Ecological characterization of the tree hole habitats of Culicoides species (Diptera: Ceratopogonidae) and the potential vertical transmission of orbiviruses in these insects in southeastern Louisiana

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Abstract

Tree holes serve as important habitats for certain *Culicoides* species (biting midges: Diptera: Ceratopogonidae) that transmit epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) to ruminants. However, very little is known regarding the environmental characteristics influencing *Culicoides* emergence from these habitats. In this study, we characterized the tree hole habitats of *Culicoides* species and examined these insects for the potential vertical transmission of EHDV/BTV. Tree holes (*n* = 33) were surveyed at Bob R. Jones Idlewild Research Station, Louisiana, USA, and various physical/environmental characteristics of these habitats were recorded. In addition, substrate samples were incubated for adult insect emergence. The emerged *Culicoides* adults were identified and processed for EHDV/BTV detection using Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR). A total of 133 *Culicoides* adults emerged from the samples: *C. hinmani* (44.4%, 59/133), *C. guttipennis* (22.6%, 30/133), *C. arboricola* (18.0%, 24/133), *C. nanus* (7.5%, 10/133), *C. paraensis* (3.8%, 5/133), *C. debilipalpis* (3.0%, 4/133), and *C. beckae* (0.8%, 1/133). *Culicoides* species emerged from only 42.4% of the tree holes (14/33), with midge emergence being the highest from American elm (*Ulmus americana*) (12.0 ± 0.0 [mean ± SE]) and sweetgum (*Liquidambar styraciflua*) (6.4 ± 4.5), followed by Chinese privet (*Ligustrum sinense*) (4.5 ± 3.5) and water oak (*Quercus nigra*) (3.8 ± 2.0). The presence of standing water, tree species, tree hole direction, and tree hole orientation had a significant effect on the presence and abundance of *Culicoides* species. However, the environmental variables influencing midge abundance varied between *Culicoides* species. No *Culicoides* adults emerged were positive for EHDV/BTV. Our findings suggest that some tree holes are more productive midge habitats than others, likely reflecting a variation in their environmental characteristics. Vertical transmission of EHDV/BTV appears to be unlikely in tree hole *Culicoides* species. However, further studies across a larger spatial and temporal scale will be needed to ascertain the validity of these findings. Overall, our study provides new insight into the habitat characteristics of tree hole *Culicoides* species and provides valuable information towards the identification of productive midge habitats in Louisiana that can be targeted for biting midge control in/around livestock facilities.

Introduction

Culicoides species (Diptera: Ceratopogonidae), commonly referred to as biting midges, are blood-feeding insects distributed throughout the tropical and temperate regions of the world [1]. This genus is represented by over 1400 species with around 110 species occurring in the US [2]. Adult biting midge females seek blood meals from various vertebrate hosts, which can result in major annoyance, hypersensitivity reactions, and pathogen transmission to susceptible hosts. Among the several pathogens *Culicoides* species transmit, epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) (genus *Orbivirus*, family Sedoreoviridae) are of utmost importance to the US animal agriculture. These viruses cause hemorrhagic disease in sheep, cattle, white-tailed deer, and other ruminants, thereby exerting a significant economic impact in animal agriculture in the country [3]. In the United States, *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz are the confirmed vectors of BTV/EHDV [4–7]. However, *C. stellifer* Coquillett, *C. venustus* Hoffman, *C. debilipalpis* Lutz and other species may also be involved in virus transmission in the southeastern US [8, 9].

The immature stages of *Culicoides* species are usually found in various organically enriched habitats including the edges of ponds, streams, seepages, manure pats, and tree holes with the larvae feeding on resident microorganisms, detritus, and other small invertebrates [10, 11]. Much of our knowledge on the larval habitat ecology of *Culicoides* species, particularly of those involved in *Orbivirus* transmission in North America, arises

from studies on artificial wastewater ponds and various natural water bodies [12, 13]. However, the tree hole (or tree cavity) habitats of *Culicoides* species have received very little attention to date [14–16]. This is important because some midge species that develop in tree holes (often referred to as “tree hole species”), such as *C. debilipalpis*, are putative vectors of EHDV/BTV in the southeastern US [9, 17–19].

Arboviruses are maintained in nature mainly through horizontal transmission between infected arthropods and vertebrate hosts. However, during unfavorable conditions such as winter/dry seasons, some arboviruses can persist in the environment by being vertically transmitted from infected adult females to offspring or by surviving in the overwintering arthropod adults [20, 21]. In the U.S.A., hemorrhagic disease (EHDV/BTV) is endemic. However, very little is known regarding the overwintering mechanisms of EHDV/BTV in this region. Previous studies from California suggested that BTV can survive in long-lived *C. sonorensis* parous females infected the previous year [22]. More recent studies reported vertical transmission of EHDV in colonized *C. sonorensis* [23]. However, field evidence for vertical transmission is lacking. Interestingly, field-collected larvae of *C. sonorensis* and *C. crepuscularis* from Colorado were found to harbor BTV RNA [24], suggesting the possibility of vertical transmission of orbiviruses in some *Culicoides* species. Louisiana’s livestock sector contributes $2.5 billion to the states’ economy [25]. With BTV/EHDV exerting a significant impact on animal agriculture, a better understanding of the habitat ecology of *Culicoides* species associated with *Orbivirus* transmission will be beneficial towards identifying the productive habitats of these insects to be targeted for biting midge control. In this study, we examined the physical and environmental characteristics of tree holes associated with *Culicoides* species and examined these insects for the potential vertical transmission of orbiviruses in southeastern Louisiana.

Materials and Methods

This study was conducted at Bob R. Jones Idlewild Research Station, East Feliciana parish, Louisiana, USA from March to September of 2024 (Fig. 1a). Tree holes (*n* = 33) suspected to harbor *Culicoides* immatures (eggs, larvae, and pupae) were identified using manual surveys and drone-assisted surveillance of the landscape (Fig. 1b). Various physical and environmental parameters of the tree holes were recorded including the presence of standing water (wet [standing water present] or dry [standing water absent]), presence of moss (present or absent), tree hole direction (relative to the tree trunk), tree hole orientation (horizontal [tree hole opening parallel to the ground] or vertical [tree hole opening perpendicular to the ground]), tree species identity, height of tree hole from the ground, area of tree hole opening (longest diameter of tree hole was measured and area calculated as πr^2 [r = radius]), and distance of tree to nearest permanent water body (using satellite imagery from Google Earth Pro v.7.3.7.1155). From the wet tree holes (*n* = 13), ~ 50 mL of the standing water was collected into a conical flask using a turkey baster and its pH and temperature measurements were recorded (pHep HI98128, Hanna Instruments, Capitol Scientific, Inc., Austin, TX) (pH and temperature values of dry tree holes [*n* = 20] were not measured). In addition, ~ 100 g of the tree hole substrates were collected into Ziploc bags using a trowel, brought to the laboratory, and set up in Petri dishes (100 × 25mm) for adult insect emergence [13]. Water levels across all dishes were kept at a pre-determined level by adding tap water as needed. All dishes were incubated at 26 ± 1°C, 60–80% RH and 14:10 h (L:D) cycle. The emerged *Culicoides* adults were identified using morphological keys [10, 26] and were stored at -20°C for later processing. All *Culicoides* adults were processed individually for RNA extraction and subsequent detection of BTV/EHDV RNA using multiplex Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) [23, 27].

Statistical analysis

All numerical values except 'standing water pH' and 'standing water temperature' were log(x + 1) transformed to homogenize variance. The effects of various environmental variables on the presence and total abundance of *Culicoides* species were examined using generalized linear models (GLM) under binomial and Poisson (or negative binomial) error distributions respectively. Means that were significantly different were separated using Tukey's multiple pairwise comparisons test [28]. The variables 'standing water pH' and 'standing water temperature' were not measured from dry tree holes (*n* = 20). Therefore, comparisons of pH and temperature were made only between the wet tree holes with or without *Culicoides* species, using Wilcoxon rank-sum test. Co-occurrence patterns of *Culicoides* species were examined using a probabilistic species co-occurrence analysis model by treating each tree hole as a 'site' (package *cooccur*). The effects of environmental variables on the abundance of individual *Culicoides* species were examined using GLM or bias-reduced GLM (package *brglm2*) under Poisson error distributions. Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values were computed and compared between different models, and the models with the lowest values were selected as the best fit model. Variance inflation factors (VIF) were used to check for collinearities between the environmental variables with a value of > 10 indicating strong collinearity (Zuur et al. 2010, Harrell 20102). The variables 'tree hole direction', 'tree hole orientation', and 'tree identity' had high VIF values or presented model convergence issues when included; and thus, were excluded from the full model of all *Culicoides* species. The variables included in the full model for all species, except *C. nanus*, were 'presence of standing water', 'presence of moss', 'area of tree hole opening', 'height from ground', and 'distance to water'. For *C. nanus*, the variables in the full model included only 'presence of standing water', 'presence of moss', and 'height from ground'. From the full model of each *Culicoides* species, a set of candidate models were first generated using information-theoretic approach (functions *dredge* and *get.models* with no 'fixed' term specified; package *MuMin*). Subsequently, a subset of these candidate models were selected (delta AICc < 2) and averaged to compute the magnitude of effect and the sum of weights of the important variables influencing midge abundance (function *model.avg*; package *MuMin*). All statistical analyses were conducted using R v4.6.0. [31] using packages *MASS* [32], *car* [33], *lsmeans* [34], *cooccur* [35] and *MuMin* [36].

Results

A total of 33 tree holes were sampled belonging to 10 tree species. Most of the tree holes belonged to sweetgum (33.3%, 11/33) and water oak (33.3%, 11/33) followed by winged elm (9.1%, 3/33) and Chinese privet (6.1%, 2/33) (Fig. 1c). *Culicoides* species adults emerged from only 42.4% (14/33) of the tree holes. The tree species that harbored *Culicoides* species the most were American elm (100%, 1/1) and Chinese privet (100%, 2/2), followed by sweetgum (63.6%, 7/11) and water oak (36.4%, 4/11) (Fig. 1c). However, the mean (± SE) number of *Culicoides* species emerged per tree was the highest in American elm (12.0 ± 00) and sweetgum (6.4 ± 4.5), followed by Chinese privet (4.5 ± 3.5) and water oak (3.8 ± 2.0) (Table S1).

A total of 133 biting midge adults emerged from the samples belonging to seven species: *Culicoides hinmani* (44.4%, 59/133), *C. guttipennis* (22.6%, 30/133), *C. arboricola* (18.0%, 24/133), *C. nanus* (7.5%, 10/133), *C. paraensis* (3.8%, 5/133), *C. debilipalpis* (3.0%, 4/133), and *C. beckae* (0.8%, 1/133) (Fig. 1d). Most of the *Culicoides* species emerged mainly from sweetgum and water oak samples (84.2%, 112/133), except for *C. nanus* that emerged only from American elm (Fig. 2).

The presence of standing water had a significant effect on the presence and abundance of *Culicoides* species (Table 1), with wet tree holes ~ 2.5 times more likely to harbor *Culicoides* species than dry tree holes (Fig. 3a and 3b). However, Tukey's *posthoc* test showed that this difference was significant only for *C. guttipennis* and *C. arboricola*, and not for any other *Culicoides* species emerged (Fig. S1 and S2). The pH and temperature values were not measured from dry tree holes. As such, these values could not be compared between wet and dry tree holes. Nonetheless, the overall pH values of wet tree holes with *Culicoides* species (n = 9) were not significantly different from those without *Culicoides* species (n = 4) (W = 22, P = 0.604) (Table 2). However, the pH values of wet tree holes with *C. arboricola* (n = 6) were significantly lower (6.77 ± 0.27) than those without *C. arboricola* (n = 7) (7.38 ± 0.53) (W = 36, P = 0.035) (Table 2). These differences were not significant for the other *Culicoides* species (P > 0.05) (Table 2). Similarly, the overall temperature values of wet tree holes with *Culicoides* species were not significantly different from those without *Culicoides* species (W = 11, P = 0.308). However, the temperature values in wet tree holes with *C. arboricola* (n = 6) were significantly higher (27.88 ± 0.92) than those without *C. arboricola* (n = 7) (24.46 ± 1.50) (W = 6.5, P = 0.038) (Table 2). In addition, the temperature values in wet tree holes with *C. paraensis* (n = 2) were also significantly higher (30.10 ± 0.10) than those without *C. paraensis* (n = 11) (25.30 ± 1.04) (W = 0, P = 0.026) (Table 2). Temperature differences were not significant for the other *Culicoides* species (P > 0.05) (Table 2).

Tree species had a significant overall effect on the presence and abundance of *Culicoides* species (Table 1), with American elm and Chinese privet more likely to harbor biting midges, followed by sweetgum and water oak (Fig. 3c and 3d). However, Tukey's *post-hoc* test did not detect significant differences in *Culicoides* presence/abundance between the tree species. Notably, the number of American elm (n = 1) and Chinese privet (n = 2) trees sampled in the study was very low, compared to sweetgum (n = 11) and water oak (n =11). As such, the high likelihood of American elm and Chinese privet trees harboring *Culicoides* could be an artifact of their low sample size examined. Tree hole direction had a significant effect on the presence and abundance of *Culicoides* species (Table 1), with tree holes facing the North-East more likely to harbor *Culicoides* species than those facing the other directions (Fig. 4a and 4b). However, Tukey's *post-hoc* test did not detect significant differences in *Culicoides* presence/abundance between the different directions. Tree hole orientation was another environmental variable that had a significant effect on the presence and abundance of *Culicoides* species (Table 1), with tree holes oriented horizontally (tree hole opening parallel to the ground) > 2 times more likely to harbor these insects than those oriented vertically (tree hole opening perpendicular to the ground) (Fig. 4c and 4d). The presence of moss, area of tree hole opening, height from ground, and distance to water had no significant effect on the presence and total abundance of *Culicoides* species (Table 1).

Several *Culicoides* species co-occurred in the tree holes (Table S2). However, all species pair co-occurrences (n = 21) were found to be random and no positive or negative associations were detected (Fig. S3).

Associations between *Culicoides* species and environmental variables

The total number of models generated from the full model for *C. hinmani* were 32, out of which 3 were ranked delta AICc < 2 (Table S3). Model averaging showed that *C. hinmani* abundance in the tree holes was positively associated with the 'area of tree hole opening', 'presence of moss', 'distance to water', and 'presence of standing water' (Table 3). For *C. guttipennis*, the total number of models generated were 32, out of which 4 were ranked delta AICc < 2 (Table S3). Model averaging showed that the abundance of *C. guttipennis* was positively associated with 'height from ground' and 'presence of standing water', but negatively associated with 'presence

of moss' (Table 3). The total number of models generated from the full model for *C. arboricola* were 32, out of which 2 were ranked delta AICc < 2 (Table S3). Model averaging showed that *C. arboricola* abundance in the tree holes was positively associated with 'presence of standing water' and 'area of tree hole opening' (Table 3). For *C. nanus*, the total number of models generated were 8 out of which 7 were ranked delta AICc < 2 (Table S3). Model averaging showed that the abundance of *C. nanus* was positively associated with 'height from ground' and 'presence of standing water' and negatively associated with 'presence of moss' (Table 3). The total number of models generated from the full model for *C. paraensis* were 32, out of which 4 were ranked delta AICc < 2 (Table S3). Model averaging showed that *C. paraensis* abundance was positively associated with 'presence of moss' and negatively associated with the 'area of tree hole opening' (Table 3). For *C. debilipalpis*, the total number of models generated were 32, out of which 5 were ranked delta AICc < 2 (Table S3). Model averaging showed that the abundance of *C. debilipalpis* in the tree hole was positively associated with 'presence of moss' and 'presence of standing water' and negatively associated with 'height from ground' and 'distance to water' (Table 3). Finally, the total number of models generated from the full model for *C. beckae* were 32, out of which 4 were ranked delta AICc < 2 (Table S3). Model averaging showed that the abundance of *C. beckae* was positively associated with 'presence of standing water' and 'presence of moss' in the tree hole and negatively associated with 'distance to water' (Table 3). However, the relative importance of different environmental variables and their magnitude of effect on midge abundance varied between *Culicoides* species (Table 3).

EHDV/BTV detections from emerged *Culicoides* species

All *Culicoides* species that emerged from the samples were negative for EHDV/BTV through RT-qPCR.

Discussion

Overall, our findings suggest that not all tree holes serve as habitats for *Culicoides* species. Some tree holes are more suitable than others for the oviposition and/or larval development of *Culicoides* species, likely reflecting a variation in their biotic and/or abiotic characteristics. The presence of standing water in the tree holes had a significant effect on *Culicoides* presence and abundance, which was not unexpected. The immature stages of *Culicoides* species are sensitive to desiccation and require moisture for survival [37, 38]. As such, the presence of standing water likely provides the moisture levels required for *Culicoides* larval survival. However, *Culicoides* species emerged from 25.0% (5/20) of the dry tree holes as well. It is likely that these dry tree hole substrates, although lacking standing water, had enough moisture levels to support the oviposition/larval development of *Culicoides* species. The minimum threshold level of moisture required for the survival of *Culicoides* immatures is currently unknown. Further studies will be needed to determine the minimum threshold moisture levels required to support the life history traits of *Culicoides* species and to identify other important factors associated with midge emergence. Previous studies classified *C. guttipennis* and *C. arboricola* as wet tree hole species (emerging mainly from tree holes with standing water) whereas *C. hinmani*, *C. nanus*, *C. paraensis*, and *C. debilipalpis* (reported as *C. lahillei*) were classified as dry tree hole species (emerging mainly from tree holes without standing water) [14, 15]. Our findings in the current study are consistent with *C. guttipennis* and *C. arboricola* to be emerging mainly from wet tree holes (Fig. S1 and S2). However, we found no evidence for *C. hinmani*, *C. nanus*, *C. paraensis*, and *C. debilipalpis* to be dry tree hole species as their emergence was not significantly different between the wet and dry tree holes (Fig. S1 and S2). Previous studies suggested that pH differences between wet and dry tree holes could be important in the segregation or resource partitioning strategies of *Culicoides* species [14, 15]. In the current study, pH and temperature levels in the dry tree holes

were not measured and thus, could not be compared against those in the wet tree holes. However, standing water pH and/or temperature in the wet tree holes appear to be important for the emergence of *C. arboricola* and *C. paraensis*, but not for the other *Culicoides* species (Table 2). These findings collectively suggest that the habitat selection of tree hole biting midges varies between *Culicoides* species and likely relies on multiple factors.

Tree species identity had a significant overall effect on *Culicoides* presence and abundance with the highest number of midge adults per tree emerging from American elm and sweetgum followed by Chinese privet, and water oak (Table S1). Notably, *C. debilipalpis*, a putative vector of EHDV/BTV, emerged only from sweetgum samples (100.0%, 4/4) (Table S2). As such, these findings suggest that certain tree species may play an important role in the biology/ecology of *Culicoides* species. Interestingly, there was no *Culicoides* emergence from winged elm and pecan tree hole samples in the current study. However, these trees were reported to have relatively higher numbers of *Culicoides* emergence in previous studies [16]. It is likely that these differences are due to the relatively small sample size of tree holes examined from one geographic location in the current study. In addition, each of the tree holes were sampled only once, with the sampling period spread across different seasons from March to September of 2024. As such, it is currently unknown whether the tree holes found without midge emergence in the current study were inhabited by *Culicoides* species before/after the sampling event or not, as there could be a seasonal component to the resource partitioning strategies of *Culicoides* species [14]. Therefore, further studies incorporating monthly collections of the tree hole samples will likely be needed to ascertain the validity of these findings. Nonetheless, it is possible that the cavities of certain tree species are more attractive for *Culicoides* gravid females and/or are more supportive for the larval development of biting midges. Further studies examining the associations between tree species and tree hole *Culicoides* species may provide valuable insight into their ecology that can be potentially exploited for biting midge control. Previously, sand flies (Diptera: Phlebotominae) were reported to show strong associations with certain tree species in South America [39, 40].

Tree hole direction was found to have a significant effect on *Culicoides* presence and abundance with most adult midges emerging from tree holes facing the North-East followed by those oriented towards the South-West. Currently, the reasons behind this phenomenon are unknown. However, it is possible that the tree holes facing the axis passing through North-East and South-West receive optimal dawn/dusk light, temperature, wind, and/or other environmental conditions favorable for *Culicoides* oviposition and/or larval development. Further studies will be needed to examine the importance of tree hole direction in influencing the life history traits of *Culicoides* species. Tree hole orientation was the other variable found to have a significant effect on *Culicoides* presence and abundance with most adults emerging from tree holes oriented horizontally. It is likely that the tree holes oriented horizontally collect more water and organic material than vertically oriented tree holes resulting in better nutritional and environmental conditions for *Culicoides* species.

All co-occurrence patterns of *Culicoides* species pairs in the tree holes were found to be random. These findings suggest that these *Culicoides* species are distributed independently of other species in the tree holes, with no evidence for any direct or indirect correlation between these midge species. However, this does not necessarily mean that *Culicoides* species are distributed randomly in the environment. The gravid females likely choose habitats that are more attractive and possibly result in optimal survival traits in the offspring [41, 42]. Previously, several mosquito species have been reported to exhibit strong co-occurrence patterns in Florida [43].

The key environmental variables important for *Culicoides* species abundance, in general, were 'presence of standing water', 'presence of moss', 'area of tree hole opening', 'height from ground', and 'distance to water'. However, the relative importance of these variables and their magnitude of effect varied between *Culicoides* species. The variable 'presence of standing water' was important for all species except *C. paraensis* and showed a positive correlation with midge abundance, highlighting the importance of moisture levels on *Culicoides* larval survival. The variable 'presence of moss' was important for all species except *C. arboricola*. The presence of moss had a positive effect on four species (*C. hinmani*, *C. paraensis*, *C. debilipalpis*, and *C. beckae*) and a negative effect on two species (*C. guttipennis*, and *C. nanus*). Currently, the role of moss in influencing the life history traits of tree hole *Culicoides* species is unknown. However, previous studies on ground dwelling species showed that moss from the larval habitat elicited a strong oviposition response in *C. stellifer* and *C. impunctatus* under laboratory conditions [41, 44]. Moreover, several *Culicoides* species were found to show strong spatial correlations with certain types of vegetation in the field [10, 45–47]. Whether moss in the tree holes serves as an important oviposition cue for *Culicoides* species is currently unknown. Further studies will be needed to experimentally evaluate the functional role of moss on the oviposition and larval development of tree hole *Culicoides* species. The other important variables 'area of tree hole opening', 'height from ground', and 'distance to water' were important for three species each, showing positive relationships with some midge species and negative relationships with others. The role of these environmental factors in influencing the life history traits of *Culicoides* species is currently unknown. Further studies will be needed to determine their importance in influencing the habitat associations of tree hole biting midges. Further studies will also be needed to characterize other biotic/abiotic factors of the tree holes such as moisture levels of the substrate, organic matter, nutrients, microbial communities, and other physicochemical properties to gain a better understanding of the habitat requirements of *Culicoides* species.

Several *Culicoides* species that emerged from the samples are of high medical and veterinary importance in North America. *Culicoides debilipalpis* is widely distributed from the southeastern US extending all the way to Mexico, Central America, and Argentina [10]. This species feeds heavily on white-tailed deer [48], can support EHDV/BTV infections under laboratory conditions [17, 49], and is one of the highly abundant species during disease outbreaks in the southeastern US [9, 18], making *C. debilipalpis* a strong candidate species for EHDV/BTV transmission, particularly in the southeastern US. Moreover, this species is also a vector of *Mansonella* spp. filarial nematodes affecting humans in South America [50]. *Culicoides paraensis* is widely distributed from the northeastern US to Mexico, Central America, and Argentina [10]. This species is a confirmed vector of Oropouche virus (OROV) and *Mansonella* spp. filarial nematodes affecting humans, which are endemic mainly in South America, Central America, and some islands of the Caribbean. In the recent years, OROV has expanded beyond its native range and has caused outbreaks in several countries, including Haiti, Cuba, and the US, increasing concern for public health [51, 52]. *Culicoides arboricola* is distributed primarily in the southern and eastern US [10]. This species mainly bites birds and plays a secondary role in the transmission of *Haemoproteus* spp. protozoan parasites to domestic turkeys and other bird species [3, 53]. Moreover, field collected individuals of *C. arboricola* from Louisiana were found to be positive for West Nile virus [54]. *Culicoides hinmani* is distributed mainly in the eastern US where it also plays a secondary role in the transmission of *Haemoproteus* spp. to domestic turkeys and other birds [3, 10]. Given their overall importance, further studies on the biology, ecology, and control of tree hole *Culicoides* species are warranted.

Orbiviruses were not detected in any of the emerged *Culicoides* species in the current study, suggesting that vertical transmission of EHDV/BTV is unlikely in tree hole *Culicoides* species. However, it should be noted that

the sample size of biting midges examined in this study, particularly for *C. debilipalpis*, was very low (*n* = 4). As such, these conclusions should be interpreted cautiously. Further studies across a larger spatial and temporal scale will be needed to ascertain the validity of our conclusions. Further studies will also be needed to examine the potential vertical transmission of EHDV/BTV in other important ground dwelling midge species such as *C. sonorensis*, *C. stellifer*, *C. venustus*, and *C. insignis*, which may provide valuable clues towards the endemic nature of hemorrhagic disease and the overwintering mechanisms of EHDV/BTV in North America.

Conclusions

Overall, our study suggests that some tree holes are more productive *Culicoides* habitats than others, which likely reflects a variation in their environmental characteristics. The presence of standing water, pH, temperature, tree species, tree hole direction, and tree hole orientation, in general, influenced the emergence of *Culicoides* species from these habitats. However, the environmental variables important for midge abundance varied between individual *Culicoides* species. Further studies will be needed to experimentally evaluate the importance of these variables in influencing the oviposition/larval development of tree hole *Culicoides* species. Collectively, our findings provide new insight into the ecological characteristics of tree hole *Culicoides* species and provide valuable information towards the identification of productive midge habitats in southeastern Louisiana. These habitats can eventually serve as foci for establishing effective *Culicoides* control strategies in/around livestock facilities in the state. Vertical transmission of EHDV/BTV appears to be unlikely in tree hole *Culicoides* species. However, further studies across a larger spatial and temporal scale will be needed, particularly for *C. debilipalpis*, to ascertain the validity of our conclusions.

Abbreviations

EHDV: epizootic hemorrhagic disease virus; BTV: bluetongue virus; L:D: light:dark; RH: relative humidity.

Declarations

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Author contributions

DE and LDF conceptualized and designed the study. DZ and DE collected the data. DE wrote the manuscript. LDF was deceased before manuscript was written. All other authors approved the final manuscript.

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Availability of data and materials

All data generated and analyzed are included in this article and its supplementary files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

None.

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Tables

Table 1. Summary of the effects of various environmental variables on the presence and abundance of tree hole *Culicoides* species.

Environmental variable	*Culicoides* occurrence	*Culicoides* abundance
Presence of standing water	$LR\chi^2$ = 6.4, df = 1, ***P* = 0.011**	$LR\chi^2$ = 4.3, df = 1, ***P* = 0.037**
Presence of moss	$LR\chi^2$ = 0.3, df = 1, *P* = 0.5783	$LR\chi^2$ = 0.1, df = 1, *P* = 0.758
Tree identity	$LR\chi^2$ = 16.1, df = 9, ***P* = 0.064**	$LR\chi^2$ = 18.3, df = 9, ***P* = 0.031**
Tree hole direction	$LR\chi^2$ = 13.9, df = 7, ***P* = 0.053**	$LR\chi^2$ = 19.2, df = 7, ***P* = 0.007**
Tree hole orientation	$LR\chi^2$ = 11.4, df = 1, ***P* = 0.001**	$LR\chi^2$ = 3.3, df = 1, ***P* = 0.068**
Height from ground	$LR\chi^2$ = 1.7, df = 1, *P* = 0.187	$LR\chi^2$ = 0.2, df = 1, *P* = 0.642
Area of tree hole opening	$LR\chi^2$ = 0.9, df = 1, *P* = 0.345	$LR\chi^2$ = 0.4, df = 1, *P* = 0.520
Distance to water	$LR\chi^2$ = 0.1, df = 1, *P* = 0.769	$LR\chi^2$ = 0.1, df = 1, *P* = 0.783

Table 2. Standing water pH and temperature values (mean ± SE) in the wet tree holes of *Culicoides* species.

	Standing water pH		Standing water temperature	
	Emerged	Not emerged	Emerged	Not emerged
Total *Culicoides*	7.16 ± 0.29a (9)	6.98 ± 0.87a (4)	26.93 ± 0.76a (9)	24.03 ± 2.79a (4)
C. hinmani	7.97 ± 0.86a (2)	6.94 ± 0.33a (11)	27.60 ± 2.60a (2)	25.75 ± 1.13a (11)
C. guttipennis	7.22 ± 0.45a (6)	7.00 ± 0.47a (7)	26.43 ± 0.90a (6)	25.70 ± 1.78a (7)
C. arboricola	6.77 ± 0.27a (6)	7.38 ± 0.53b (7)	27.88 ± 0.92a (6)	24.46 ± 1.50b (7)
C. nanus	8.83 ± 0.00a (1)	6.96 ± 0.30a (12)	25.00 ± 0.00a (1)	26.13 ± 1.09a (12)
C. paraensis	7.08 ± 0.04a (2)	7.11 ± 0.37a (11)	30.10 ± 0.10a (2)	25.30 ± 1.04b (11)
C. debilipalpis	7.04 ± 0.00a (1)	7.11 ± 0.34a (12)	30.00 ± 0.00a (1)	25.71 ± 1.04a (12)
C. beckae	7.10 ± 0.00a (1)	7.10 ± 0.34a (12)	25.10 ± 0.00a (1)	26.12 ± 1.09a (12)

Letters following the means represent significant differences between the '*Culicoides* emerged' and 'not emerged' groups (Wilcoxon rank-sum test) ($P < 0.05$). Values in parentheses following the means represent the number of tree holes sampled.

Table 3. Model averaged summary (full-average) showing the effect of various environmental variables on the abundance of *Culicoides* species.

Species	Environmental variable	Estimate	Standard Error (SE)	Adjusted SE	z value	Pr (> |z|)	Sum of weights	N containing models
C. hinmani	(Intercept)	-9.75	3.67	3.81	2.56	0.011		
	Area of tree hole opening	1.00	0.51	0.53	1.89	0.059	1.00	3
	Moss present: yes	2.87	1.20	1.25	2.29	0.022	1.00	3
	Distance to water	0.12	0.32	0.33	0.36	0.716	0.25	1
	Standing water present: yes	0.17	0.56	0.58	0.30	0.764	0.21	1
C. guttipennis	(Intercept)	-4.54	1.65	1.72	2.65	0.008		
	Height from ground	0.87	0.39	0.41	2.14	0.032	1.00	4
	Standing water present: yes	0.58	0.74	0.75	0.77	0.442	0.54	2
	Moss present: yes	-0.23	0.52	0.53	0.43	0.670	0.33	2
C. arboricola	(Intercept)	-3.59	2.21	2.27	1.59	0.113		
	Standing water present: yes	1.95	0.83	0.86	2.27	0.023	1.00	2
	Area of tree hole opening	0.22	0.35	0.35	0.62	0.537	0.43	1
C. nanus	(Intercept)	-4.99	3.57	3.66	1.37	0.172		
	Height from ground	0.59	0.86	0.88	0.67	0.504	0.47	3
	Standing water present: yes	0.79	1.35	1.38	0.58	0.565	0.42	3
	Moss present: yes	-0.52	1.13	1.16	0.45	0.656	0.33	3

C. paraensis	(Intercept)	1.15	3.79	3.84	0.29	0.765		
	Area of tree hole opening	-0.83	0.79	0.81	1.03	0.302	0.65	2
	Moss present: yes	1.03	1.41	1.44	0.71	0.476	0.56	2
C. debilipalpis	(Intercept)	-1.43	2.26	2.31	0.62	0.537		
	Height from ground	-0.35	0.63	0.65	0.54	0.589	0.36	2
	Moss present: yes	0.33	0.96	0.98	0.34	0.734	0.22	1
	Distance to water	-0.08	0.31	0.32	0.25	0.807	0.13	1
	Standing water present: yes	0.17	0.65	0.67	0.26	0.795	0.11	1
C. beckae	(Intercept)	-2.51	2.52	2.58	0.97	0.330		
	Standing water present: yes	0.33	0.98	1.01	0.33	0.745	0.21	1
	Distance to water	-0.16	0.50	0.52	0.32	0.751	0.19	1
	Moss present: yes	0.17	0.76	0.79	0.22	0.829	0.16	1

Figures

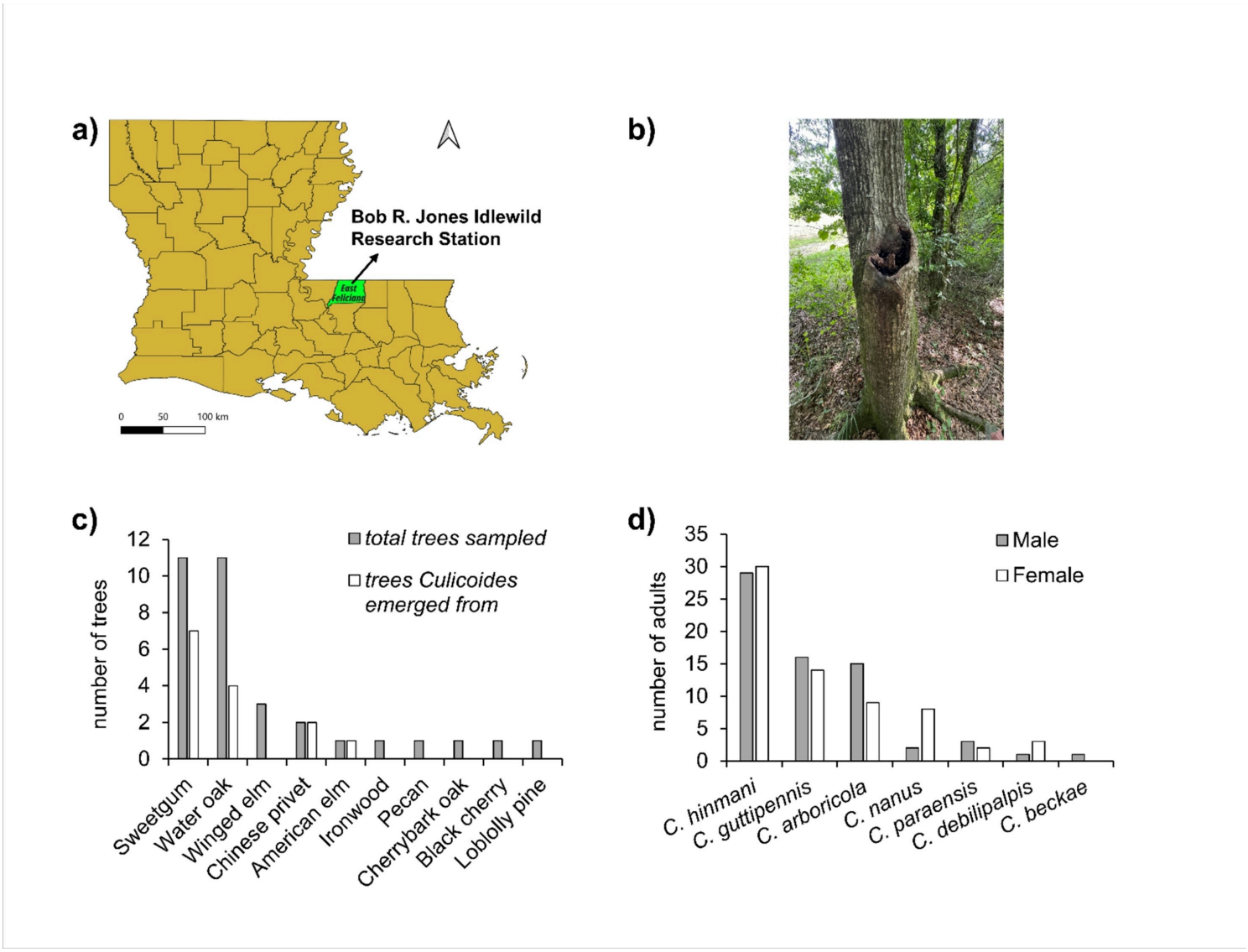

Figure 1

a) Location of Bob R. Jones Idlewild Research Station in East Feliciana parish, Louisiana, USA, b) A representative tree hole habitat of *Culicoides* species, c) Total number of tree holes *Culicoides* species emerged from, and d) Total emergence of *Culicoides* species from the samples, by sex.

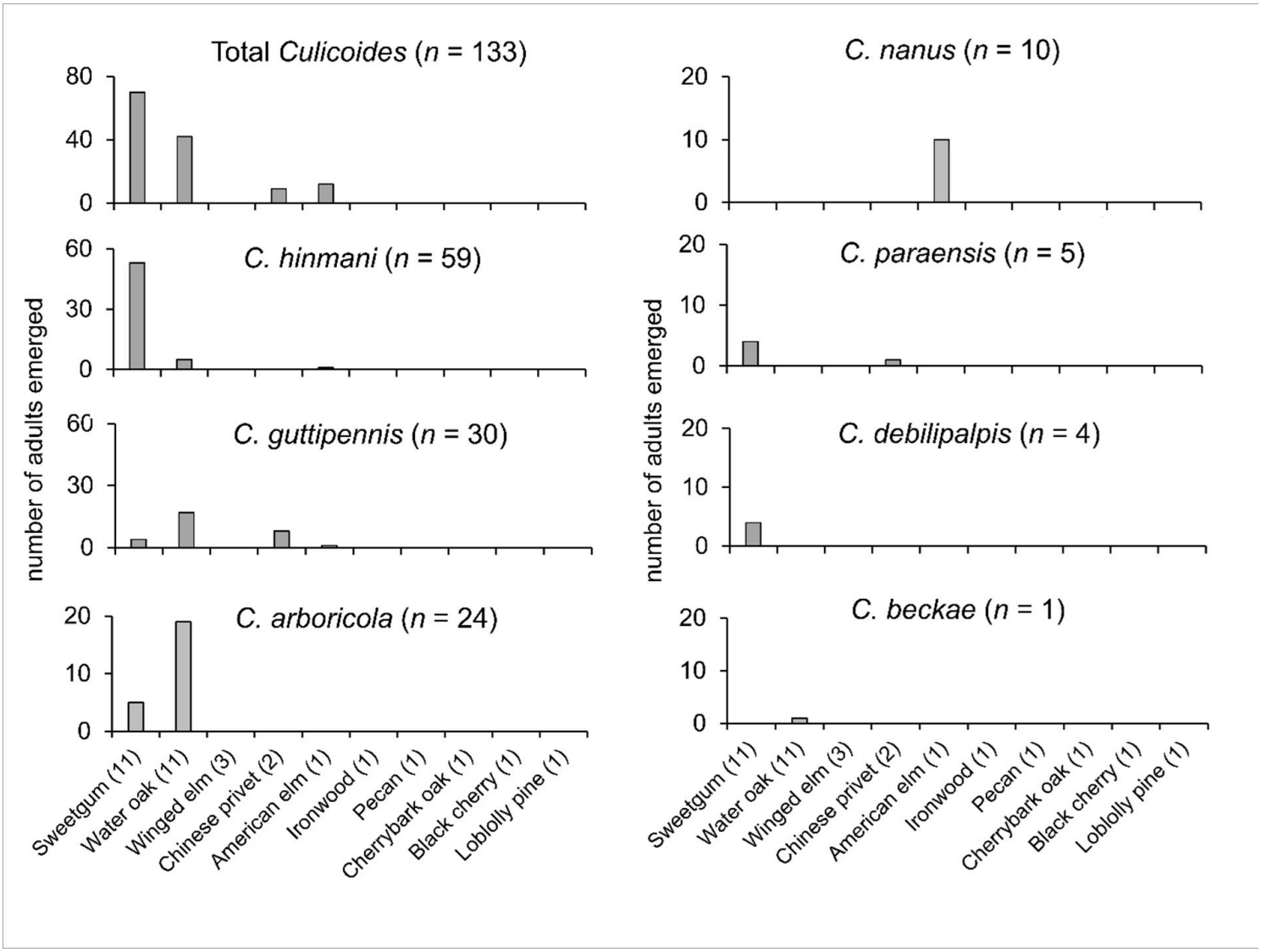

Figure 2

The emergence of *Culicoides*species from different tree species. Values in parentheses along x-axis represent the number of tree holes sampled.

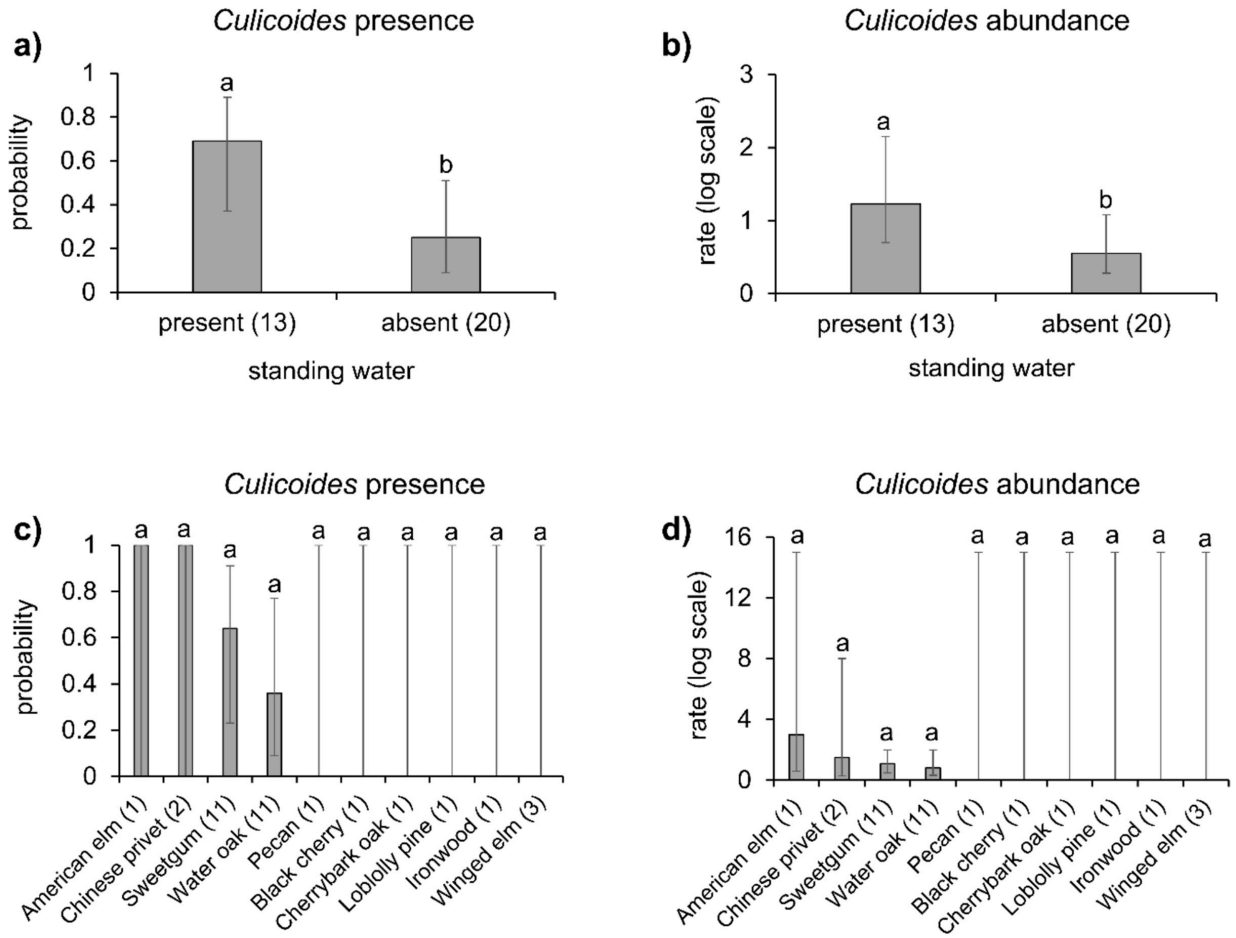

Figure 3

The effect of standing water on the, a) presence, and b) abundance of total *Culicoides* species. The effect of tree species identity on the, c) presence, and d) abundance of total *Culicoides* species. Values in parentheses along x-axis represent the number of tree holes sampled.

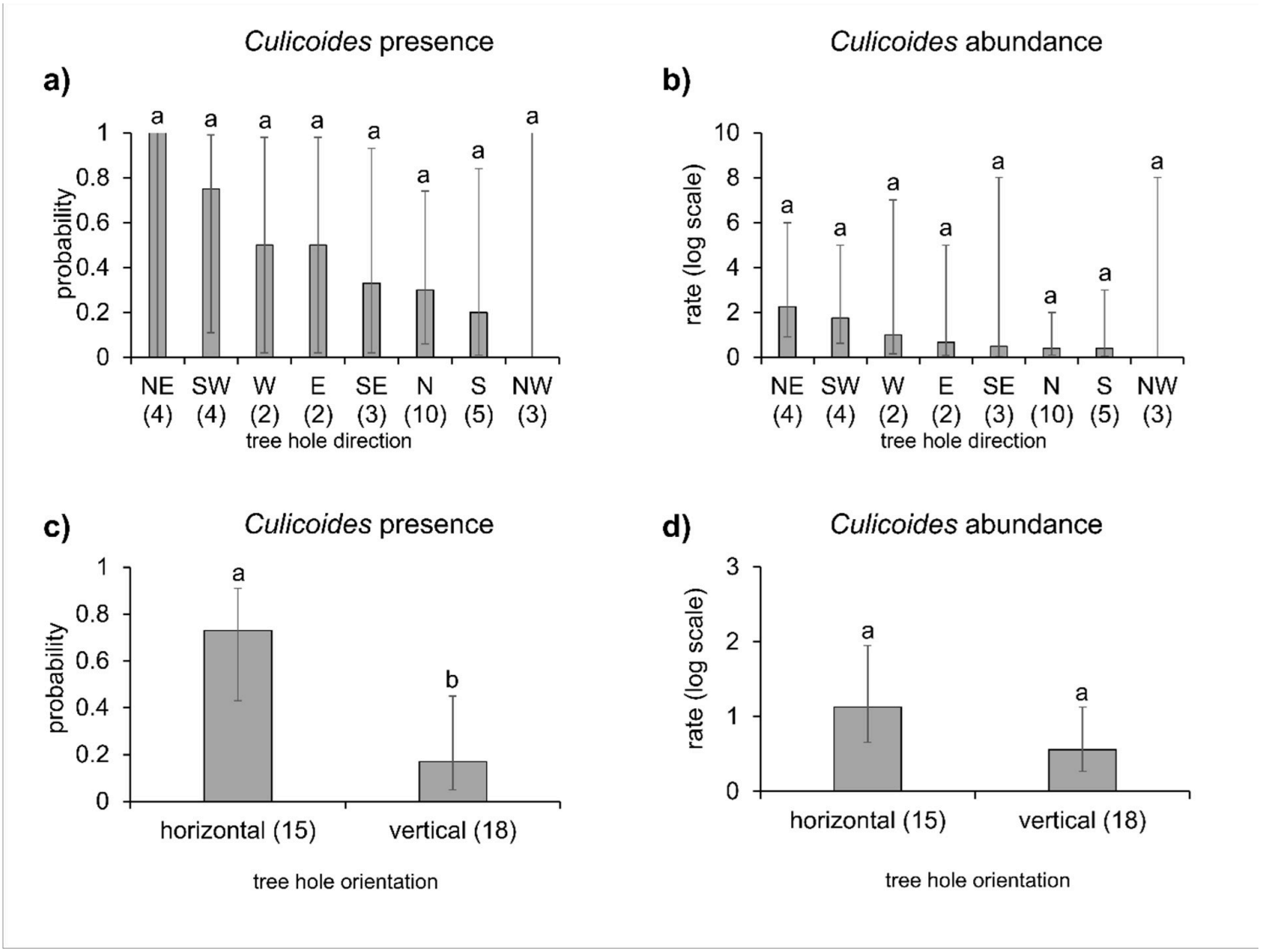

Figure 4

The effect of tree hole direction on the, a) presence, and b) abundance of total *Culicoides*species. The effect of tree hole orientation on the, c) presence, and d) abundance of total *Culicoides* species. Values in parentheses along x-axis represent the number of tree holes sampled.

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