Thermal Limits for Flight Activity of Field-collected Culicoides in the United Kingdom Defined Under Laboratory Conditions

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Research

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Abstract

Background

Culicoides biting midges (Diptera: Ceratopogonidae) are biological vectors of internationally important arboviruses and inflict biting nuisance on humans, companion animals and livestock. In temperate regions, transmission of arboviruses is limited by temperature thresholds, both in replication and dissemination of arboviruses within the vector and in the flight activity of adult *Culicoides*. This study aims to determine the cold-temperature thresholds for flight activity of *Culicoides* from the United Kingdom under laboratory conditions.

Methods

Over 18,000 *Culicoides* adults were collected from the field using 4W down-draught miniature ultraviolet Centers for Disease Control traps. Populations of *Culicoides* were sampled at three different geographical locations within the United Kingdom during the summer months, and again in the autumn at one geographical location. Activity at constant temperatures was assessed using a bioassay that detected movement of adult *Culicoides* towards an ultraviolet light source over a 24-hour period.

Results

The proportion of active adult *Culicoides* increased with temperature but cold temperature thresholds for activity varied significantly according to collection season and location. Populations dominated by the subgenus *Avaritia* collected in South East England had a lower activity threshold temperature in the autumn (4°C), when compared with populations collected in the summer (10°C). Within the subgenus *Avaritia*, *Culicoides scoticus* was significantly more active across all temperatures tested than *Culicoides obsoletus* within the experimental setup Populations of *Culicoides impunctatus* collected in the North East of England*,* were only active once temperatures reached 14°C. Preliminary data suggested flight activity of the subgenus *Avaritia* does not differ between populations in South East England and those in the Scottish Borders.

Conclusions

These findings demonstrate seasonal changes in temperature thresholds for flight and across different populations of *Culicoides.* This data, alongside that defining thresholds for virus replication within *Culicoides,* provides a primary tool for risk assessment of arbovirus transmission in temperate regions. In addition, the study also provides a comparison with thermal limits derived directly from light-suction trapping data, which is currently used as the main method to define adult *Culicoides* activity during surveillance.

Background

Culicoides biting midges (Diptera: Ceratopogonidae) are biological vectors of a range of internationally important arboviruses of companion animals, livestock and wild mammals including bluetongue virus (BTV), African horse sickness virus (AHSV) and Schmallenberg virus (SBV) [1, 2]. In addition, *Culicoides* transmit Oropouche virus (OROV), which causes a febrile illness in humans, and play a poorly defined role in the transmission of a range of other zoonotic arboviruses [3, 4]. Within northern Europe, both BTV and SBV have a significant impact on livestock production through clinical disease and also ruminant movement controls and trade restrictions imposed to reduce virus spread [5, 6]. The risk of major shifts in the global distribution of *Culicoides*-borne arboviruses is currently considered to be high [7, 8], as illustrated by the recent unprecedented emergence of AHSV in Thailand [9].

In temperate regions, such as North-Western Europe, temperature has a fundamental influence on the transmission and persistence of *Culicoides*-borne arboviruses [10]. The rate of arbovirus replication and dissemination within a biological vector is usually highly dependent on environmental temperature, as most arthropods are poikilothermic. In the case of *Culicoides*: BTV interactions, the threshold for infection and replication lies within a range of 11-15°C [11]. It is clear, however, that the temperature threshold for *Culicoides* active flight is below this range and therefore could allow survival of the virus through winter periods when replication does not occur but when infected adults are able to survive, fly and feed on hosts [12, 13]. It has also been demonstrated that *Culicoides* infected with AHSV at 25°C and then immediately held at a constant temperature of 10°C for 35 days develop full infections following a return to 25°C for 3 days [14]. Hence, resumption of temperatures conducive to virus replication could also lead to transmission during transient warmer periods within winter.

Temperature-related thresholds to activity in adult *Culicoides* can be assessed through direct analysis in the laboratory and through a variety of field-based methods. Direct studies of activity use laboratory-based bioassays based on a phototactic response while they are held at a known temperature. A recent study in the Republic of South Africa (RSA) examined the major afrotropical vector of arboviruses, *Culicoides imicola* Kieffer, and measured flight response using a white fluorescence light stimulus and horizontal flight over a distance of approximately 15cm [15]. At 10°C and 13°C, *C. imicola* phototactic response was less than 5% of the insects introduced, while at 17°C, more than 25% responded. At temperatures of more than 17°C, the phototactic response in *C. imicola* did not exceed 28% of those introduced into the bioassay system. An earlier study, based in Japan, examined activity using a dedicated bioassay system based on a requirement for 8cm of horizontal flight in response to an ultraviolet light source [16]. In both *Culicoides oxystoma* Kieffer and *Culicoides maculatus* Shiraki, active flight was recorded in less than 10% of individuals at temperatures below 16-17°C, although a few individuals were recorded as being active even at the minimum temperature of 6°C. In contrast to the study in RSA, over 80% of individuals exhibited a phototactic response at temperatures of greater than 25°C.

Activity of adult *Culicoides* can also be measured in the field by collection in parallel with measurement of environmental temperature. The most epidemiologically relevant method of assessing thermal limits to activity is to measure adult *Culicoides* blood-feeding behaviour on a relevant host. A key issue is that

these studies are generally carried out at times of peak adult seasonal abundance, where thermal limits to activity are not usually reached, and are also limited by the availability of daylight [17]. As an example, collections of adult *Culicoides* from sheep made in the United Kingdom (UK) using a drop-trap design reported all species within the subgenus *Avaritia* as active across the entire temperature sampling range of 11.8-29.0°C between the daylight hours of 18:00 and 21:30 [17]. Truck trapping has also been carried out in the UK using a vehicle driven over a set course with a net mounted on its roof [18]. This samples populations of adult *Culicoides* actively flying at a set height of approximately 1.5m without an attractant. *Culicoides* of the subgenus *Avaritia* were collected over a range of 8.6-30.0°C, with larger catches associated with higher temperatures, although reductions in activity were recorded when the temperature exceeded 21°C [18]. Both of these approaches are usually only deployed at single sites on a local scale due to their complex logistics.

Thermal limits to *Culicoides* adult activity can also be inferred from less labour-intensive trapping approaches. Semiochemical-based traps for livestock-associated vector *Culicoides* in northern Europe have received only limited attention [19], with the majority of research targeting the nuisance biting species *Culicoides impunctatus* Goetghebuer [20, 21]. In contrast, ultraviolet (UV) light-suction traps are used in Europe for both research activities and surveillance of *Culicoides* during arbovirus outbreaks [22, 23, 24]. While the intrinsic biases in *Culicoides* populations sampled through the use of light-suction traps are well known [17, 25, 26], they are by far the most commonly deployed means of detecting activity due to their ease of use, although data collected from winter periods examining temperature thresholds for activity remains scant.

In France, adult *Culicoides* activity was inhibited, as measured by an absence of individuals in trap collections, on nights when the maximum temperature fell below 10°C [27], and a similar result was found in Germany where activity was limited to days with a maximum temperature of ≥10.8°C [28]. A field study in the UK was able to collect adult *Culicoides*, using UV light-suction traps, in February and March when the maximum temperature for both months was 10.1°C [29]. Results from a large dataset of *Culicoides* collections across Europe indicate that the onset of the vector season can occur at mean temperatures as low as 1°C in the most northern latitude tested, whereas mean temperatures of 10°C are required for *Culicoides* activity in countries in the southern latitudes [24]. Key variation in experimental methods include the method by which temperature is inferred (e.g. *in situ*, using national weather station-based monitoring or remotely sensed data) and the fact that light-suction trap collections are not representative of biting rate on hosts in this region [17].

Surveillance of adult *Culicoides* flight periods is currently adopted in Europe to determine the time period during which the probability of arbovirus transmission is very low, defined as the seasonal vector-free period (SVFP) [30]. Following the BTV-8 outbreak in northern Europe in 2006, the European Commission enforced bluetongue surveillance programs in member states which involved entomological surveys of *Culicoides* vectors using light-suction traps [31]. Using this surveillance data, a SVFP was identified in the winter of 2006/2007 in the UK, enabling over 70,000 susceptible livestock movements [32]. The determination of the SVFP is dependent on the use of light-suction trap surveillance despite their

limitations which include the underestimation of host-seeking activity compared to other methods [33, 34].

Understanding the temperature threshold for flight activity in *Culicoides* is critical in evaluating the risk of transmission following an incursion, in addition to understanding their role in arbovirus overwintering in the UK [12]. The implementation of trap surveillance, if not already in place, is time-consuming resulting in a delay of active surveillance from the point of initial incursion. Applying easily accessible temperature data, however, to evaluate the activity rates of *Culicoides* populations and thus infer the risk of transmission by active *Culicoides* could overcome the issues associated with post-incursion surveillance. The aim of this study was therefore to determine a cold temperature threshold for flight activity under experimental conditions for field-caught UK *Culicoides* species, collected at different seasons and geographical locations.

Methods

Study sites

Field collections of adult *Culicoides* were conducted across five sites in the UK (Figure 1) at different times of the year (Additional File 1: Table S1). In South East England, three equine holdings (site 1: 51°08’46.5”N, 0°36’51.6”W; site 2: 51°17'26.2"N, 0°39'09.0"W; site 3: 51°17'00.3"N, 0°36'24.6"W) were used for collections in June – August 2017 (South East England summer cohort; SES) and again for collections in September – October 2017 (South East England autumn cohort; SEA). All three sites were predominately used for grazing, with animal stabling and muck heaps present at each site. In North East England, collections were made from a forest campsite (site 4: 55°14'13.1"N, 2°35'12.1"W) during July 2018 (North East England summer; NES). During the same month, a collection was also made at a mixed farm in the Scottish Borders (site 5: 55°15'44.5"N, 2°41'16.4"W) (Scottish Borders summer cohort; SBS).

Collection methods

Insects were collected using down-draught miniature blacklight (UV) Centers for Disease Control (CDC), model 912 traps (John W Hock Co, Gainesville, FL, USA). Each CDC trap uses a 4W UV tube and is powered by a 12V lead acid sealed battery (Yuasa, Japan). At each site, between four and eight traps were used in order to catch sufficient *Culicoides* for the trials and were positioned at least 50m apart [35]. Traps were suspended at a height of approximately 1.5m above ground. Traps were set up at least two hours before sunset and run overnight before collection within two hours of sunrise the following day. Insects were collected live into a 340ml cardboard collection cup containing a cotton pad soaked in 10% sucrose to provide a sugar source and paper towel cut into long, thin strips to provide shelter from the trap fan downdraft. Cardboard collection cups were secured to the CDC trap using a CDC sleeve and elastic bands (Additional File 2: Figure S1). The following morning, sleeves from each trap were removed and tied with a clip-lock tie to contain the insects within the collection cup and sleeve.

Flight activity experiments

Insects collected were transferred and released into a large plastic, black box (42cm (l) x 35cm (w) x 32cm (h)) with a translucent funnel (14cm in length) attached on one side to allow for insects to exit. Clear plastic tubing (6cm in length) was secured to the translucent funnel to visualise and count approximately 150-250 active, phototactic adult *Culicoides* exiting the box and entering a 'flight activity pot' (Figure 2). The flight activity pots were modified 340ml cardboard collection cups with a small plastic funnel (66mm top diameter; 7.4cm in length) covered in aluminium foil fitted at the top, and a fine mesh covering the bottom of the pot. The total height of the flight activity pot from the base of the cardboard cup to the tip of the funnel was 14cm. A cotton wool bung within the funnel retained all insects within the flight activity pot until the start of the experiments.

Once eight flight activity pots had been filled, they were numbered at random from one to eight. Pots one to six were placed into a 'test' temperature-controlled incubator (MIR-254 Panasonic, UK) at an initial temperature of 20±1°C, while pots seven and eight were placed in a second 'maintenance' incubator also set at 20±1°C as a control for activity of *Culicoides* in the sample. Both incubators contained a UV light source (4W blue-blacklight tube; John W Hock Co, Gainesville, FL, USA) suspended within the incubator that acted as an attractant and no other light was provided within the incubators. The test incubator was then set to the desired temperature (2°C – 14°C) and insects were allowed to acclimatise for two hours. Following the acclimatisation period, the cotton wool bung at the top of the flight activity pot funnel was removed and a 7.5cm diameter pill box (Watkins and Doncaster, UK) with a fine mesh lid was secured on top of the funnel allowing active insects to fly towards the UV light stimulus into the attached pill box (Figure 2E). All flight activity pots were placed with the mesh lid faced downwards onto a tray to ensure darkness, so that the UV light was the only light source available above the flight activity pots. A small cotton pad soaked in 10% sucrose was placed underneath each flight activity pot on the tray, rather than within the pot, so that insects could still access the sugar-meal through the fine mesh but were prevented from sticking to the pad.

At regular intervals the collection pill boxes were replaced (0.5, 1, 2, 4, 6, 8, 10- and 24-hour intervals) and the collected *Culicoides* were killed by freezing at -20°C for a minimum of 2 hours. All remaining inactive individuals in the flight activity pots were also killed by freezing after 24 hours. The percentage of *Culicoides* flying after 24 hours was determined by calculating the mean cumulative proportion of active *Culicoides* across the six flight activity pots maintained at each test temperature. In each temperature trial, collections were also made at the same intervals from pots seven and eight, which were maintained at 20±1°C throughout the experiment to ensure the population tested were active without the constraint of cold temperatures. A flow diagram of the full experimental design is provided in Additional File 3: Figure S2. In the SES cohort, five temperatures were tested (6, 8, 10, 12 and 14°C) and the SEA cohort a further five temperatures were tested (2, 4, 6, 8 and 10°C). Four temperatures were tested in the NES cohort (8, 10, 12 and 14°C) and only one temperature of 12°C was tested in the SBS cohort.

Sample identification

Only individuals that were tested in activity experiments were identified, all surplus insects that remained in the initial black plastic sorting box were not used for experimental study. *Culicoides* from the activity experiments were sorted morphologically under a dissecting microscope using characteristic wing patterns with the aid of an identification key [36]. Adult *Culicoides* were grouped into six categories: subgenus *Avaritia*, *Culicoides pulicaris* Linnaeus*, Culicoides punctatus* Meigen*, Culicoides achrayi* Kettle and Lawson*, C. impunctatus* and other *Culicoides*. Female *Culicoides* were also identified to physiological state by examination of the abdomen [37] and assigned to one of the following categories: unpigmented, pigmented, gravid and blood-fed.

For both South East England cohorts (SES and SEA), a sub-sample of females within the subgenus *Avaritia,* were identified further to species level (*C. obsoletus* and *C. scoticus* only) using an adapted multiplex polymerase chain reaction (PCR) method targeting the internal transcribed spacer (ITS) 1-5.8S-ITS2 region [38]. All individuals belonging to the subgenus *Avaritia* from one flight pot from each temperature trial was chosen at random for molecular analysis and used as a representative sample for each population at each temperature trial. *Culicoides* were transferred to individual reaction tubes with 200µl of tissue digest solution containing 100nM Tris-HCl pH8 (Thermo Fisher, UK), 200mM NaCl (Sigma-Aldrich, UK), 0.2%(w/v) SDS (Thermo Fisher), 5mM EDTA (Thermo Fisher), 200µg/mL proteinase K (Thermo Fisher), and nuclease-free water (Thermo Fisher). Following an overnight incubation in tissue digest solution at 37°C, individual *Culicoides* specimens were transferred to tubes containing 70% ethanol for storage. The *Culicoides* DNA was then extracted from 100µl of tissue digest solution and eluted into 100µl buffer using the KingFisher Flex automated extraction platform and the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher) according to manufacturer's instructions.

Two microliters of each sample DNA was added to each well on a PCR plate (Life Technologies, UK) each containing 8µl of mastermix which consisted of 1x TaqMan Fast Advanced MasterMix (Thermo Fisher), 0.3µM of each primer [38], 0.2µM of each probe [38] and diluted to a total reaction volume of 10µl using nuclease free water (Thermo Fisher). Negative extraction controls consisted of elution from wells which did not contain any *Culicoides* specimen in the extraction plate and negative PCR controls contained just nuclease free water. At least three negative controls and at least three positive controls, using DNA extracted from males from the same study morphologically identified as either *C. obsoletus* or *C. scoticus*, were added to each plate.

The PCR thermal profile used consisted of 2 mins at 50°C for activation of uracil-DNA-glycosylases (UDG), an initial denaturation step of 2 mins at 95°C, followed by 40 cycles of 95°C for 3 secs and 60°C for 30 secs and was carried out using an Applied Biosystems 7500 Fast instrument (Thermo Fisher). Each plate was analysed using the ViiA7 Real Time PCR system software (Thermo Fisher). Determination of species for each individual specimen was based on the cycle threshold (C_t) value for each species-specific primer-probe pairing. Negative samples were defined as having a $C_t \geq 35$ and positive samples were defined as having a $C_t \leq 25$. Samples with a C_t between >25 and <35 were regarded as undetermined and were repeated. If samples remained undetermined following re-examination, samples were defined as unknown and were removed from analysis.

Statistical modelling

Generalised linear mixed models (GLMMs) were used to investigate the relationship between temperature and the proportion of *Culicoides* flying and how this relationship differed amongst cohorts. Specifically, a binomial family GLMM with a logit link function was constructed with the proportion of *Culicoides* flying as the response variable. Model selection proceeded by stepwise deletion of non-significant (P>0.05) terms (as judged by likelihood ratio tests), starting from a model including temperature (°C), cohort and an interaction between them as fixed effects and pot as a random effect (to allow for between-pot variation). The models were implemented using the lme4 package [39] in R (version 3.6.1) [40].

Separate models were constructed for total *Culicoides* (all *Culicoides*, unpigmented females and pigmented females), the *Avaritia* subgenus (all *Culicoides*, unpigmented females and pigmented females) and *C. impunctatus* (all *Culicoides* and pigmented females). Sample sizes were insufficient to examine relationships for: (i) *C. pulicaris*, *C. achrayi* or other *Culicoides*; or (ii) blood fed females, gravid females or males for any species/groups. In addition, the SBS cohort was excluded from this analysis as activity was only assessed at a single temperature (12°C) for this cohort.

Further models were constructed for *Culicoides* flight activity at 12°C to compare activity amongst the SES cohort, the NES cohort and the SBS cohort. A similar approach to that described above was used, except the model included cohort as a fixed effect and pot as a random effect. Flight activity in the cohorts were compared using Tukey multiple comparisons.

For the SES and SEA cohorts, there was a sufficient number of *Culicoides* caught to allow two further analyses. First, to compare flight activity of unpigmented and pigmented females of the *Avaritia* subgenus a GLMM was constructed including pigmentation state (i.e. unpigmented or pigmented) as a fixed effect, as well as two- and three-way interactions between it and the other fixed effects (i.e. temperature and cohort). Model selection was carried out as described above. Second, to compare flight activity of *C. obsoletus* and *C. scoticus*, a binomial family generalised linear model (GLM) with a logit link function was constructed. The proportion of *Culicoides* flying was the response variable and temperature, cohort and species were fixed effects. Model selection proceeded as described above.

Results

For both the SES and SEA cohorts, a total of five collections were made to test five temperatures in each cohort. Each collection, made across multiple sites (sites one to three), was used to test one temperature at a time. In the SES cohort, a total of 5,586 *Culicoides* were collected across the five collections to test five temperatures (6, 8, 10, 12 and 14°C). In the SEA cohort, a total of 5,309 *Culicoides* were collected across the five collections to test five temperatures (2, 4, 6, 8 and 10°C). A total of 7,228 *Culicoides* were tested in the NES cohort from a total of four collections from site 4 only, to test four temperatures (8, 10, 12, 14°C). One collection was made in the SBS cohort to test one temperature (12°C) in which 585 *Culicoides* were tested (Additional File 4: Table S2).

In the South East of England, >97% of *Culicoides* in both the summer (5,463 of the 5,586 individuals) and autumn (5,259 of the 5,309 individuals) cohorts belonged to the subgenus *Avaritia.* In contrast, >99% of *Culicoides* in North East England were identified as *C. impunctatus* (6,611 of the 6,643 individuals). There were insufficient numbers collected of other species (*C. pulicaris, C. punctatus, C. achrayi* and other *Culicoides*) from all cohorts for further analyses to be conducted on these species. (Additional File 4: Table S2). Collections were also dominated by unpigmented and pigmented female *Culicoides* and no further analysis was conducted on males, gravid or blood fed females (Additional File 4: Table S2).

In all cohorts where multiple temperatures were tested, the proportion of active *Culicoides* increased as temperature increased within the range tested (Figure 3). This relationship was observed for total *Culicoides* (all adults, unpigmented females and pigmented females), the subgenus *Avaritia* (all adults, unpigmented females and pigmented females) and *C. impunctatus* (all adults and pigmented females) in both South East England cohorts (SES and SEA) and the NES cohort (Figure 3). Moreover, the rate at which *Culicoides* activity increased with temperature was the same amongst the three cohorts tested (i.e. there was no significant (P>0.05) interaction between temperature and cohort) (Additional File 5: Table S3).

The season and geographical location of *Culicoides* collection influenced the temperature at which *Culicoides* activity began and the levels of activity reached at each temperature (Figure 3, Additional File 5: Table S3). The cold temperature threshold for activity, as measured by the minimum temperature at which >5% of the *Culicoides* population is active, was significantly higher for *Culicoides* from the NES cohort compared with *Culicoides* from the two south east England cohorts, SES and SEA. Collections made from North East England primarily comprised of *C. impunctatus*, whereas collections made in South East England were dominated by *Culicoides* of the *Avaritia* subgenus. In the NES cohort, the cold temperature threshold was 14°C, in which only 9% of the *Culicoides* population were active (Figure 3). The two South East of England cohorts had substantially higher activity rates at all temperatures compared to NES (Figure 3). Yet, adult *Culicoides* in the SEA cohort had a lower cold temperature threshold for activity (4°C) compared with those collected in the SES cohort (10°C), though the two populations demonstrated similar activity levels at 14°C (Figure 3). This pattern was similar for both the unpigmented and pigmented females in these cohorts (Figure 3). Indeed, there were no significant (P = 0.66) differences in flight activity between unpigmented and pigmented females of the *Avaritia* subgenus for both the SES and SEA cohorts. In all temperature trials, *Culicoides* from the same populations maintained at 20±1°C within control pots were active, with average activity levels in control pots of 86% and 78% for the SES and SEA cohorts respectively, indicating that any reduction in activity is a result of the colder temperatures in temperature trial pots.

A single collection was subsequently made at a site in the Scottish Borders in the summer (SBS) which consisted of a greater proportion of livestock-associated species of *Culicoides* compared with collections made in the NES cohort, collected less than 5 miles away; a total of

210 of the 585 individuals tested from the SBS cohort were classified in the subgenus *Avaritia* whereas only16 of the 6,643 individuals tested from the NES cohort were classified in the subgenus *Avaritia*. Adult *Culicoides* collected in the SBS cohort had a significantly greater flight activity at 12°C, the only temperature tested, compared with *Culicoides* collected nearby in the NES cohort, when measuring total *Culicoides* and pigmented females (P<0.001). Flight activity for *C. impunctatus* specifically at 12°C, however, was not significantly different between the NES and SBS cohorts (P=0.34). Flight activity at 12°C was not significantly different between the SES cohort and the SBS cohort for total *Culicoides* (P=0.99), total unpigmented females (P=0.73) and total pigmented females (P=0.35) (Figure 4, Additional File 6: Table S4). For adults belonging to the subgenus *Avaritia* specifically, flight activity at 12°C was higher for the SBS cohort compared with the SES cohort, for pigmented females only (P=0.007). For all other physiological states within the subgenus *Avaritia*, activity levels between the SES and SBS cohorts were not significantly different.

Within the South East of England, the two cryptic species within the subgenus *Avaritia, C. obsoletus* and *C. scoticus* differed in their flight activity (P<0.001), with a greater proportion of *C. scoticus* than *C. obsoletus* active at all temperatures (Figure 5, Additional File 7: Table S5). As seen in the previous analyses, adults of each species from the SEA cohort were more active at lower temperatures than those from the SES cohort (Figure 5). Additionally, there was a greater number of individuals from the subgenus *Avaritia* subsample identified as *C. scoticus* later in the season in the SEA cohort (84%) when compared to collections made earlier in the season in the SES cohort (37%).

Discussion

This study has identified significant differences in temperature thresholds for flight activity of adult *Culicoides* according to species and season under laboratory conditions. In the populations examined in South East England, which were dominated by *Culicoides* of the subgenus *Avaritia*, the

cold temperature activity threshold was higher for populations tested earlier in the season (SES: 10°C), in comparison to those collected later in the season (SEA: 4°C), despite similar activity rates being recorded at higher temperatures. In addition, species specific differences in activity were recorded at an interspecific level with greater activity being recorded across the temperatures tested for *C. scoticus* relative to *C. obsoletus*. In addition, *C. impunctatus* collected in North East England (NES: 14°C) demonstrated significantly reduced activity across all temperatures despite an identical process of acclimatisation and test of flight fitness prior to use in the bioassay.

Studies carried out in South East England utilised two separate populations taken from summer (June-August) and autumn (September-October). The number of generations occurring each year in *Culicoides* in Northern Europe remains unclear, although bi- or trivoltinism has been suggested in populations of *C. impunctatus* [41] and the subgenus *Avaritia* [42]. This study highlights a significant difference in thermal tolerance of populations sampled in the same year and site using identical methods of selection, acclimatisation and measurement of activity. The underlying mechanism enabling this adaptation is

unknown, although previous studies carried out in the United States of America (USA) demonstrated rapid cold hardening in adult *Culicoides sonorensis* through short term exposure to temperatures of less than 5°C [43]. This study highlights the lack of knowledge that currently exists in the drivers of differences in behaviour, biology and ecology between generations of *Culicoides*.

Despite this apparent rapid adaptation to activity at cooler temperatures observed in populations of the subgenus *Avaritia* in South East England, testing of *C. impunctatus* at a more northern latitude led to equivocal results. While cited as a putative vector of arboviruses [44], this species differs significantly in biology and ecology from individuals belonging to the subgenus *Avaritia*. Firstly, it is autogenous [41, 45], mating facultatively and laying its first egg batch a few days following emergence from development sites without host seeking [17, 46]. This results in a flying adult female population that is dominated by parous individuals for the majority of the season, as reflected in the results of the current study where this life stage comprised over 95% of individuals tested. The high temperature threshold for activity in *C. impunctatus* was primarily driven by a limited flight response in populations tested within the bioassay. This was despite the fact that adults capable of flight towards light following trapping were pre-selected by the experimental design and conditions used were identical to the trials in South East England. The average flight activity response at 20°C for total *Culicoides* collections across the SES and SEA cohorts was 86% and 78% respectively. In contrast, a greatly reduced flight activity level of 20% was observed in *Culicoides* populations collected in the NES cohort maintained at 20°C. In addition, it was demonstrated through the trap collection conducted in the nearby Scottish Borders that this was a species-specific response, rather than a broader effect of sampling location. The reasons for this lack of response remain unclear but could represent a reduced phototactic response in this species, or a species-specific impact on behaviour caused by initial capture using light-suction trapping.

The temperature thresholds for flight of subgenus *Avaritia* populations in this study (4°C in SEA and 10°C in SES) were similar or slightly above those recorded for the temperate *C. oxystoma* in Japan (6°C) [16], but were lower than the afrotropical *C. imicola* (14°C) in RSA [15]. One major difference in experimental design between the current study and these was the avoidance of a sorting step that necessitated cold anaesthesia prior to testing activity. This was viewed as preferable to pre-exposing the *Culicoides* to cold, given previous evidence of cold hardening [43]. The experimental trade-off for avoiding this step was a lack of harmonisation of density in pots due to the difficulties of estimating numbers of *Culicoides* introduced. While this could have led to variation in density-dependent disturbance between flight activity pots, this was not evident in the study and would have been minimal in cooler temperatures where flight was restricted to a small proportion of the midges introduced. In addition, the requirement for vertical flight, rather than horizontal, enabled a more straightforward measure of activity, potentially reducing this factor.

In terms of wider policy implications of the study, the European Food Safety Authority (EFSA) has reported previously that, based on light-suction trapping the threshold temperature required to initiate *C. obsoletus* complex adult activity was 10°C, with temperatures of ≤4°C for longer than 10 days leading to an end to adult activity [47], although differentiation between survival and activity in this report is not

well defined. The disparity between predictions of activity made from light-suction trapping and direct examination of flight could result from a range of factors including the fact that light-suction traps are usually set at 1.5-2m from ground level and have a limited effective range [35]. In addition, the period where the temperature range most often limits adult flight occurs during late autumn, winter and early spring, when risk of arbovirus incursion, and hence systematic trapping, is limited.

The SVFP is determined from collections made through vector surveillance programs which, when limited, can underestimate the vector activity. The use of temperature to determine optimal times to deploy surveillance trapping would provide a better assessment of transmission risk, particularly in the event of an incursion of an arbovirus. In this scenario, the deployment of entomological surveillance in affected areas can take considerable time. In the period where there is an absence of active vector surveillance, predicting the activity of *Culicoides* achieved using temperature-related baseline parameters contributes significantly to arbovirus risk assessments and policy decision making in the immediate aftermath of an outbreak.

Vector surveillance using UV light suction traps can actively select for host seeking female *Culicoides* due to their increased attractiveness to light, therefore underestimating the number of *Culicoides* from the total population [48, 49] and also underestimate the levels of activity particularly of non-host seeking *Culicoides.* This study confirmed that there was no significant difference in the flight response towards a UV light stimulus between unpigmented and pigmented females of the subgenus *Avaritia* collected in either the summer or autumn from the same geographical location in the South East of England. This conclusion has implications when determining the SVFP, which is defined as <5 parous (pigmented) *Culicoides* specifically caught in a UV light-suction trap collection over one night, despite both unpigmented and pigmented females being equally as active and both host seeking.

Whilst both collections from South East England consisted predominately of individuals belonging to the subgenus *Avaritia*, there was a far greater abundance of *C. scoticus* present in the SEA cohort (84%) compared with the SES cohort (37%). In this study we show, for the first time, that *C. scoticus* are significantly more active, under these laboratory conditions, at any temperature compared with *C. obsoletus* in both the summer and autumn, although this increased activity of *C. scoticus* could also have been influenced by differences in the species attractiveness to light. Due to difficulties separating these two species by morphological methods [50] differences in their behaviour as well as their vector competence have not yet been fully explored. Nevertheless, the differences in phototactic activity and seasonality between *C. obsoletus* and *C. scoticus* observed in this study could have implications for disease management especially when determining the transmission season which could vary between species.

It has been suggested that *Culicoides* adults are able to survive the winter in an inactive, dormant form, and following an increase in temperature are able to become active and subsequently transmit virus, albeit in climates far warmer than those in northern Europe [14, 51]. As demonstrated for *C. imicola*, activity may cease at sub-optimal temperatures (<4°C), but complete mortality is only observed at much

lower temperatures (-3°C) [52] with one study showing that *Culicoides* can survive 15 days at -1.5°C [53]. Hence, temperatures below the activity threshold will render *Culicoides* inactive, but individuals may still be able to survive. These individuals will go undetected during surveillance, and if fully infected represent a risk of transmission with consequences for arbovirus overwintering. The accidental import of viraemic ruminants during trade may also lead to uncertainty regarding the infection of active and host-seeking *Culicoides* at temperatures above the threshold for flight activity (4°C), but below that required for virus replication (11-15°C). Therefore, studies of initial infection at the range of 4-11°C, followed by a realistic increase in temperature simulating a change to warmer conditions would be useful to quantify this risk pathway. Furthermore, it is not known whether the small proportion of active individuals at low temperatures found in this study can remain active for longer than 24 hours, although *Culicoides* have previously been shown to recover after 10 days at 4°C [54]. Further investigations into the effect of prolonged exposure to cold temperatures on adult *Culicoides* activity are required to assess the effect of cold temperatures at the population level in northern Europe.

Conclusions

The data presented here define cold temperature thresholds for flight activity in a range of UK *Culicoides* species and populations. Variation was observed in the subgenus *Avaritia* populations tested at different times of the year suggesting an effect of season on the activity of adult *Culicoides* whereby populations emerging later in the season possess a greater degree of cold tolerance. Further investigations are required to determine the effect of prolonged exposure to cold temperatures on *Culicoides* activity and survival to fully understand the consequence of cold winter temperatures on UK *Culicoides* populations and their ability to act as vectors of arboviruses.

Abbreviations

AHSV African horse sickness virus

BTV Bluetongue virus

CDC Centers for Disease Control

EFSA European Food Safety Authority

EIP Extrinsic incubation period

GLMM Generalised linear mixed model

GLM Generalised linear model

IBH Insect bite hypersensitivity

ITS Internal transcribed spacer

NES North East England summer cohort

PCR Polymerase chain reaction

SBS Scottish Borders summer cohort

SBV Schmallenberg virus

SEA South East England autumn cohort

SES South East England summer cohort

SVFP Seasonal vector free period

UDG Uracil-DNA-glycosylases

UK United Kingdom

USA United States of America

UV Ultra-violet

Declarations

Ethics approval and consent to participate

Not applicable. No technique used during the trial required ethical approval.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LT performed studies, wrote and approved the submission. ME was a major contributor in writing and revising the submission and provided taxonomic expertise. SGu carried out statistical analyses and edited and approved submission. CS, JStok and JSton contributed to data acquisition. SC, KD, SGr and AB supervised the study and edited and approved the submission. LT, SC and CS contributed to development of experimental design. All authors read and approved the final manuscript.

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Figures

Figure 1

Map of study sites. A total of five study sites were used; sites 1, 2 and 3 in south east England, site 4 in north east England and site 5 in the Scottish Borders. The red points on the map indicate each study site from where Culicoides were collected.

Figure 2

Photographs of equipment used for collecting and sorting Culicoides collections for the flight activity study. Cardboard collection cup (340ml) containing a sucrose-soaked cotton wool pad and paper towel strips (A), collection cup and sleeve removed from trap and secured using clip-lock tie (B), black plastic box with funnel at one end with attached clear plastic tubing (C), insects entering flight activity pots from the dark plastic box via the funnel and clear tubing (D), flight activity pots 1-6 were transferred into dark incubators and specifically were placed with the mesh downwards onto a tray to ensure darkness within the flight activity pot. A small sucrose-soaked cotton wool pad was placed underneath and a 7.5cm diameter pill box, with a fine mesh lid, was secured on top of the funnel allowing active insects to fly towards the UV light stimulus into the attached pill box. (E).

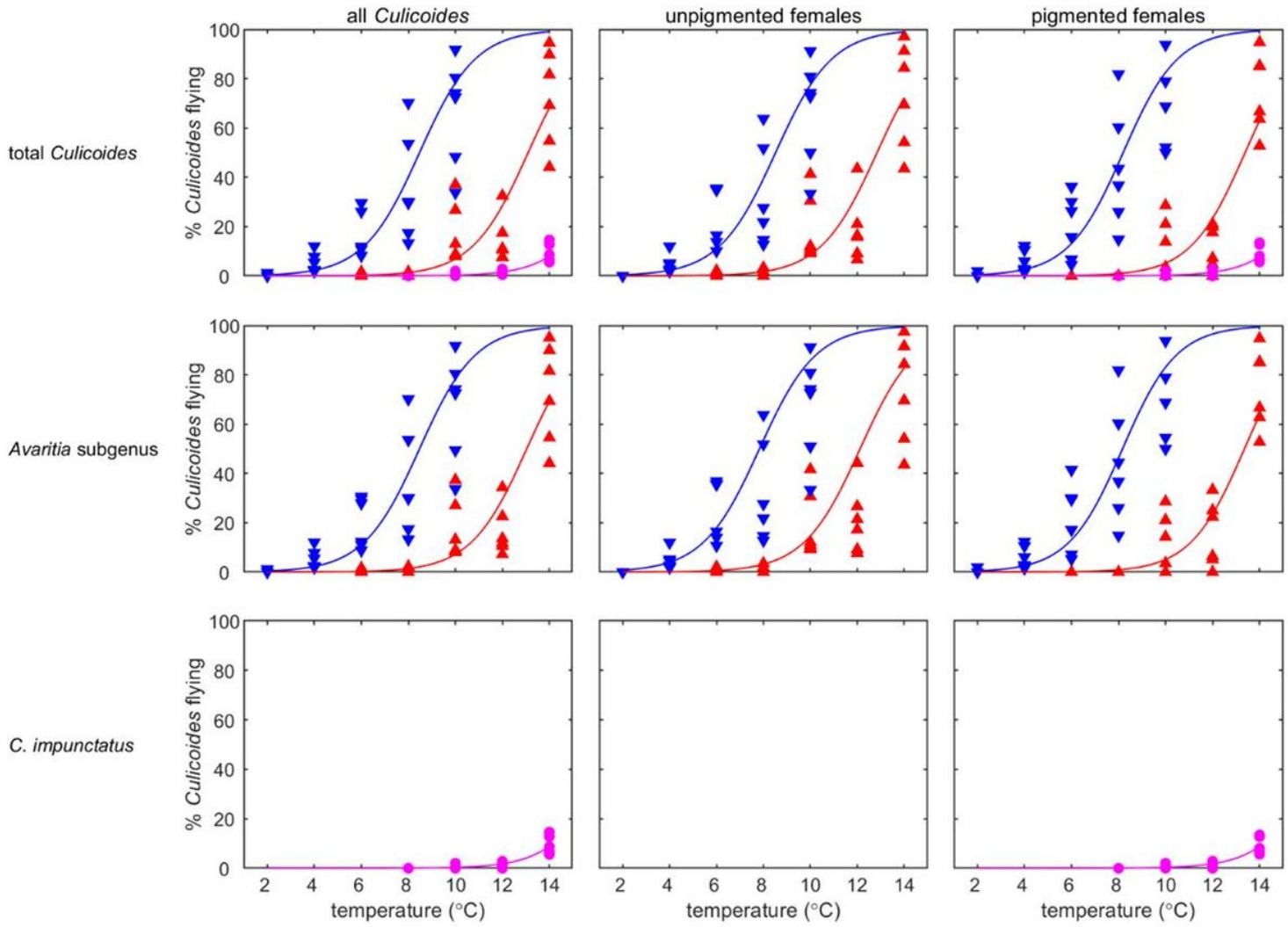

Figure 3

Percentage of Culicoides collected in the flight pill boxafter 24 hours at defined temperatures. Rows show results for total Culicoides (top row), the Avaritia subgenus (middle row) and Culicoides impunctatus (bottom row). Columns show results for all Culicoides (left), unpigmented females (centre) and pigmented females (right). Symbols show the observed percentage of Culicoides flying and the curve is the fitted model (using the estimated fixed effects only). Cohorts are indicated by the curve/symbol colour: SES (red); SEA (blue); and NES (magenta). If data are not shown for a cohort, insufficient specimens of that species/group were caught for it to be included in the analysis.

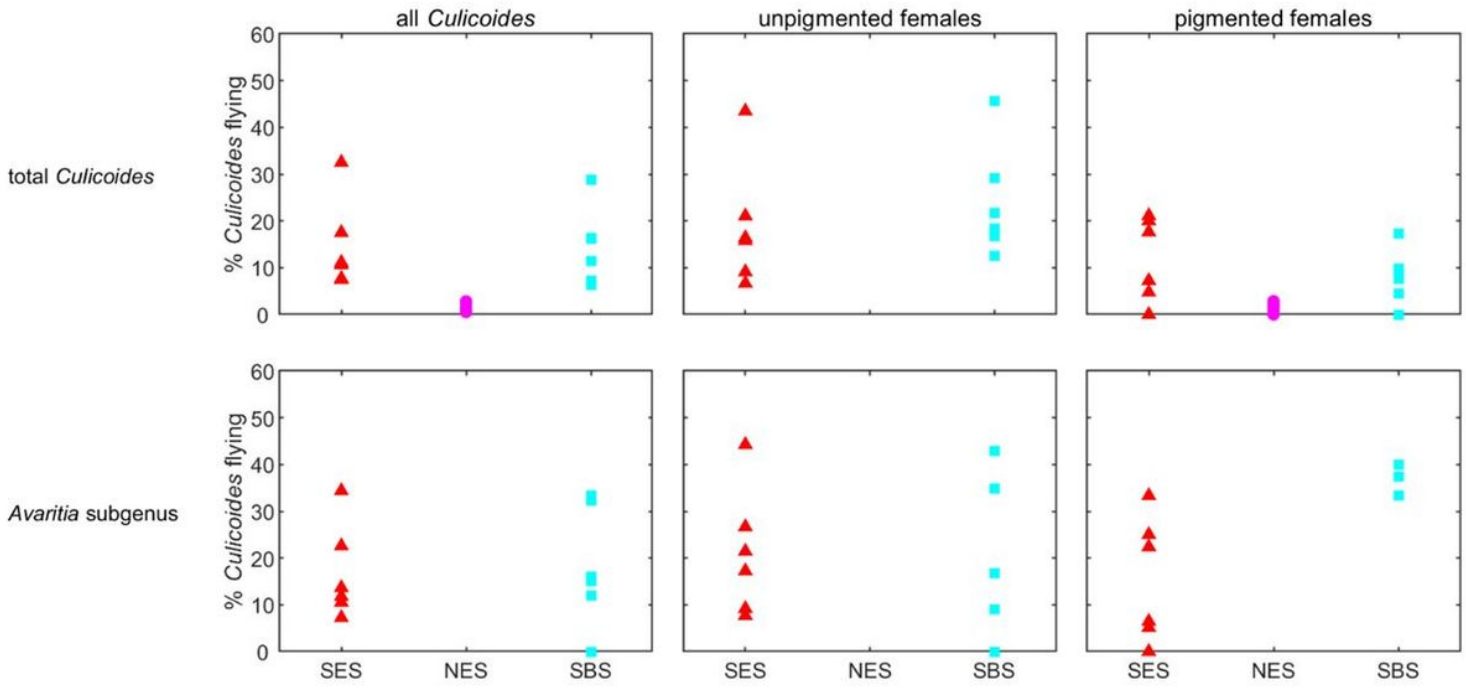

Figure 4

Percentage of Culicoides collected in the flight pill box after 24 hours at 12°C. Rows show results for total Culicoides (top row) and the Avaritia subgenus (bottom row). Columns show results for all Culicoides (left), unpigmented females (centre) and pigmented females (right). Symbols show the observed percentage of Culicoides flying, with cohorts indicated by the curve/symbol colour: SES (red); NES (magenta); and SBS (cyan). If data are not shown for a cohort, insufficient specimens of that species/group were caught for it to be included in the analysis.

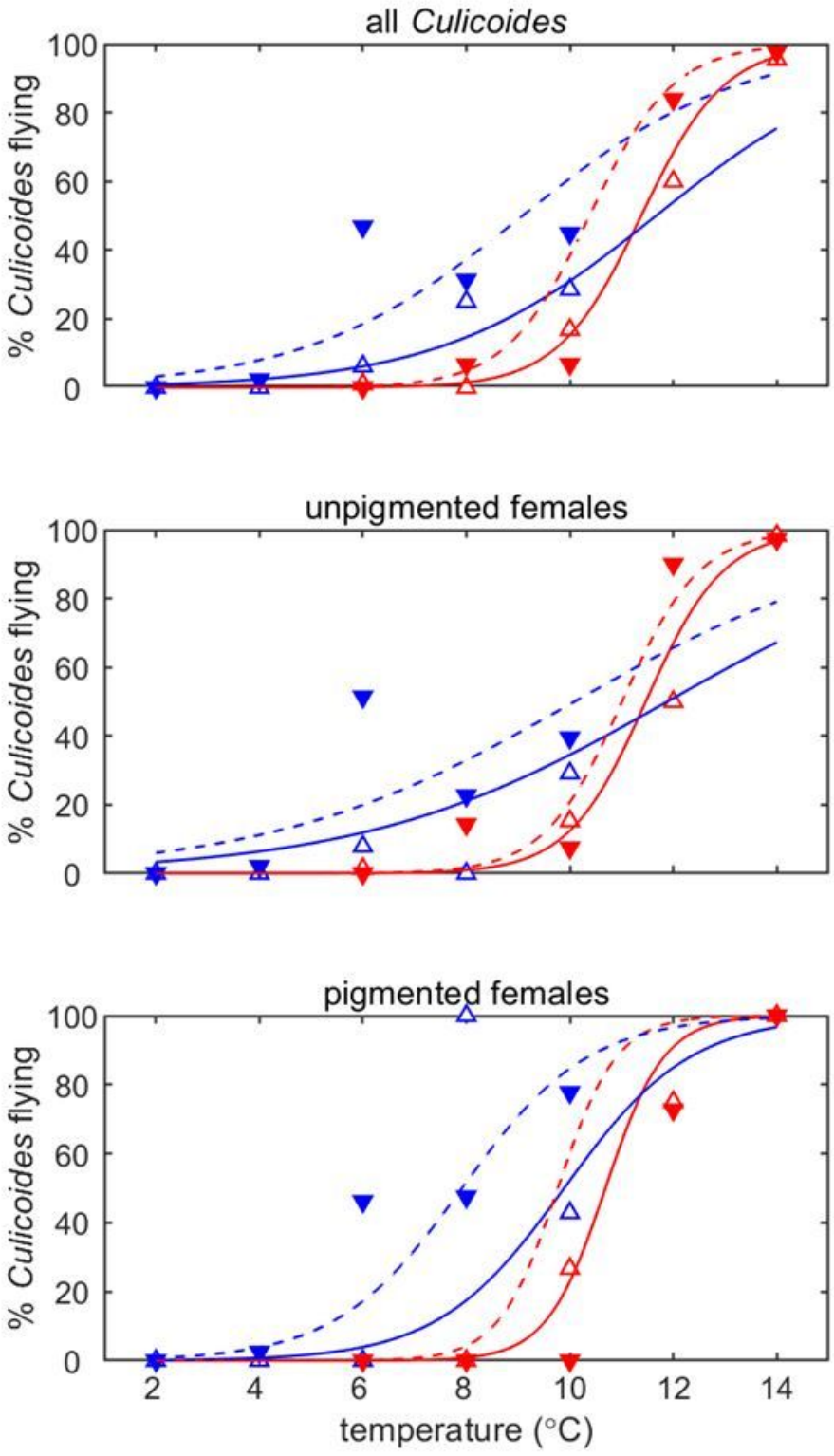

Figure 5

Percentage of Culicoides obsoletus and Culicoides scoticus collected in the flight pill box after 24 hours. Flight activity is shown for total Culicoides (top row), unpigmented females (middle row) and pigmented females (bottom row). Symbols show the observed percentage of midges flying and the curve is the fitted model for C. obsoletus (open triangles and solid lines) and C. scoticus (filled triangles and dashed lines). Cohorts are indicated by the curve/symbol colour: SES (red) and SEA (blue).

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