19:40				~	0.396	0.778	0.117	0.527	0.325	0.229
20:00					~	0.308	0.041	0.143	0.074	0.097
20:30						~	0.351	0.696	0.239	0.279
21:30							~	0.424	0.961	0.909
22:30								~	0.606	0.481
23:30									~	0.963
00:30										~

Clock		17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
	17:30	~	0.772	0.835	0.426	0.791	0.203	0.126	0.152	0.031	0.01
	19:00		~	0.916	0.308	0.646	0.095	0.063	0.135	0.009	0.008
	19:20			~	0.297	0.728	0.037	0.009	0.037	**0.00106**	**0.00052**
	19:40				~	0.53	0.174	0.045	0.174	0.005	0.004
	20:00					~	0.068	0.02	0.079	0.002	**0.00066**
	20:30						~	0.782	0.963	0.339	0.181
	21:30							~	0.832	0.66	0.273
	22:30								~	0.339	0.226
	23:30									~	0.791
	00:30										~

cycle		17:30	19:00	19:20	19:40	20:00	20:30	21:30	22:30	23:30	00:30
	17:30	~	0.386	0.061	0.019	0.011	0.013	**0.000133**	**0.00006**	**0.000002**	**0.00003**
	19:00		~	0.415	0.267	0.303	0.205	0.006	0.001	**0.00008**	**0.00082**
	19:20			~	0.794	0.94	0.661	0.042	0.004	**0.00031**	0.002
	19:40				~	0.861	0.624	0.031	0.004	**0.00038**	0.003
	20:00					~	0.66	0.049	0.005	**0.00028**	0.005
	20:30						~	0.131	0.02	0.003	0.017
	21:30							~	0.245	0.173	0.207
	22:30								~	0.521	0.462
	23:30									~	0.696
	00:30										~

Table S4: Mean activity per phase in DD (continual darkness) over the course of the experiment. Activity is calculated as the sum of active windows per time interval (2 minutes), divided by the number of individuals. Significance level for the comparison of the two treatments within phase is indicated as: * significant (p<0.05), ** very significant (p<0.01), *** highly significant (p<0.001), ns = not significant.

	phase				
DD	1	2	3	4	5
all (SL)	***	***	***	***	***
control	0.295	0.209	0.076	0.314	0.172
twilight	0.114	0.074	0.050	0.234	0.200
Females (SL)	***	***	**	***	***
control	0.307	0.252	0.097	0.329	0.202
twilight	0.208	0.120	0.085	0.352	0.365
Males (SL)	***	***	***	***	***
control	0.333	0.189	0.062	0.359	0.171
twilight	0.116	0.084	0.054	0.278	0.225

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Chapter III

City-dwellers and country folks: population differentiation along an urban-rural gradient in the mosquito *Culex pipiens*

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Keywords: population differentiation, *pipiens, molestus,* Culicidae, microsatellites, species inventory

Abstract

Urban areas combine a suite of characteristics that differ in many ways from natural habitats. Green areas are patchily distributed within highly built-up areas and differ greatly depending on how they are managed (e.g., as parks or left as derelict land). Urban areas also contain an array of features produced by humans, such as pollution, noise and light, differing markedly from natural habitats. Mosquitoes occur around the globe and some species are particularly widespread, which is due to a large flexibility in habitat requirements. Additionally previous work suggests that light, as an important environmental cue for circadian and seasonal timekeeping, strongly influences circadian clock expression, daily activity and egg production. In a first step, we investigated which mosquito species occur in the city of Berlin, Germany and genetically analysed the common *Culex pipiens* across an urban-rural gradient by means of 10 microsatellite markers. We found that one panmictic population covered the whole study area. In order to analyse population differentiation as a result of adaptation to urban habitat, we advise future studies to consider genome scans or candidate genes rather than increasing the number of microsatellite markers.

Introduction

The human population is growing worldwide and this leads to the establishment of new settlements and the spreading of existing ones. City life brings a multitude of challenges such as noise, pollution (air and water bodies), a scarcity of suitable breeding habitat and artificial light (street lights and house lighting) to its inhabitants (Longcore & Rich 2004; Grimm *et al.* 2008). On the other hand, the habitat structure is heterogeneous and rich in interfaces of different structure types (Grimm *et al.* 2008). These characteristics are encountered in rural areas to a much lesser extent, because, for example, the different land cover types occur in larger patches reducing the number of interfaces. Many species are not capable of dealing with this situation (for example forest species), while others may actually profit from the resulting heterogeneous habitats (f.e. birds: Grimm *et al.* 2008).

Among those species successfully populating urban areas are mosquitoes (Diptera:Culicidae). Mosquitoes can be found in a wide variety of habitats, ranging from tropical to arctic climates, from ponds to forests and meadows. Some species have strong ties to other species (for example *Culex* and the catchfly *Silene otites*: Jhumur *et al.* 2008; Aedes aegypti and humans (Lima-Camara *et al.* 2011). Other species are more generalists in their ecology and feeding behaviour, such as *Aedes albopictus* (Paupy *et al.* 2009). The challenges urban mosquitoes need to overcome are: higher incidences of pollution in urban habitats, which might influence the larval mosquito population much more than the adults. Also, noise can interfere with mosquito mating, as the recognition of conspecifics in mating swarms is largely driven by wing beat frequency (Gibson & Russell 2006; Robert 2009). Previous studies revealed that light at night, a common feature of human settlements of all sizes, can strongly influence for example daily activity patterns (Honnen *et al.submitted*) or prolong host seeking, hence blood feeding, activity (Chadee & Martinez 2000).Given the specific set of ‘urban’ habitat characteristics in addition to each species’ habitat requirements it can be assumed that few species are capable of living in the city. Schumann (2010) reports a total of 39 Culicid species in Berlin and Brandenburg (46 in Germany), based on museum specimen and a literature survey, but there is no data available on which species actually occur in urban habitats. Townroe and Callaghan (2014) report only 3 species occurring in urban experimental sites in England, *Cx. pipiens*, *Anopheles claviger* and *An. plumbeus.* Container-breeding species seem to be better suited for urban habitats as they can find more ovipostion sites, for example on cemeteries (Vezzani 2007). Vezzani (2007) reports a total of 31 species in 16 countries of the New World and Japan. Apart from one study specifically analysing the presence of *Cx. pipiens pipiens, pipiens molestus* and *torrentium* in two German metropolitan areas (Rudolf *et al.* 2013), we are not aware of any study assessing

the species inventory of a large city. The results of this study were then used to determine a suitable target species for genetic analysis.

The common house mosquito *Culex pipiens* is one of the most widespread mosquito species in the Palaearctic, which is also reflected in their breeding strategy of using any kind of small water-bearing structure for oviposition (Vezzani 2007; Becker *et al.* 2012; Townroe & Callaghan 2014). It predominantly feeds on avian hosts, but is known to switch hosts upon infection with West-Nile-Virus (Kilpatrick *et al.* 2006). Due to these characteristics this species can be found in cities as well as in natural habitats. *Culex pipiens* is also the eponymous species of a species complex to which belong, for example, *Cx. quinquefasciatus* and *Cx. torrentium*. Additionally there are two bioforms of *Cx. pipiens:* f. pipiens *and* f. molestus with different ecological and genetic features despite being morphologically identical (Becker *et al.* 2012). Both types can hybridise in areas of sympatric occurence but this seems to be the case in low rates (Fonseca *et al.* 2004; Kothera *et al.* 2010; Gomes *et al.* 2013).

Microsatellites have been employed widely to study mosquito populations (Fonseca *et al.* 2004; Keyghobadi *et al.* 2006; Hemme *et al.* 2010; Kothera *et al.* 2010). Interestingly, a study investigating the differentiation between two ecotypes of *Cx. pipiens,* f. pipiens (dependent on bloodmeal) and f. molestus (independent of bloodmeal) in New York and Chicago revealed that the *pipiens* forms of both cities fall into the same genetic cluster (based on 8 microsatellites, Kothera *et al.* 2010) This is surprising since these two cities are well over 1200 km apart. If this was the result of ongoing gene flow, a continuous mosquito population must span these two cities. Another possible scenario explaining this phenomenon would be parallel adaptation to similar parameters occurring in urban areas. In contrast, a study of Hemme and colleagues (2010) in Trinidad was able to detect population differentiation in *Aedes aegypti* along a highway, which posed an efficient barrier to gene flow. If populations differentiate from each other across geographical ranges depends strongly on the dispersal capacity of the individual and whether the landscape allows for dispersion, i.e. no physical (as opposed to physiological, i.e. mating barriers) barriers hinder dispersal. Dispersal on the other hand is influenced by a range of parameters, habitat suitability among them (Service 1997; Verdonschot & Besse-Lototskaya 2014). Service (1997) suggests that species occurring in more open habitats (i.e. meadows, rural areas) face a greater probability to be caught and drift-dispersed by wind than woodland or urban species. Furthermore, if this is the case, there should be less gene flow between local populations of woodland or urban species (Service 1997). Motivated flights of mosquitoes (search for resting and oviposition sites, nectar or blood sources and mates) are usually short (1-5km, Service 1997). Verdonschot & Besse-Lototskaya (2014) give an overview of studies regarding flight distances for a number of mosquito species from different genera (f.e. maximum average

flight distances for 105 species from 7 genera). They obtained an average flight distance for *Culex* mosquitoes (in mark-recapture studies) of 921m per day (SD 613.1m) and estimated average maximum dispersal distance of 9695m (Verdonschot & Besse-Lotoskaya 2014). Therefore the range analysed here (max. distance between sampling sites 100.82km), should allow for structuring.
For a number of species wind-assisted dispersal across very large distance is known (for an overview see: Verdonschot & Besse-Lototskaya 2014). A highly built-up urban area may hinder normal dispersal flight, but also wind-assisted dispersal. Additionally habitat availability and suitability differs dramatically from natural landscapes.
In this study we investigate which species occur in the city and ask, whether the city-dwellers (within city borders) of the most common species have developed into discrete subpopulations that differ from the adjacent peri- or suburban (just outside city borders and small towns) and rural sites (single or no buildings). The results will provide insights on if and how quickly local populations adapt to urban habitats or whether ongoing gene flow would prevents differentiation between populations. Using microsatellite analysis we aim to detect whether or not a subset of the populations is contributing to the gene pool associated to urban habitats.

Material and Methods

Sampling

Volunteers sampled adult mosquitoes in the summer of 2011 in their houses and gardens in and around the city of Berlin, Germany (Figure 1A). The animals were collected alive with aspirators consisting of flexible rubber tubes, covered with mesh on one side. Sampled individuals were stored at -20°C. Since the samples have mainly been collected from private households sites are named after the municipal entity. For the species inventory samples have been classified as follows: 1) urban: SP (Spandau), F (Friedrichshain), FH (Friedrichshagen), M (Mahlsdorf), PB (Prenzlauer Berg), WH (Wilhelmshagen), B (Französisch-Buchholz), L (Lichtenberg), K (Kreuzberg), H (Hönow), NK (Neukölln), M (Mahlsdorf), Kö (Köpenick), Z (Zehlendorf); 2) peri-urban: SE (Schöneiche), E (Eberswalde), N (Neuenhagen), KM (Klein Machnow), R (Rangsdorf), D (Dabendorf); 3) rural: (Störitzsee [ST], Gollinsee [G], Westhavelland [WHL], Freienbrink [FB]) sites have been sampled with sweep nets and aspirators (Fig. 1A). We used only *Culex pipiens* for genetic analysis because this was the most abundant species in all classes. Since the number of specimen collected varied greatly between sites we were not able to obtain a sufficient number for all sites. Therefore we were forced to adapt the classification in the following way: 1) urban: F (Friedrichshain), PB (Prenzlauer Berg); 2) peri-urban: FH (Friedrichshagen), M (Mahlsdorf),

SP (Spandau), WH (Wilhelmshagen); rural: Störitzsee [ST], Gollinsee [G], Westhavelland [WHL] (Fig.1B).

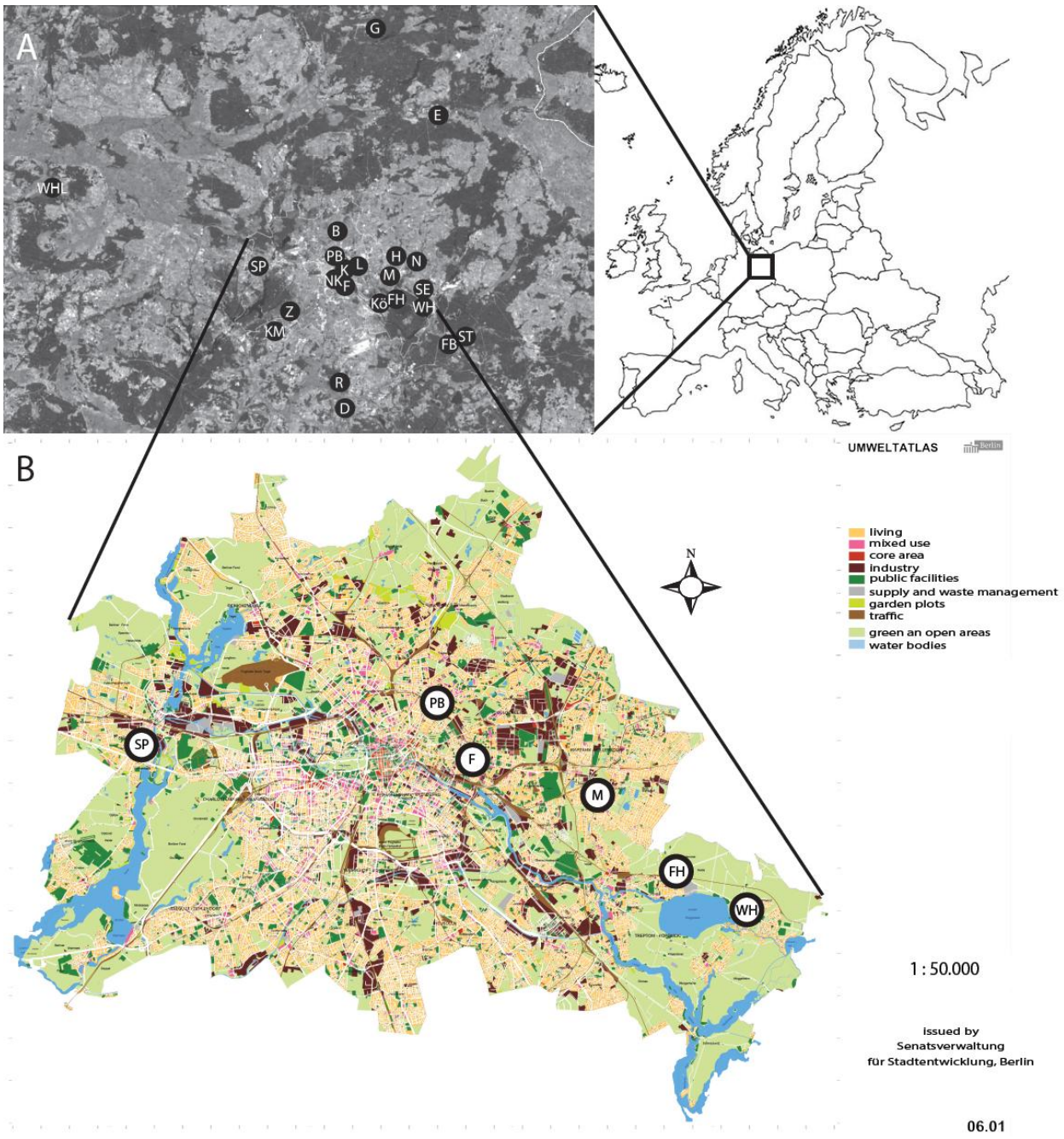

Fig.1. A) Map of sampling locations used for the species inventory, the samples WHL, G, E, KM, R, D; FB and ST are outside of the city borders of Berlin. B) Map of Berlin with samples of microsatellite study. The map also shows the building structure and uses. This map was kindly provided by Berlin senate of communal development (Senatsverwaltung für Stadtentwicklung) and has been modified to specify the sampling locations.

Individuals were identified to species level using the computer-based multicriterial identification program of Schaffner *et al.* (2001). The individuals were classified based on the sampling location as urban, peri-urban and rural.
We included 11 individuals of *Cx pipiens* f. molestus from a laboratory colony (Honnen *et al.submitted*) in the analysis as a means to test whether any of the sampled and identified *Cx. pipiens* would be assigned to the *molestus* population by means of genetic similarity. This was done to validate the morphological identification. Additionally, some (CxpGT4/EMMA), CxpGT20, CxpGT46, CxpGT53, Appendix Table S2) of the microsatellite loci used in this study are known not to amplify in *Cx. torrentium;* a species morphologically closely resembling *pipiens pipiens* with overlapping ranges, also in urban habitats (Rudolf *et al.* 2013).

DNA extraction

DNA was extracted after tissue disruption using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following the manufacturers' protocol with the lysis step being done over night. DNA was eluted to a final volume of 80µl. Primer sequences were obtained from: Fonseca *et al.* (1998) (CQ26), Keyghobadi *et al.* (2004) (CxpGT40, CxpGT46, CxpGT51, CxpGT9, CxpGT53, CxpGT4 (EMMA hereafter), CxpGT20) and Smith *et al.* (2005) (CxqGT4, CxqGT6b). Multiplex PCR was done for three primer mixes (1) CxpGT40, CxpGT51, CxqGT4, CxpGT46; 2) CQ26, CxpGT6b, CxpGT9; 3) CxpGT53, CxpGT4/EMMA, CxqGT20: see appendix S1). Genotyping was done on a 24-capillary 3500xL Genetic Analyzer and alleles were scored with GeneMapper® software v4.1 (both Applied Biosystems®).

Statistical analysis

Prior to all analyses the data were tested for genotyping errors (stutter bands, large-allele dropout, null alleles) using the Micro-Checker software (Van Oosterhout *et al.* 2004). Null alleles can occur as a result of a number of processes: I) an un-detected substructuring of the data (i. e. Wahlund effect); II) insufficient primer binding in non-focal species; III) preferential amplification of short alleles or IIII) slippage during PCR (for an overview see: Chapuis & Estoup 2007). A likelihood test of linkage disequilibrium between pairs of loci and a probability test of deviations from Hardy-Weinberg equilibrium was performed using Genepop ver. 4.2 with dememorisation of 10000, 1000 batches and 10000 iterations per batch (Raymond & Rousset). As the Micro-Checker programme specifically tests for those technical errors we tested for substructuring using the Structure software (Pritchard *et al.* 2000). This model-based Bayesian clustering analysis groups individuals based on their genotypes into K clusters under the assumption that these clusters meet the conditions of

Hardy-Weinberg and linkage equilibria. Furthermore, we allowed for admixture and correlated allele frequencies (Falush *et al.* 2003) and used the locprior model implemented in the software (Hubisz *et al.* 2009). While the 'model with prior population information' embedded in Structure assumes that the probability for an individuals' assignment to a cluster may vary among populations, it still relies on highly informative (near exact) location information, the locprior model is placing more weight on clustering outcomes correlated with location information and is thus more suitable for data with few individuals and loci or low divergence between them (Hubisz *et al.* 2009). We tested our data for K=1 to K=10 with 20 iterations per K. The burn-in period was set at 50,000 steps in the Markov Chain Monte-Carlo procedure followed by 100,000 replications. For each of these runs, the most probable number of K was also evaluated with the ad-hoc statistic ΔK (Evanno *et al.* 2005) as implemented in the online tool Structure Harvester (Earl & von Holdt 2012). Additionally, we tested for substructuring using BAPS Version 6.0, which is also a model-based Bayesian clustering algorithm with the advantage that the number of clusters K is not fixed (Corander *et al.* 2003). We tested K =2-25 with 10 iterations each, first based on individuals and second based on groups using sampling site as prior (Corander *et al.* 2006). The second option is recommended especially when few loci are studied and when loci are not highly informative thus information on spatial proximity greatly facilitates clustering (Corander *et al.* 2008). Expected and observed heterozygosities were calculated using Arlequin version 3.11 (Excoffier & Smouse 1994). Allelic richness, a measure of allelic diversity corrected for sample sizes and calculated with a rarefaction approach, was determined with FStat version 2.9.3.2. (Goudet *et al.* 1996). Allele frequencies for each locus and sampling site were also determined with FStat. Private alleles were obtained with CONVERT ver. 1.31 (Glaubitz 2004). Mosquitoes can display a high prevalence of null alleles (Lehmann *et al.* 1997). Therefore we used the FreeNA software to calculate the null allele frecuency with the EM algorithm developed by Dempster et al (1977, implemented in FreeNA, S3) and F_{ST} values with ENA correction, as suggested by Chapuis and Estoup (2007). Additionally, we calculated global F_{ST} and pairwise differentiation between sites as well as an AMOVA (on the individual level and between sites) using Arlequin. Isolation by distance (IBD) among groups was tested for with Mantel tests on matrices of genetic and geographic distances using IBDWS (Jensen *et al.* 2005; http://ibdws.sdsu.edu/~ibdws/) We used linearised FST-values calculated as FST/(1-FST) and logarithmic geographic distance matrices based on the minimum overland Euclidean distances between each pair of sampling sites 'measured' using Google Earth.

In order to further validate whether our field collected specimen were indeed *Cx. pipiens pipiens* we conducted assignment test using GeneClass2 version 2.0 (Piry *et al.* 2004) with

allele-frequency based assignment computation (Paetkau *et al.* 1995) and the probability criterion of (Paetkau *et al.* 2004).

Results

Urban mosquito species composition

A total of 686 individuals were caught in 2011. Of these, 555 individuals could be identified to the species level. We found a total of 24 species constituting six genera in the study area: 7 species in urban, 9 in peri-urban and 21 in rural locations. By far the most common species was *Culex pipiens* (*ca.* 75 % of all catches) (Table 1). The species found in the city of Berlin were *Aedes vexans* (14) *Culiseta annulata* (7), *Coquillettidia richiardii* (4), *Ochlerotatus flavescens* (2) and *Culiseta fumipennis* (1). The most numerous and the only species found in the city center was *Culex pipiens* (293, Table 1). All other species were found exclusively in the sections at the city border, built-up predominantly with familiy homes with gardens (Fig. 1B).

Table 1: Species inventory in the study area per class and overall.

Species	urban	peri-urban	rural	total
Aedes cinerus	0	0	4	4
Ae. vexans	14	12	9	35
Culex pipiens	293	30	94	417
Cx. territans	1	0	4	5
Cx. modestus	0	0	3	3
Culiseta alaskaensis	0	0	4	4
Cs. annulata	7	3	3	13
Cs. fumipennis	1	0	0	1
Cs. morsitans	0	0	5	5
Cs. ochroptera	0	0	4	4
Ochlerotatus flavescens	2	1	34	37
Oc. cyprius	0	1	2	3
Oc. cantans	0	0	3	3
Oc. caspius	0	1	0	1
Oc. excrucians	0	0	1	1
Oc. geniculatus	0	2	1	3
Oc. leucomelas	0	0	2	2
Oc. punctor	0	1	0	1
Oc. pullatus	0	0	2	2
Oc. intrudens	0	1	1	2
Coquillettidia richiardii	4	0	2	6
Anopheles maculipennis s.l.	0	0	2	2
An. plumbeus	0	0	1	1

Microsatellite analysis

There was no linkage between any two pairs of loci (Bonferroni-adjusted p= 0.001) hence all loci were used in subsequent analyses. The assumptions underlying Hardy-Weinberg

Equilibrium were violated in the overall data set as expected and observed heterozygosity differed significantly in 6 out of 10 loci. Stutter bands occurred at six loci in the overall data set, in those cases the electropherograms were re-examined by eye. In the case of ambiguous scoring the genotype was excluded from further analysis. Large allele drop out was not the case at any of the loci. However, signs of null alleles were detected at all but two loci (CxqGT6b, CxqGT4). Splitting the data into populations based on sampling sites and re-running the analysis revealed that the number of populations displaying null alleles at any of the analysed loci ranged from 0 to 9. The global F_{ST} corrected for null alleles (ENA) was 0.016 (95% CI: 0.009 - 0.023) and thus slightly lower but not very different from the uncorrected F_{ST}: 0.018, CI: 0.009 - 0.025. It was therefore valid to use all loci in subsequent analysis.

All 10 microsatellite loci were polymorphic, the number of alleles ranging from 4 to 49 and allelic richness ranged from 2.49 to 15.24. Comparing the number of alleles (A) and allelic richness (A_R) shows that both measures are in the same range for all sampling groups (A=94-143; A_R=8.25-9.39), with the exception of the LAB (i.e. *molestus*) group (A=48; A_R=4.75). In this group number of alleles and allelic richness were considerably lower (Table S2). The number of observed heterozgotes (H_O) was overall (global value) and at each site high, we still detected an excess of homozygotes (i.e. H_E was higher, see Appendix Table S2). The inbreeding coefficient (F_{IS}) within sites ranged from 0.180 – 0.285 (Appendix Table S2).

Private alleles were detected at all sampling sites and varied in number from 2 (SP, LAB) to 10 (G).

The most probable number of K clusters detected after ΔK calculation is 5, but the supporting value is low and very close to K=2 (K=5: ΔK=2.48; K=2: ΔK= 1.63, Fig. 2 bottom and top, respectively). Those 5 potential clusters are composed of individuals across all sampling sites and do not correspond to geographical proximity or habitat type of sampled sites. The *molestus* individuals represent a distinct group in the data set while all other mosquitoes form one group with few individuals harbouring moderate to high proportion of a different genotype (Figure 2). Running the analyses without the *molestus* group but otherwise identical parameters did not produce a better distinction of clusters (data not shown). Using BAPS to test for structuring resulted in a most probable number of K=22 (probability=0.92) for individual clustering (i.e. without information on sampling location, data not shown). Using the grouped clustering option K was calculated to be 2 (LAB and all others) with a probability of 1 (data not shown).

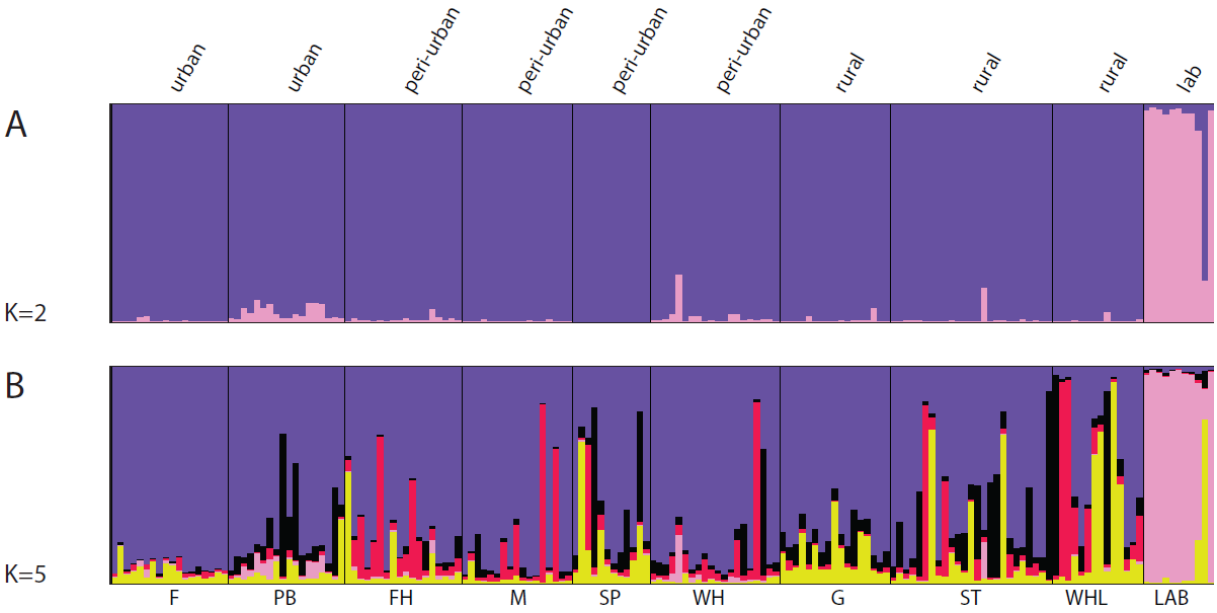

Fig. 2: Clustering results of *Cx. pipiens* populations from Berlin and surroundings based on the Structure program. Each individual is represented by a single bar, the colours denote the proportion of the genotype belonging to the respective cluster using parameters K=2 (A) and K=5 (B). The program distruct (Rosenberg 2003) was used to create the figure based on the structure output.

Calculation of pairwise F_{ST} values between sites only yielded significant results for the comparison with the molestus (LAB) group, there was virtually no differentiation in any other comparison (Table 2). We did not detect any patterns of IBD between sites (one-sided p=0.0654, R^2=0.140)

Table 2: Paiwise F_{ST} values for the comparison of sites. Significance level was 0.001 after Bonferroni-correction, significant values are indicated in bold. Tests were done with Arlequin.

	F	FH	G	M	PB	LAB	SP	ST	WH	WHL
F	-									
FH	0.002	-								
G	-0.002	0.004	-							
M	0.000	0.009	0.011	-						
PB	-0.004	0.003	0.008	0.005	-					
LAB	**0.132**	**0.125**	**0.144**	**0.132**	**0.117**	-				
SP	-0.007	0.007	-0.005	0.012	0.003	**0.137**	-			
ST	-0.001	0.004	0.004	0.006	-0.002	**0.128**	0.004	-		
WH	-0.001	0.002	0.004	0.004	0.001	**0.113**	0.002	0.002	-	
WHL	0.010	0.011	0.006	0.012	0.010	**0.148**	0.000	0.012	0.006	-

According to the main question of the study, I) a comparison between urban (F, FH, M, PB, SP, WH) and rural (ST, G, WHL) populations; II) a comparison of urban (F, PB, SP) vs. peri-urban (FH, M, WH) vs. rural (ST, G, WHL) population pairwise F_{ST} were also calculated among groups but yielded no significant differentiation (data not shown).
No *pipiens* individual was assigned to the *molestus* laboratory group and vice versa in the assignment tests (probabilities ranging between 0.0 and 0.8 % in both cases).

Discussion

We recorded the mosquito species inventory in the city of Berlin and analysed the genetic differentiation of mosquito populations along an urban-rural gradient. The number of species declines towards the inner city and is thus in accordance with previously published data of Southern England (Townroe & Callaghan 2014).In a literature review Vezzani (2007) found 31 species breeding on cemeteries in 16 countries. Of these, 23 species observations were restricted to a single country, often reported based on a single study. This highlights that it strongly depends on whether there are mosquito species present, that are capable to use urban structures for breeding. Also, urban species composition differs from country to country and region to region, therefore an urban species inventory proved to be valuable information.
The most common species we found is the ornithophilic *Culex pipiens*, which we further investigated by means of microsatellite analysis. There is no clear differentiation into genetic clusters coinciding with urban or rural origin. Furthermore, all *pipiens pipiens* individuals fall into a single genetically highly admixed group, suggesting that all individuals were derived from a single continuous panmictic population. In contrast, the forms *molestus* and *pipiens* were grouped into clearly separated clusters, which was also reflected by the F_{ST} across all populations that was low but, as pairwise testing revealed, solely driven by the single different group sampled from the laboratory colony. This finding was further underlined by the results from the assignment tests, suggesting that there are also no hybrids in the data set. Interestingly, one individual from the LAB population was grouped into the *pipiens pipiens* cluster (Fig. 2). When we re-analysed the data with different parameters (i.e. burn-in of 100,000; 500,000 MCMC steps and 50 interations) this individual clustered with the *molestus* cluster (data not shown). Analysing the data with fewer loci, in order to evaluate, whether these play a central role in detecting the observed global and pairwise F_{ST} values and clustering patterns did not yield different results (data not shown). As this particular individual was sampled from an environment with no connection to other mosquito populations and from a strictly autogenous population, we can be confident, that it was indeed a *molestus*

specimen. The biology and also the genetic architecture of the two *Culex pipiens* ecotypes are very different and the single species status is still under debate (Becker *et al.* 2012). However, the long-held assumption is, that *molestus* diverged from *pipiens pipiens* multiple times at different locations and the former thus represents a genetic subset of the latter (Fonseca *et al.* 2004, Kothera *et al*. 2010). It is notable, that our laboratory colony was begun from individuals from Karlsruhe in Southern Germany, approximately 525km away. The sampling sites of this study span a range of 75 km from North to South and 83 km from West to East with a maximum distance of 100.82 between sites (WHL to ST).Yet, no pattern of IBD was detected, which suggests that local mosquito populations are well connected across large distances. Hence, the differentiation between *pipiens pipiens* and *pipiens molestus* is likely due to the differing ecotypes rather than distance alone. This is also in line with the findings of Kothera *et al.* (2010) who found no differentiation between *pipiens pipiens* from New York and Chicago.

Motivated flight in mosquitoes is usually restricted to distances of less than 5km, independent of the flying ability of the species (Service 1997). However, there are exceptions to this rule in some *Aedes* species (Service 1997). The only means by which long distances are covered is wind-assisted dispersal or transportation by humans or their devices, but these flights are not controlled by the mosquito itself (Service 1997). Distances covered intentionally are highly influenced by weather conditions and availability of feeding ground and breeding sites, i.e. if there are suitable sites nearby dispersal may be limited (Service 1997, Verdonschot & Besse-Lototskaya 2014). Based on an average maximum dispersal of 9695m for *Cx. pipiens*, and given the prevalence of natural water bodies in the City of Berlin and the surrounding federal state of Brandenburg, it is not unreasonable to assume that long-distance dispersal is not the most prevalent way of dispersal in the population and thus differentiation is generally possible. Yet, the fact that there is none suggests, that a multitude of breeding ground exist within and around the city (Townroe & Callaghan 2014, Vezzani 2007) and thereby facilitates gene flow on a large scale.

All field collected specimens were identified based on morphological characters which can be erroneous as many species differ morphologically only by few characters (or none, as is the case with *pipiens* and *molestus*). We do not believe that incorrect identification of specimen led to an inflated measure of genetic diversity because we used a computer-based multi-criteria key that allows using multiple characters to identify the specimen (Schaffner *et al.* 2001). This way, the absence of a single character does not hinder identification, as can be the case in dichotomous presence/absence keys. However, *torrentium* and *pipiens* can only be distinguished morphologically with some confidence in males (Weitzel *et al.* 2011). The distribution of *pipiens* and *torrentium* overlaps in central Europe (Hesson *et al.* 2014), also in urban habitats (Rudolf *et al.* 2013). Therefore we included microsatellite markers that are

known to amplify in *Cx. pipiens* and not in *Cx. torrentium* (CxpGT4, CxpGT20, CxpGT46, CxpGT53, Smith *et al.* 2005). Furthermore, to distinguish between *pipiens pipiens* and *pipiens molestus* individuals from an autogenous laboratory colony were added to the analysis. We did not find a general failure in genotyping any of the aforementioned loci, nor did we find individuals that assigned to the laboratory samples or LAB samples that assigned to *pipiens*, therefore we are confident about our species identification. Nevertheless, there remain four cases in the species inventory where we cannot rule out misidentification due to the single occurrence of the species (urban: *Cs. fumipennis*; peri-urban: *Oc. caspius*; rural: *Oc. excrucians*). *Culiseta fumipennis* and *Ochlerotatus pullatus* (found 2 times in WHL) have not been previously described for Brandenburg but both species occur in Germany (Schumann 2010). These specimens could either have been carried longer distances by wind, or, more likely, misidentified. The fact, that we did not detect *Cx. torrentium* can be caused by misidentification leading to an overestimation of *Cx. pipiens* as well. While this is a notable option for the specimens not analysed genetically, the results of the microsatellite analysis suggest, that there might be differences either in habitat use or in the ability to compete in urban areas in the two species. Townroe & Callaghan (2014) did not detect any *torrentium* specimen in their urban sites whereas Rudolf *et al.* (2013) report them in two metropolitan areas in Germany. It has to be mentioned that in the former study larvae have been collected and eggs in the latter. So we cannot make any assumptions on potential competition between early larval stages or the hatching success of the two species. Also, Rudolf *et al.* (2013) sampled either very small towns surrounded by large open agricultural areas or, in the case of Hamburg, green and moderately build-up areas adjacent to or outside the city borders. In the present study most if these site would be classified as peri-urban, not urban. Taken together this suggests that *Cx. torrentium* prefers the more open areas, whereas *pipiens* successfully breeds in highly build-up areas.

Despite the fact, that urban habitat characteristics differ from natural mosquito habitat and likely do favour particular phenotypes over others, we did not detect any differentiation between mosquito populations. This may in part be attributed to the low number of markers used, but previous studies were able to successfully detect differentiation on a small geographical scale with a comparable set of 11 microsatellite markers (Hemme *et al.* 2010). Also the sampling size of the analysed sites might have influenced the power to detect population differentiation. But with only 11 individuals of *molestus* we obtained significant F_{ST} values. This therefore indicates that the study area harbours indeed one population with overlapping dispersal ranges of individuals, which thus facilitate gene flow, even across potential barriers such as highways (Hemme *et al.* 2010).

In order to elucidate, whether city life indeed favours particular phenotypes or genotypes, future studies should focus on candidate genes or genome scans.

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Appendix

S1: Multiplex PCR protocol for microsatellite analysis of *Culex pipiens*

Primercombinations

I)

CxpGT40	169-231	FAM	0.08µl
CxpGT51	108-174	NED	0.15µl
CxqGT4	152-156	HEX	0.12µl
CxpGT46	260-286	Fam	0.15µl
H_2O			3.92µl

II)

CQ26	178-220	HEX	0.12µl
CxpGT6b	128-136	HEX	0.12µl
CxpGT9	108-142	NED	0.30µl
H_2O			3,84µl

III)

CxpGT53	230-325	FAM	0.60µl
CxpGT4/EMMA	139-153	HEX	0.10µl
CxqGT20	110-155	NED	0.15µl
H_2O			3,22µl

Mastermix:

buffer	1µl
$MgCL_2$	1µl
BSA	1µl
dNTP	1µl
taq	0.08µl

Touchdown PCR profile

Initial denaturation	
94°C	2min
5 cycles	
94°C	30sec
64-60°C	30sec
72°C	30sec
25 cycles	
94°C	30sec
60°C	30sec
72°C	30sec
Terminal Elongation	
72°C	10min
4°C	∞

Table S2: Variability parameters for each site and locus (accession no. and reference are given here). Number of alleles (A), allelic richness (A_R*, based on 10 individuals) and inbreeding coefficient (F_{IS}) have been calculated with FSTAT, observerd (H_O) and expected (H_E) heterozygosity have been calculated using Arlequin.

			F	**N=18**				**FH**	**N=18**				**G**	**N=17**			
Locus	**Accession No.**	**reference**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**
CxpGT40	AY423742	Keyghobadi et al. 2004	18	12.889	0.778	0.933	0.171	21	13.848	1.000	0.952	-0.052	16	11.103	0.824	0.877	0.063
CxpGT46	AY423743	Keyghobadi et al. 2004	8	6.959	0.778	0.844	0.081	11	8.373	0.778	0.863	0.102	9	6.859	0.588	0.788	0.259
CxqGT4	AY958074	Smith et al. 2005	3	2.365	0.167	0.160	-0.041	3	2.477	0.222	0.208	-0.071	4	3.014	0.235	0.223	-0.058
CxpGT51	AY423744	Keyghobadi et al. 2004	20	13.919	0.778	0.954	0.189	17	12.386	0.722	0.944	0.241	18	13.495	0.813	0.948	0.147
CxqGT6b	AY958075	Smith et al. 2005	7	5.508	0.667	0.656	-0.017	4	3.426	0.412	0.522	0.217	6	4.85	0.471	0.558	0.161
CQ26	AF075423	Fonseca et al. 1998	9	7.223	0.611	0.800	0.241	10	8.255	0.667	0.844	0.215	8	6.825	0.765	0.750	-0.02
CxpGT9	AY423739	Keyghobadi et al. 2004	11	8.373	0.500	0.863	0.428	13	9.275	0.500	0.870	0.432	13	10.203	0.529	0.895	0.416
CxpGT53	AY423745	Keyghobadi et al. 2004	24	15.798	0.722	0.971	0.262	18	13.000	0.556	0.941	0.417	20	14.962	0.706	0.971	0.28
Emma	AY423738	Keyghobadi et al. 2004	9	7.559	0.667	0.797	0.167	10	8.591	0.833	0.883	0.057	9	7.586	0.765	0.781	0.021
CxpGT20	AY423741	Keyghobadi et al. 2004	14	10.681	0.833	0.916	0.093	15	10.857	0.833	0.897	0.073	14	10.331	0.824	0.893	0.08
Total			123					122					117				
Mean			12.30	9.13	0.65	0.79	0.16	12.20	9.05	0.65	0.79	0.16	11.70	8.92	0.65	0.77	0.13

continued			**M**	**N=17**				**PB**	**N=18**				**LAB**	**N=11**			
Locus	**Accession No.**	**reference**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**
CxpGT40	AY423742	Keyghobadi et al. 2004	12	8.891	0.824	0.827	0.004	13	10.116	0.944	0.917	-0.03	7	6.636	0.455	0.723	0.383
CxpGT46	AY423743	Keyghobadi et al. 2004	9	7.762	0.824	0.861	0.045	10	8.529	0.889	0.884	-0.006	3	3	0.455	0.593	0.242
CxqGT4	AY958074	Smith et al. 2005	2	1.939	0.176	0.166	-0.067	4	2.921	0.222	0.211	-0.054	2	1.996	0.000	0.173	1
CxpGT51	AY423744	Keyghobadi et al. 2004	16	12.297	0.529	0.943	0.446	18	13.072	0.722	0.951	0.246	7	7	0.800	0.847	0.059
CxqGT6b	AY958075	Smith et al. 2005	8	6.481	0.706	0.750	0.061	6	5.045	0.556	0.656	0.156	2	2	0.364	0.312	-0.176
CQ26	AF075423	Fonseca et al. 1998	9	7.91	0.647	0.863	0.256	11	8.382	0.444	0.827	0.47	5	5	0.200	0.632	0.695
CxpGT9	AY423739	Keyghobadi et al. 2004	11	8.84	0.875	0.877	0.002	15	10.416	0.611	0.890	0.32	7	7	0.600	0.768	0.229
CxpGT53	AY423745	Keyghobadi et al. 2004	18	13.506	0.412	0.955	0.577	21	14.908	0.611	0.970	0.377	6	6	0.500	0.747	0.343
Emma	AY423738	Keyghobadi et al. 2004	10	8.462	0.875	0.833	-0.053	10	8.538	0.722	0.883	0.186	4	3.909	0.545	0.671	0.195
CxpGT20	AY423741	Keyghobadi et al. 2004	13	10.645	0.867	0.910	0.05	12	9.416	0.833	0.890	0.066	5	4.996	0.636	0.810	0.222
Total			108					120					48				
Mean			10.80	8.67	0.67	0.80	0.13	12.00	9.13	0.66	0.81	0.17	4.80	4.75	0.46	0.63	0.32

continued			SP	N=12				ST	N=25				WH	N=20			
Locus	**Accession No.**	**reference**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**
CxpGT40	AY423742	Keyghobadi et al. 2004	13	11.764	0.917	0.931	0.016	20	12.133	0.920	0.924	0.005	16	11.192	0.800	0.892	0.106
CxpGT46	AY423743	Keyghobadi et al. 2004	7	6.498	0.583	0.815	0.294	9	6.722	0.560	0.831	0.331	10	8.755	0.650	0.892	0.277
CxqGT4	AY958074	Smith et al. 2005	3	2.957	0.333	0.304	-0.100	3	2.311	0.208	0.194	-0.075	3	2.447	0.250	0.229	-0.092
CxpGT51	AY423744	Keyghobadi et al. 2004	12	10.913	0.750	0.909	0.182	24	14.275	0.840	0.962	0.129	21	14.02	0.850	0.960	0.117
CxqGT6b	AY958075	Smith et al. 2005	6	5.498	0.583	0.638	0.089	8	5.825	0.760	0.658	-0.159	5	4.203	0.550	0.624	0.122
CQ26	AF075423	Fonseca et al. 1998	8	7.308	0.583	0.736	0.214	10	7.882	0.560	0.840	0.338	11	8.398	0.600	0.851	0.301
CxpGT9	AY423739	Keyghobadi et al. 2004	11	10.242	0.500	0.909	0.461	14	10.215	0.760	0.907	0.165	14	9.406	0.500	0.867	0.429
CxpGT53	AY423745	Keyghobadi et al. 2004	17	14.931	0.750	0.967	0.233	25	14.371	0.640	0.962	0.339	22	14.78	0.550	0.968	0.438
Emma	AY423738	Keyghobadi et al. 2004	7	6.619	0.750	0.732	-0.026	10	8.324	0.680	0.878	0.229	11	8.725	0.850	0.864	0.017
CxpGT20	AY423741	Keyghobadi et al. 2004	10	9.141	0.750	0.877	0.150	20	11.887	0.720	0.927	0.226	16	10.373	0.750	0.888	0.159
Total			94					143					129				
Mean			9.40	8.59				14.30	9.39				12.90	9.23			

continued			WHL	N=14			
Locus	**Accession No.**	**reference**	**A**	**A_R***	**H_O**	**H_E**	**F_{IS}**
CxpGT40	AY423742	Keyghobadi et al. 2004	10	8.674	0.643	0.857	0.257
CxpGT46	AY423743	Keyghobadi et al. 2004	7	6.261	0.615	0.782	0.220
CxqGT4	AY958074	Smith et al. 2005	3	2.64	0.214	0.204	-0.054
CxpGT51	AY423744	Keyghobadi et al. 2004	16	12.691	0.786	0.934	0.164
CxqGT6b	AY958075	Smith et al. 2005	5	4.566	0.643	0.643	0.000
CQ26	AF075423	Fonseca et al. 1998	8	7.268	0.250	0.659	0.631
CxpGT9	AY423739	Keyghobadi et al. 2004	11	9.418	0.500	0.899	0.453
CxpGT53	AY423745	Keyghobadi et al. 2004	17	14.446	0.615	0.966	0.373
Emma	AY423738	Keyghobadi et al. 2004	6	5.818	0.571	0.778	0.273
CxpGT20	AY423741	Keyghobadi et al. 2004	13	10.7	0.786	0.878	0.109
Total			96				
Mean			9.60	8.25	0.56	0.76	0.24

Table S3: Null allele frequencies for each site and locus calculated with the EM algorithm (Dempster et al, 1977) implemented in FreeNA (Chapuis and Estoup, 2007)

	CxpGT40	CxpGT46	CxqGT4	CxpGT51	CxqGT6b	CQ26	CxpGT9	CxpGT53	EMMA	CxpGT20
F	0.056	0.000	0.000	0.076	0.032	0.094	0.182	0.111	0.022	0.010
FH	0.000	0.014	0.000	0.107	0.049	0.091	0.173	0.189	0.016	0.025
G	0.000	0.059	0.000	0.070	0.014	0.004	0.189	0.128	0.000	0.018
M	0.000	0.000	0.000	0.203	0.000	0.123	0.000	0.269	0.000	0.018
PB	0.000	0.000	0.000	0.101	0.007	0.203	0.124	0.173	0.080	0.000
LAB	0.131	0.112	0.195	0.000	0.000	0.271	0.089	0.125	0.027	0.093
SP	0.000	0.115	0.000	0.056	0.013	0.098	0.203	0.093	0.000	0.054
ST	0.000	0.139	0.000	0.054	0.000	0.141	0.053	0.154	0.106	0.098
WH	0.013	0.113	0.000	0.036	0.001	0.122	0.186	0.206	0.000	0.054
WHL	0.127	0.072	0.000	0.053	0.044	0.244	0.200	0.163	0.125	0.000

General discussion

Rationale

Artificial light at night is a commonplace phenomenon in all but the most remote human settlements. Only recently, studies have tried to assess the influence of light on non-human city-dwellers. Most of these studies focussed on the explanation of easily visible phenomena, such as the attraction of insects to artificial light sources (Eisenbeis & Hassel 2000) and predators exploiting disoriented insects (Blake *et al.* 1994). However, light is an environmental cue triggering subtle behavioural changes, such as timing of activity (*e.g. Daphnia* vertical migration, Moore *et al.* 2000) and life history traits; e.g. eclosion in *Anopheles gambiae* (GILES, 1902) (Reiter & Jones 2009) or diapause in *Daphnia* (Stross & Hill 1968). There are receptors and pathways collecting and translating the information provided by light into physiological responses that inevitably begin with the expression of genes. As a result artificial light potentially acts as selective force favouring certain behaviours. Despite widespread interest, potential evolutionary aspects have been largely neglected.

Effects of artificial light on *Culex pipiens* f. pipiens and *pipiens* f. molestus

Effects on the individual

Gene expression

Light provides information for an organism, most prominently on the time of day. It thus induces rhythmic expression of genes and, accordingly, rhythmic regulation of physiological processes (Panda *et al.* 2002). Previous studies suggest that rhythmic gene expression in mosquitoes can be produced either by the internal clock or by the presence of light (Rund *et al.* 2011, 2013). Usually these studies compare light-dark cycles with constant darkness or, rarely, constant light. However, a switch from light cycles to constant conditions occurs neither in nature nor in urban habitats. Outside lighting is switched on when natural light decreases and thus artificially prolongs the day. To gain an overview of whether this prolonged lit period is capable of influencing physiological processes, I assessed the gene expression on the genome level for two different light regimes in *Cx. pipiens* f. molestus. I sequenced the transcriptome, which is the entirety of transcribed genes at a given time point, using RNAseq. There was a pronounced difference in the gene expression between males and females. A little over 50% of the gene expression is significantly different between males

and females regardless of the light regime, while the expression of the remaining genes does not differ significantly between sexes (Chapter I). This may be the result of different life styles of males and females, in particular regarding diet and finding a suitable food source. Sex-specific gene expression is common for a range of taxa, including insects, nematodes, birds mammals (Ellegren & Parsch 2007 and references therein) and cichlid fish (Böhne *et al.* 2014). This further highlights, that sex-specific differences need to be taken into account when analysing the response to a treatment. I also found remarkable differences between sexes in response to the light treatment. While females do not seem to be influenced by different light regimes, I found strong differences in males. Most strikingly there were fewer genes differentially expressed in the extended light treatment (Chapter I). In particular, genes related to immune response, gametogenesis and lipid metabolism and transport were less expressed with additional light.

The circadian clock is directly linked to environmental light. A photo-receptor, CRYPTOCHROME-1 in the case of mosquitoes, collects light at specific wavelengths. This starts a reaction cascade within the central feedback loop and thus enables the time setting of the inner clock (Chapter II, Fig.1). Although the circadian rhythm is, by definition, independent of external light information to approximately keep its 24h rhythm (Meireles-Filho & Kyriacou 2013), this synchronization ensures a correct functioning of the clock. It also enables collecting information on day length and thus helps timing of seasonal events (Panda *et al.* 2002). Given the clock's direct connection to ambient light and its feedback loop nature I assumed that influence of artificial light at night can be detected within the loop, namely in the expression of its genes. Indeed, I discovered significant differences between the light-dark and the extended light treatment (chapter II). This difference was mainly driven by two genes: *period* and *clock*, which were down-regulated in extended light phase over all data points and for males. In females *period* was also down-regulated but *cycle* was up-regulated, revealing a sex-specific response.

Daily activity

The clock system plays a key role as timer of downstream processes; therefore a disruption should be reflected by a shift in downstream functions. One of these functions, controlled by the circadian clock, is daily locomotor activity in a number of taxa ranging from flies to humans (Panda *et al.* 2002). A change or disruption in one or more clock components should consequently result in aberrant movement patterns.

I found that the daily activity patterns were indeed impacted by the light regime and that this influence was different for males and females (chapter II). Light intensity changes rapidly at dawn and dusk and this seems to be an important cue triggering locomotor activity for *Cx.pipiens* f. molestus, too. *Culex pipiens* is a nocturnal species and commenced activity at

very low light levels and in the dark, shortly after lights-off and before lights-on. This general pattern was consistent in both experimental treatments. But the propensity to be active was much lower in the treatment with additional light and remained so throughout the day. Observing the activity in constant darkness revealed that, without light cues, activity was triggered by the internal clock alone. Overall activity was significantly lower in the treatment group, despite the lack of light cues in constant darkness. Also, the differences between males and females prevailed here, which suggests that the effects of artificial light at night persisted until after the end of the exposure.

Effects on the population

Fecundity

On two levels, i.e. gene expression and daily activity, males and females exhibited different responses to light at night. This may lead to changes in the mating behaviour of males and females, evoking population level effects, e.g. reduced reproductive output. This could, in the long run, negatively influence population viability. I was able to show that the number of eggs per raft and the diameter of individual eggs were smaller in the light treatment, indicating that the prolonged lit period exerted a negative influence on fecundity (chapter II). This can be caused by a reduced number of successful matings in the treatment leading to fewer fertilized eggs but not necessarily to a smaller diameter. Another reason may be a poorer physical condition, e.g. fewer fat reserves of the females that do not allow for the production of many and/or large eggs. Each one or a combination of these explanations may be responsible for the observed pattern.

Urban population differentiation

In the experimental light treatment fecundity was reduced which shows that the following generation may also be influenced. The number and the size of the larvae is expected to be smaller from the light treatment due to the smaller sized eggs. This clearly affects population size and thus the production of offspring per generation. A size reduction in local populations would in turn lead to a loss of connectivity between populations. Reduced connectivity would then be reflected by differentiation between populations and could be measured by means of microsatellite analysis.

I investigated the population of *Cx. pipiens*, which proved to be the most abundant species in and around Berlin (chapter III). The population analysed in this study showed a high degree of genetic diversity and no structuring into subpopulations that coincide with urban or rural origin. This indicates a high rate of genetic exchange via gene flow between local populations (chapter III). Gene flow keeps the diversity high and thus a great variety of

alleles is kept or constantly brought back into the urban population. Therefore it is difficult to evaluate whether light at night is a selective force potentially leading to adaptation. It may be that adaptation to urban habitats is in fact buffered or counteracted by gene flow. Future work is needed to disentangle the effects of individual plasticity, light as selective force and gene flow.

Implications and outlook on the effects of artificial light at night

For the first time a transcriptome in response to light at night was analysed. Only few studies deal with gene expression in different light environments, so the interpretation of the results remains speculative and calls for further investigation.
Lipid metabolism covers lipid transmembrane transport and storage as well as utilisation. Genes associated with this were expressed to a lesser extent in the treatment with additional light in males. Usually lipids are metabolised when the carbohydrate storage, the preferred energy source, is depleted, as is the case in long-distance flying (Clements 1955). This result coincides with overall reduced activity observed in this treatment, i.e. potentially reduced need for utilisation of energy reserves, suggesting a connection between activity and lipid metabolism. This might also be interconnected with the reduced expression of gametogenesis-related genes. These genes are mostly connected to mitosis and meiosis, where energy provision of the cells is necessary. Genes related to immune response were also amongst the genes less expressed in additional light. A web of science search for the terms “mosquito”, “male”, “immune”, “gene*” with and without the additional term “mating” (performed on 16.09.2014) yielded no results, which makes it difficult to interpret this outcome. If, for example, immune genes were up-regulated in anticipation of mating, then the fact that these genes are expressed to a lesser extent could mean mating activity is not anticipated. These ideas and connections are mere speculation and remain to be tested in future studies, for example by exposing individuals to immune challenges in different light regimes.

Clock gene expression was significantly different when the two treatments were compared, but the difference remained stable throughout the experiment indicating that clock function itself remained stable. This is also underlined by overall similar activity patterns (i.e. main active times coincide with darkness) in both treatments, which were maintained in constant darkness, despite the significant difference between treatments (see Fig.4, chapter II). This suggests that clock gene expression is flexible within a certain range. The experimental set-up (see methods, chapter II) did not disrupt clock function as a whole. Gene expression and

transcript abundance of the clock genes is not the only driver of the circadian rhythm. Although the expression of clock genes is strongly correlated (chapter II), the absolute abundance of transcripts of each gene varies at any given time which influences the timing of protein complex formation. This would clearly not result in a stable rhythm. To maintain stability there are a number of post-transcriptional and post-translational processes at work that regulate transcript abundance and thus play an important part in shaping the actual timing in protein abundance (Hardin & Panda 2013). There is still a gap in knowledge on how exactly these regulatory components help maintaining the ~24h cycle (Hardin & Panda 2013), therefore it is difficult to estimate whether they are capable of buffering the fluctuations in gene expression created by my experimental light regime.
On the other hand, clock genes are thought to control activity and daily movement patterns (Gentile *et al.* 2009; Meireles-Filho & Kyriacou 2013) such as foraging, mate search and oviposition. To date, it is unclear which of the genes of the central feedback loop are participating in locomotor control. So far, *cryptochrome-2* (Gentile *et al.* 2009) and timeless have been suggested as candidates (Gentile *et al.* 2013). We found the *cryptochrome-2* expression to vary only slightly across the times sampled (Fig.2 chapter II) and there were no significant differences between treatments. The experimental sampling time points coincide with the main active time of C*ulex pipiens* (Veronesi *et al.* 2012). If *cryptochrome-2* played a role in regulating locomotor activity, I would expect the significant differences in activity between treatments (Fig.4, chapter II) to be reflected by different *cryptochrome-2* abundances in each treatment. But this is not the case, suggesting that *cryptochrome-2* abundance alone is not regulating activity. In an RNAi knockdown assay the *timeless* gene was found to influence activity in *Aedes aegypti* (LINNAEUS, 1762) (Gentile *et al.* 2013). Knockdown mosquitoes displayed a reduced activity on day 4 after infection (Gentile *et al.* 2013). But the authors also mention that it may be highly species-specific which clock genes regulate activity. In this study I found the genes *period* and *clock* to be down-regulated in the presence of additional light (Table 1, chapter II). Together with reduced activity in the additional light treatment (Fig.4, chapter II) this indicates that these genes might be involved in locomotor control in *Cx. pipiens* f. molestus. However, this is different for males and females as in males *clock* is down-regulated but in females this gene is unaffected by the treatment, instead, *cycle* is up-regulated. This difference might be responsible for the sex-specific activity patterns observed in this study. Based on these findings I propose to analyse these genes and their involvement in locomotor control in the future. Furthermore, it is a promising starting point for investigating sex-specific timing. However, post-transcriptional and -translational processes may shape this relation which would not be detected with this experimental set-up but which needs to be considered in future studies.

A night shortened by artificial light might decrease the amount of time available for a nocturnal animal to forage and to find a mate. In mosquitoes, this relationship was indeed found, just in the opposite direction. Diurnal *Aedes aegypti* can utilise artificial light at night to prolong host-seeking time. In urban Trinidad (night time brightness ca. 53-107 lux, converted from foot-candles (5-10fc)) 10% of human landing captures occurred at night, but virtually no captures were made at the same time in dark rural areas (Chadee & Martinez 2000).
I found that artificial light may alter activity patterns throughout the day, not only the timing of onset and end of activity. The reduced activity in twilight condition (i.e. prolonged resting periods) may hence be followed by a reduction in foraging time and thus decreased nutrient uptake and smaller fat reserves. Additionally, a shortened active period may lead to fewer mating encounters. Fewer matings, undernourished females or both would lead to fewer eggs laid. This is exactly what I found. With additional light, there were fewer eggs per raft and they were smaller in diameter, but the number of rafts was comparable. This suggests that the number of successful matings was comparable in both treatments and insufficient fat reserves in females are responsible for the fewer and smaller eggs. This points to a link between the two sets of findings. However, I did not measure other key variables (e.g., total feeding time, sugar consumption, growth rate). Therefore future work is needed to underpin the observed results measuring these variables along with eggs per female and female size.
Egg size is a predictor of desiccation resistance in *Aedes* mosquitoes (the larger, the more resistant; Sota & Mogi 1992), which means that larger eggs may have a fitness advantage in surviving unfavourable conditions (e.g. non-permanent larval habitats predominant in cities) and hence a greater probability to hatch. In contrast, smaller eggs might also have a smaller probability to hatch, but larval mortality might be influenced, as smaller larvae might have higher mortality rates than larger ones. An evaluation of these relationships would also provide important insights on the influence of artificial light at night on mosquito ecology.
The influence of artificial light on the adult population is thus carried on to the next generation, as effective population size (N_E) might be significantly smaller than in the generation before. This in turn might lead to smaller local populations which may then lose their connection to one another, inducing divergence between populations.
An analysis of the genetic structure of urban compared to rural local populations of *Cx. pipiens* revealed that there is a single panmictic population spanning the whole study area (see Fig 1, chapter III). Gene flow is not hindered between local populations and a high variability is maintained. However, it is not clear whether the gene pool of urban, artificial light-experienced populations is constantly replenished from the outside, or whether artificial light along with other urban habitat characteristics instead selects for a greater variability. These are two exciting questions to be followed up upon.

Interactions of further urban habitat characteristics with artificial light at night

Artificial light at night is only one out of many characteristics of urban mosquito habitats. Interactions of these features may either add up, or the different aspects might buffer each other's effects. Additional factors influencing urban habitats are briefly discussed in the following section.

Pollution is a broadly defined term and can include many areas and polluting agents. To my knowledge, the effect of air pollution on mosquitoes has not yet been studied. There is one study evaluating the repellent effect of indoor smoke from biomass fuels on mosquitoes (Biran *et al.* 2007). The authors found no effect of smoke on the biting propensity of female mosquitoes unless plants traditionally used as repellents had been burned. On the other hand, pollution can also come in the shape of particulate matter (Friedlander 1973). Particles in the atmosphere can increase sky glow in clear nights (Kyba *et al.* 2011). Additionally, depending on their absorption properties, they can change the reflection of light by cloud cover (Kyba *et al.* 2011). Smog, for example, intensifies reflection by clouds such that at an urban site reflection is increased by factor 10.1, while 32km away from the city centre reflection was increased only by factor 2.8 (Kyba *et al.* 2011).
Apart from the aerial habitat of the adult, also the aquatic larval habitat may be polluted. Larval habitats may strongly influence adult fitness, for example high larval density can reduce adult longevity (Alto 2011). Light intensity in the water has been found to interact with terrestrial carbon input influencing the numbers of emerging *Culex* mosquitoes (Kraus & Vonesh 2012). Urban breeding sites may be polluted by various compounds along with precipitation (Friedlander 1973). This may then influence the microbial community (Röling *et al.* 2001) in the breeding habitats. Egizi *et al.* (2014) evaluated the influence of the microbial community on larval competition between two species (*Ochlerotatus japonicus* (THEOBALD, 1901)*, Cx. quinquefasciatus* (SAY, 1823)) at different temperatures. They found that the composition of bacterial community was an important factor shaping intra-specific competition (Egizi *et al.* 2014). Hence light intensity and water pollution may act together to determine mosquito species composition in urban habitats. This in turn would have implications for pathogen transmission in urban areas. Future work could include testing different concentrations of pollutants under different light regimes and measuring e.g. hatching success and eclosion.

Noise is another habitat characteristic in urban habitats, which has been shown to strongly impact birds (Luniak 2004; Nordt & Klenke 2013). In mosquito ecology sound plays an

important role in mating, as sex and species recognition is based on wing beat frequency (Gibson *et al.* 2010). How urban noise impacts mosquitoes has not been studied yet. Only one study assessed the perceptibility of the sound produced by a mosquito swarm (*Aedes taeniorhynchus* (WIEDEMANN, 1821)*,* Mankin 1994). The author found that in a quiet environment the swarm can be heard 10-50m away, but is no longer detectable in a typical urban environment (Mankin 1994). This study was based on the human ear or human-made devices for collecting frequencies and does not allow evaluating the mosquitoes' ability to pick up wing-beat frequencies. However, this study does suggest that urban soundscapes may create a background noise that interferes with mating for species that mate in swarms, for example *Cx. pipiens.* To escape background noise levels birds sing more loudly and later in the day (Luniak 2004; Nordt & Klenke 2013). A mosquito can alter its wing-beat frequency over a very limited range, as it is a means of species recognition, but a shift in timing of swarm formation could be an option for avoiding background noise. This may be facilitated also by the availability of artificial light as it alters activity patterns (chapter II). A laboratory experiment observing swarming activity under different noise levels would provide insights to the affects of ambient sound on mosquito mating biology.

Urban Heat Islands (UHI) describe the effect that in cities air and surface temperatures differ, i.e. are higher, than in their rural surroundings (Kim 1992; Grimm *et al.* 2008). Especially the wide variety of surface structures in cities (e.g. buildings, asphalt, bare soil) contribute to patchy microclimates and an overall rise in air temperature of up to 10°C in summer compared to surrounding woodlands (Kim 1992). This leads to water bodies and air warming earlier and cooling later in the year, thus favouring reproductive output which potentially leads to higher numbers of generations per year. If this is the case, the presence of high ambient light levels may actually buffer this effect of increased productivity caused by warmer temperatures, as high light levels in larval habitats can lead to decreased adult emergence (Kraus & Vonesh 2012). Additionally, higher temperatures in water bodies may alter the microbial community, which in turn could affect competition between mosquito species (see above). Temperature is already known to have strong effects on the development of mosquito larvae and consequently also on the adults (Dodson *et al.* 2012). A mesocosm experiment with different light regimes, temperatures and monitored microbial community would help to elucidate how these factors interact. Additionally field collections would present an overview of the range of temperatures and microbial communities in urban mosquito habitats.

Summary of the main findings

There are pronounced differences between males and females in gene expression and activity patterns (**chapter I&II**), suggesting sex-specific regulation of expression and also behaviour. This may have implications on the interaction of both sexes in mating and reproduction. The facts that male mosquitoes express fewer genes related to immune function, gametogenesis and lipid metabolism and transport in an extended light period (**chapter I**) underlines how intersexual interaction may be influenced and reproductive success may suffer consequences from artificial light at night. Altered activity patterns (**chapter II**) may lead to further consequences e.g. in individual fitness as reduced activity until after the end of the experiment may significantly reduce foraging time for prolonged periods. In line with the abovementioned is the finding of reduced egg production with light at night (**chapter II**), indicating less female investment in eggs, possibly due to a suboptimal nutritional status.
Urban mosquitoes are genetically well connected to surrounding populations (**chapter III**), which may prevent that the adverse effects of light observed in the laboratory experiments act as selective force in urban habitats.

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Statement of academic integrity

I hereby certify that the submitted thesis “Evolutionary implications of artificial night-time lighting for the mosquito *Culex pipiens*: insights from genetics, physiology, behaviour and population structure” is my own work, and that all published or other sources of material consulted in its preparation have been indicated. Where any collaboration has taken place with other researchers, I have clearly stated my own personal share in the investigation (see thesis outline, page 15). I confirm that this work, in the same or a similar form, has not been submitted to any other university or examining body for a comparable academic award.

Berlin, 09.10.2014

Curriculum Vitae

For reason of privacy protection the CV is not included in the online version of the thesis