Relative abundance and geographical variation of *Culex pipiens* and *Culex torrentium* (Diptera: Culicidae) in CO_2-baited traps in Denmark

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European *Culex pipiens* and *Culex torrentium* are morphological fairly similar mosquito species with potentially different vector competences for pathogenic viruses. The relative abundance of the two species is therefore important for quantifying the potential for disease transmission in Denmark. Mosquitoes were sampled from 74 different sites in Denmark with CO_2 and octenol-baited suction traps. A total of 285 *Culex* specimens were identified to species using a restriction enzyme assay. *Culex pipiens* was the dominating species with 220 (77%) specimens caught at 22 different sites, while 65 (23%) specimens were identified as *C. torrentium* and only caught at 4 sites. The ratio of the two species differed significantly between sites with *C. torrentium* dominating in just a single location. Both mosquito species were predominantly caught late in the Danish mosquito season, from mid-August and onwards.

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1. Introduction

Culex pipiens Linnaeus, 1758 and *Culex torrentium* Martini, 1925 (Diptera: Culicidae) are morphologically fairly similar species with an overlapping range in northern Europe (Hesson *et al.* 2014). Accurate morphological identification of the two species can only be done on the basis of male hypopygial characters (Service 1968), whilst wing morphology can be used to discriminate between female adults with an accuracy of more than 90% (Börstler *et al.* 2014). *Culex pipiens* and *C. torrentium* are both perennial species that hibernate as adults. The larvae of the two species often occur together, and are found in semi-permanent waters such as edges of slow running streams, in vegetation at borders of lakes, semi-permanent pools, marshy areas, man-made containers and reservoirs of sewage plants (Becker *et al.* 2010). Females of the two species are both anautogenous and ornithophilic and rarely bite humans (Becker *et al.* 2010).

Culex pipiens and *C. torrentium* can maintain transmission of zoonotic viruses between birds. *Culex pipiens* can serve as a vector of several arboviruses including Japanese encephalitis, Sindbis virus, Usutu virus and West Nile virus (WNV) (Francy *et al*. 1989, Farajollahi *et al*. 2011). Less is known about the vector capability of *C. torrentium*, probably due to it mistakenly being confused with *C. pipiens,* its holarctic distribution, and its late recognition (Harbach 1985). However, *C. torrentium* is a known vector of Sindbis virus (Lundström *et al*. 1990) and was recently also found susceptible to WNV (Leggewie *et al*. 2016). During the last 40 years, both Sindbis virus and WNV have been isolated from wild-caught female mosquitoes in Europe (Lundström 1994, Nicolescu 1998, Jöst *et al*. 2010, Hesson *et al*. 2015a) and outbreaks of Sindbis virus and WNV in humans have occurred (Lundström 1999, A Sirbu *et al*. 2011). *Culex torrentium* appears to be a more efficient vector of Sindbis virus than *C. pipiens* (Lundström 1990), and recently *C. torrentium* was found to be highly susceptible for dissemination of WNV even at low temperatures, and with higher infection rates than *C. pipiens* (Leggewie *et al*. 2016). Because of the potential differences in vector competences, research related to their distribution, ecology and genetics has been conducted in European countries, and different molecular methods have been developed to separate females of *C. torrentium* from *C. pipiens* (Hesson *et al*. 2010, Rudolf *et al*. 2013).

In Denmark, the prevalence of *C. pipiens* and *C. torrentium* is relatively unknown, because they were previously considered one species. *Culex torrentium* was first discovered in Denmark in 1971 (Iversen 1971). Since then, little research on the distribution and occurrence of *C. torrentium* has been done. Hesson *et al*. (2014) conducted a study on the larval prevalence of the two species throughout Europe and included six artificial larval sampling sites in Denmark. The study found both species occurring together in some artificial containers and separately in others, but they were on average equally abundant. Hesson *et al*. (2014) found that *C. torrentium* dominates in northern Europe whereas *C. pipiens* dominates south of the Alps. Contrary, another study found that less than 5% of the *Culex* population belonged to *C. torrentium* and 95% to *C. pipiens* in the northern part of Germany close to Denmark (Rudolf *et al*. 2013).

Exotic mosquitoes are spreading in Europe and recently the competent WNV bridge vector *Culex modestus* Ficalbi, 1947 was discovered in England and Denmark (Medlock *et al*. 2012, Bødker *et al*. 2014). Denmark is located on the migration route of many Palaearctic-African migratory birds and antibodies against WNV have been found in migratory birds in Denmark, with 30 seropositive songbirds among 1,056 examined from 2011 to 2015 and with positive birds detected in confirmatory tests of all five years (Lohse *et al*. 2016). In the same period of five years, 3,037 *Culex* mosquitoes from the Danish vector surveillance program (www.myggetal.dk) were all tested negative for WNV (Lohse *et al*. 2016).

Here we present the first study of the relative abundances of adult *C. pipiens* and *C. torrentium* and their geographical variation in Denmark. This study is targeting adult mosquitoes collected in private gardens, in public parks, and on animal production farms. The temporal and spatial abundance of the adult vectors on these locations are assumed to be the main drivers of the potential for disease transmission from birds to humans and to production animals in Denmark.

2. Materials and methods

Mosquitoes were collected in 2012 using Mosquito Magnet Independence CO_2-baited traps with octenol (Mosquito Magnet, Litiz, PA, USA) from a total of 74 sites of randomly generated X–Y coordinates from three types of land use. The land use categories were urban gardens, recreational land use, and agricultural sites, which were further divided into four categories, pig, poultry, cattle and horse farms. At agricultural sites, the stable building or animal grazing fold nearest to the random coordinate was selected, and the trap was placed either fully outside or in the opening of a stable. Two of the 74 sites were

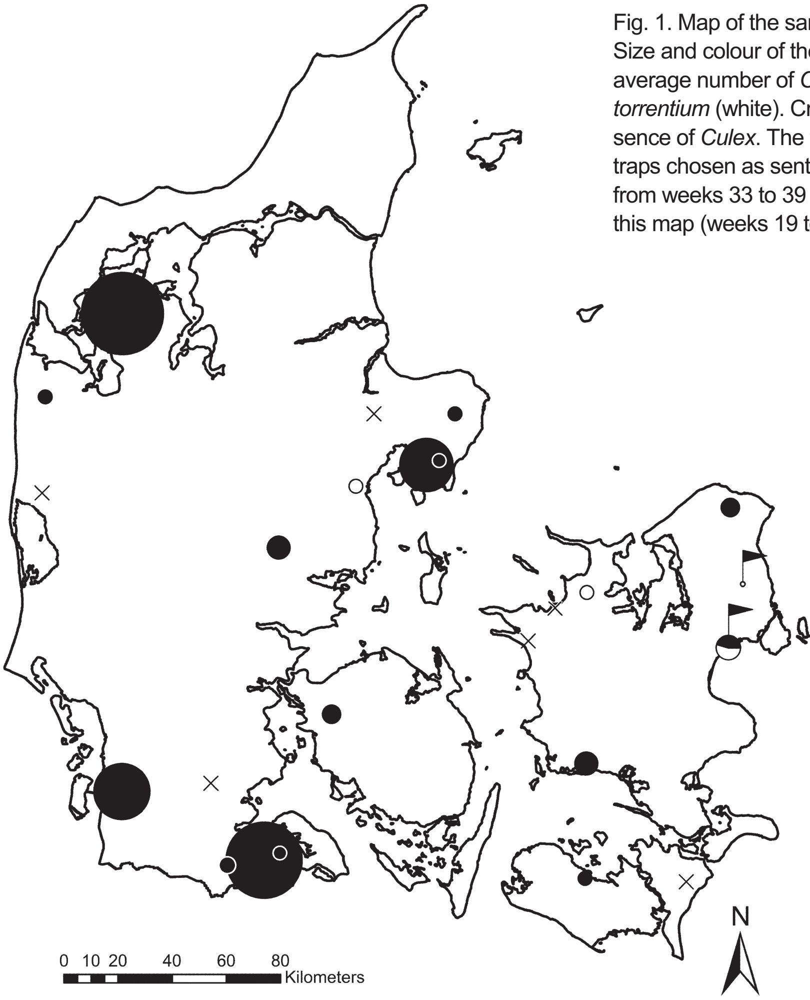

Fig. 1. Map of the sampling sites in Denmark. Size and colour of the dots represent the daily average number of *C. pipiens* (black) and *C. torrentium* (white). Crosses represent an absence of *Culex*. The two flags represent the traps chosen as sentinels. Only the catches from weeks 33 to 39 (272 exx.) are included in this map (weeks 19 to 32 with only 13 exx.).

selected as sentinel sites. The southern sentinel site was sampled daily from week 18 to 39 and the northern one weekly from week 21 to 39. These sentinel sites were therefore sampled more than the other 72 sites and these two therefore represented 29% and 26% of all collection nights. Because of a limited number of traps and the aim to sample a large number of different areas of Denmark, the traps were moved between the remaining sites every week (temporary traps). This was done in a total of 72 different temporary sites from week 21 to 37, where traps were left at each site for three consecutive days, constituting the remaning 45% of all collections nights. Each temporary collection site was only sampled once, and between three and seven new collection sites were sampled each week. Species were identified using the identification key by Becker *et al.* (2010).

Species of the morphological fairly similar *C. pipiens* and *C. torrentium* were separated by a restriction enzyme assay according to Hesson *et al.* (2010) with modifications. DNA extraction was performed with Qiagen DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) using the manufacturer's protocol for insect DNA extraction. Mosquito legs and the lower part of thorax were used for DNA extraction, and a microtube pestle was used to homogenize the sample, as this procedure yielded the best results. The PCR reactions consisted of: 6 µL DNA, 5 µL Buffer (10 x mM $(NH_4)_2SO_4$), 5 µL $MgCl_2$ (2.0 mM), the primer pair 0.5 µL TL2-N-3014 and 0.5 µL C1-J-2183 (Simon *et al.* 1994), 0.5 µL DNTP

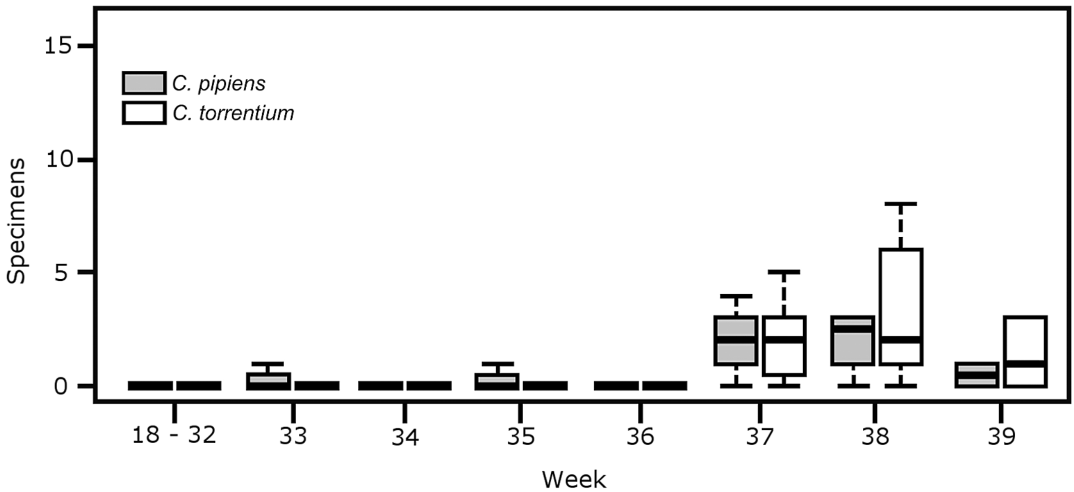

Fig. 2. Box and whisker plots of the daily catches of two *Culex* species from the southern sentinel CO_2-baited collection site. Catches are based on seven collection nights per week. Number of specimens with minimum, maximum, median, 25th and 75th percentiles are shown for each week.

(0.1 µM per sample), 0.20 µL Taq (0.02 U per µL), and 32.3 µL H_2O. Thermocycler conditions were: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 46 °C for 30 s, 72 °C for 1 min, and final extension of 72 °C for 7 min. Restriction enzyme digestion consisted of: 10 µL PCR reaction mixture, 18 µL nuclease-free water, 1 µ 10 X Buffer tango, 0.1 µL FspBI (Thermo Scientific, Waltham, MA, USA). After a gentle mixing, it was incubated at 37 °C for 3 h, followed by an inactivation of FspBI by incubation at 65 °C for 20 min. Restrictions were run on a 2% agarose E-Gel® (Invitrogen, Carlsbad, CA, USA).

Statistical analyses and box and whisker plots were made with R-Studio (R Core Team 2013). Statistical analyses were performed on weekly accumulated mosquito catches. Box and whisker plots were made as the daily catches of *C. torrentium* and *C. pipiens* (based on seven nights for the southern sentinel trap and three nights for the temporary traps) accumulated for each week. The map was made in ArcMap 10.3 (ESRI).

3. Results

A total of 285 female *Culex* specimens from the entire season were analyzed using the restriction enzyme assay. Of these, 220 specimens were identified as *C. pipiens* and 65 as *C. torrentium*. From week 19 to 32 a total of 10 *C. pipiens* and 3 *C. torrentium* were collected from 7 out of 50 sites. While this represents 2/3 of the collection period, it only constitutes 4.6% of the collected *Culex* specimens. During the last 1/3 of the collection period from week 33 to 39 a total of 210 specimens of *C. pipiens* were caught from 15 out of 24 different sites, and 62 specimens of *C. torrentium* were caught from 4 out of 24 different sites (Fig. 1). The spatial variation in the ratio of the two species differed significantly with Fisher's exact test between the collection sites at week 34 ($p = 0.032$, $df = 4$), 35 ($p = 0.012$, $df = 6$), 36 ($p = 0.025$, $df = 3$) and 37 ($p < 0.0001$, $df = 5$), but not at week 33 ($p = 1.0$, $df = 1$) and 38 ($p = 1.0$, $df = 1$). At week 39, mosquitoes were only captured in one of the collection sites. During the entire collection period, *C. pipiens* was caught at 22 different sites, significantly more than *C. torrentium* caught at just four sites (74 sites examined; Fisher's exact test: $p = 0.0001$, $df = 1$).

In total, 95% of all *C. torrentium* were collected at the southern sentinel collection site. This trap constituted 29% of all collection nights of the 74 traps. The southern sentinel collection site collected both species, with 61 specimens of *C. torrentium* and 51 of *C. pipiens*. This allowed for a comparison of the phenology for the two species, which showed fairly even seasonal dynamics at this site with an abundance peak from week

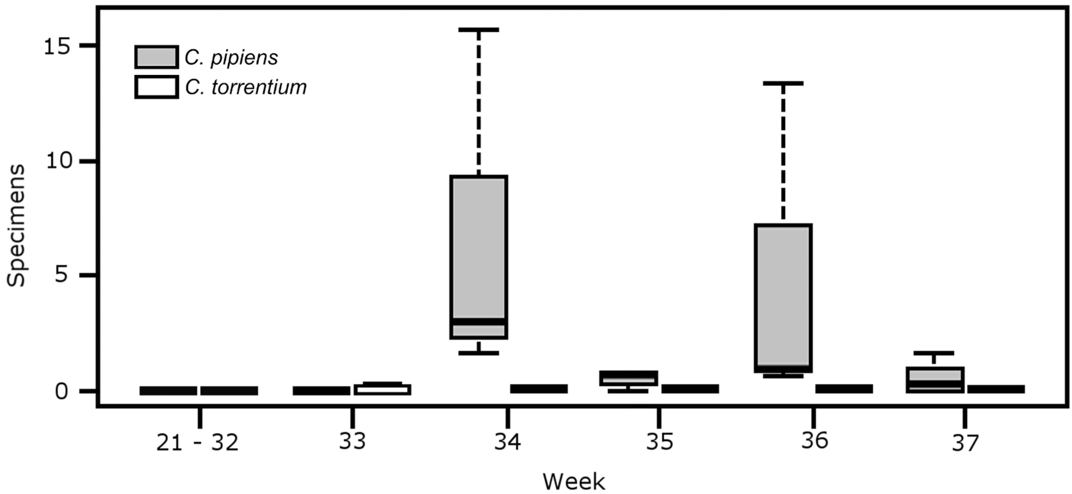

Fig. 3. Box and whisker plots of the average daily catches of two *Culex* species from three to seven weekly temporary trap sites. Each weekly catch consist of 3 consecutive collection nights. Number of specimens with minimum, maximum, median, 25^{th} and 75^{th} percentile are shown for each week.

37 to 39 (Fig. 2). The 210 specimens of *C. pipiens* collected at the temporary sites were primarily found in weeks 34 and 36 (Fig. 3) but showed a large geographical variation in abundance each week.

4. Discussion

Constituting 77% of all *Culex* specimens, this study found *C. pipiens* to be the dominant species in Denmark in octenol and CO_2-baited traps. This is consistent with the findings from the northern part of Germany, where *C. pipiens* was found to be the dominant species (Rudolf *et al*. 2013), but not with *C. torrentium* being the dominant species in northern Europe as suggested from a larger European study (Hesson *et al*. 2014). While *C. pipiens* was collected at 22 different Danish sites, *C. torrentium* was only collected at four sites. At the southern sentinel site *C. torrentium* was the dominant species with 54% during weeks 32 to 39, and this site collected 95% of all specimens of *C. torrentium*. Thus, while *C. pipiens* on average dominated nationally in both average abundance and geographical distribution, one hot spot was dominated by *C. torrentium* as expected for Northern Europe (Hesson *et al*. 2014). In general, the ratio of the two species varied significantly between the collection sites and the coincidental inclusion of an apparently rare *C. torrentium* hotspot in the survey as the sentinel site with relatively many collection nights increased the proportion of *C. torrentium* to 23%. The true national average of *C. torrentium* in Denmark is therefore likely to be lower than 23%; indeed, excluding this sentinel site lowers the prevalence of *C. torrentium* to just 1.4%. Therefore, the true national prevalence depends on the frequency of such hot spots in Denmark.

Species ratios may also be strongly influenced by sampling design with *C. pipiens* being more prevalent than *C. torrentium* in CO_2-baited traps compared to larval sampling and gravid traps (Weitzel *et al*. 2011, Hesson *et al*. 2015b). The reason for this remains unknown, but it has been speculated that *C. torrentium*, being strictly ornithophilic, spends its time around bird nests in high trees and only visits the ground to oviposit (Petrić *et al*. 1999). Both *C. pipiens* and *C. torrentium* were found to occur in high numbers late in the season. *Culex pipiens* hibernates as adults from August and slowly depletes the fat reserves and never takes blood during hibernation (Sulaiman & Service 1983). While determining *Culex* under a stereo microscope we found that many of them had developed fat reserves. This observation together with information on their hibernation biology, suggests that these mosquitoes may not have been attracted to CO_2-baited traps in search of blood meals, but rather as a result of exploring desired places for hibernation. If this is the case, differences in their hibernation biology and/or possibly phenology could also explain the low number of *C. torrentium* caught in CO_2-baited traps.

The late occurrence of *C. pipiens* and *C. torrentium* is a problem in regard to the sampling design used in this study, which was intended to catch a broad variety of mosquitoes from as many different sites in Denmark as possible. Therefore, many sites sampled early in the season that did not catch any *Culex*, would most likely have been positive for one or both of the studied species, if the sites had been sampled later in the season. Based on the results presented here, we suggest that studies investigating the ratio of *C. pipiens* and *C. torrentium* should be carried out late in the season and supplementary traps not based on CO_2 and octanol should be included in future surveys. In the present study, we used a restriction enzyme assay for species identification of 285 *Culex* specimens. For future high throughput screening of *Culex* samples, a more suitable method could be the qPCR assay for differentiation between *C. pipiens* and *C. torrentium* developed by Rudolf *et al*. (2013).

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