

Oviposition and Larval Development of *Culicoides Insignis* Lutz (Diptera: Ceratopogonidae) under Laboratory Conditions

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Research

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Abstract

Background

Culicoides insignis Lutz (Diptera: Ceratopogonidae) is a confirmed vector of bluetongue virus (BTV) throughout the American tropics and a possible vector of epizootic hemorrhagic disease virus (EHDV) in Florida. Despite its importance, fundamental information on the biology and ecology of this species is lacking. In this study, we examined the oviposition and larval development of *C. insignis* under laboratory conditions and attempted colonization of this species.

Methods

Live *C. insignis* females were collected from the field using CDC-UV-LED traps, allowed to blood-feed on live chicken, and given various natural substrates for oviposition in two-choice assays. The eggs deposited were transferred to 0.3% agar slants and the hatched larvae were provided a diet of *Panagrellus redivivus* Linnaeus nematodes.

Results

Culicoides insignis females exhibited an overall oviposition preference for dishes containing mud from their larval habitat as gravid females deposited a significantly higher number of eggs on these dishes (35.3 eggs/female) than on deionized water (DI) substrates (17.7eggs/female). The ovipositing females also deposited a higher percentage of eggs on substrates with habitat mud and other organically enriched muds ($\geq 75.2\%$) compared to DI substrates (31.0%). The larvae developed successfully to adulthood on the nematode diet, exhibiting high overall larval survival rates (85.0%). Sex-ratios of the F1 generation were male biased $\sim 3:1$ (M:F). Captive mating could not be induced in the F1 adults.

Conclusions

Mud from the larval habitat and other organically enriched muds provide strong oviposition cues to *C. insignis*. Further studies will be needed to examine whether these cues are olfactory/tactile in nature. Further studies will also be needed to characterize the larval habitat of *C. insignis* and identify the key biotic/abiotic factors influencing midge oviposition in the field. This information, in the long term, can be potentially exploited to discourage the oviposition of *C. insignis* in local habitats. The agar/nematode method is effective for the rearing of *C. insignis* larvae. However, further studies will be needed to address the issue of male-biased sex-ratios in the progeny. Further studies will also be needed to examine the mating habits/cues of *C. insignis* in nature, which may provide clues towards inducing captive mating in the F1 adults.

Background

Culicoides Latreille species (Diptera: Ceratopogonidae) transmit orbiviruses such as bluetongue virus (BTV) and epizootic hemorrhagic disease viruses (EHDV) to ruminants, exerting a significant negative

impact on animal farming industries worldwide [1, 2]. Despite their importance, very little is known regarding the biology/ecology of *Culicoides* species associated with *Orbivirus* transmission to date. In the United States (US), the confirmed vectors of BTV/EHDV are *Culicoides sonorensis* Wirth and Jones and *Culicoides insignis* Lutz [3–5]. However, other species may also be involved in virus transmission. For example, *Culicoides stellifer* Coquillett, *Culicoides venustus* Hoffman, and *Culicoides debilipalpis* Lutz are partially incriminated in *Orbivirus* transmission in the southeastern US [6, 7]. In general, very few *Culicoides* species have been fully incriminated in BTV/EHDV transmission, mainly because laboratory infection/transmission studies are essential criteria for vector incrimination. However, due to lack of *Culicoides* colonies, such studies are difficult to accomplish.

Much of our knowledge on the larval ecology of *Culicoides* vectors in North America comes from studies on *C. sonorensis*, a midge species best studied in the artificial wastewater ponds of California and also the only confirmed vector of BTV/EHDV successfully colonized to date worldwide [8–13]. As such, basic research on the biology/ecology and successful laboratory colonization of *C. insignis* and other *Culicoides* species associated with virus transmission in the southeastern US will be highly advantageous in understanding the transmission dynamics of BTV/EHDV in this region and will also be useful in the development of effective vector/disease management strategies in the long term. In this study, we examined the oviposition preferences and larval development traits of *C. insignis* under laboratory conditions and attempted colonization of this species. *Culicoides insignis* is a common vector of orbiviruses distributed across most of South America, Central America, and the Caribbean, with its northern boundary extending into Florida and the neighboring states [14, 15]. This species, 1) is frequently associated with livestock facilities [16–18], 2) is a significant biting pest of livestock [19, 20], and 3) is a confirmed vector of BTV in Florida and also likely plays a role in EHDV transmission in the state [5, 21]. Overall, our study demonstrates effective methods for the blood-feeding, oviposition, and egg collection of *C. insignis*, and provides the first insight into the oviposition preferences and larval development traits of this species. Unfortunately, male-biased sex-ratios in the progeny and difficulties in inducing captive mating in the F1 generation prevented successful colonization of this species.

Methods

Live midge collection

Live midges were collected using procedures described previously for *C. stellifer* [22]. Briefly, CDC miniature light traps fitted with UV-LED arrays and insect collection containers were set up overnight at the Archbold Biological Station's Buck Island Ranch, Lake Placid, FL, USA (27°9'16.4"N, 81°11'51.7"W). The field-collected insects were brought to the laboratory the next morning where *C. insignis* females were anaesthetized using triethylamine (TEA), morphologically identified, caged in paper cups, and provided with 10% sucrose until use.

Oviposition studies

Culicoides insignis females were starved for 6 – 12 hours after which were introduced into 50 ml conical tube feeding chambers and allowed to blood-feed on the breast of a live chicken (University of Florida IACUC protocol #7682). The partially and fully engorged females were sorted under TEA anesthesia, placed individually in paper cups provided with two different substrates for oviposition (two-choice assays) and allowed to oviposit for 14 days. The oviposition dishes were checked daily, and the number of eggs deposited on them were counted. After the end of the two-week period or after midge death, females were dissected and the number of eggs retained, if any, were also counted. The females were provided with fresh cotton pads dampened with 10% sucrose solution daily. Each oviposition dish consisted of about 1g of natural substrate (see below) placed on the bottom of a Petri dish (35 × 10 mm) and was covered with a layer of cotton and a filter paper on the top. Moisture levels across all dishes was maintained constant by adding 1.5ml deionized water and replenishing with the same volume of water in both dishes presented to midges whenever necessary. The natural sources tested for oviposition preferences were selected based on personal observations and published reports [18,23]. The larval habitat of *C. insignis* was identified on Archbold Biological Station's Buck Island Ranch, Lake Placid, FL, USA through emergence sampling from this site. Surface mud samples (top few centimeters) along the waterline from this site were collected into Ziploc bags using a trowel. Non-habitat mud was collected in the same way from a shaded puddle midge habitat located on a commercial cervid facility in Quincy, FL, USA [23]. Emergence sampling from this habitat indicated that this site was a productive habitat of *C. stellifer* and no *C. insignis* emerged from this site. Previous studies reported *C. insignis* to be associated with cattle pastures and livestock facilities [16–18]. Therefore, mud mixed with fresh cattle manure (25.0% w/w) was also tested for its oviposition attractiveness. Overall, four different two-choice assays were conducted in various combinations: 1) Deionized water (DI) vs. Deionized water, 2) DI vs. mud, 3) mud + cattle manure vs. mud, 4) habitat mud vs. non-habitat mud. Each experiment had 5 – 8 replicates using a single midge in each replicate. At least two trials were conducted per each experiment (Table 1). The eggs deposited during the oviposition studies were used for the larval rearing experiments.

Table 1

Summary of the oviposition experiments conducted on *Culicoides insignis*. Oviposition preference of *C. insignis* for mud substrates over DI substrates was marginally significant (shown in bold).

Experiment	Two-choice oviposition preference assay	Trials (replicates [n])	Blood-fed females (n)	Gravid females (n)	Ovipositing females (n)	P value
1	DI vs. DI	2 (6)	12	10 (83.3%)	4 (40.0%)	0.3500
2	DI vs. mud	3 (6–8)	21	18 (85.7%)	18 (100.0%)	0.0780
3	Mud + cattle manure vs. mud	2 (6)	12	5 (41.7%)	4 (80.0%)	0.4200
4	Habitat mud vs. non-habitat mud	2 (5–6)	11	8 (72.7%)	8 (100.0%)	0.3400

Larval rearing experiments

Midge larval rearing was conducted using methods described previously for *C. stellifer* [24]. Briefly, the eggs deposited during the oviposition studies (24 – 36 h old) were placed in Petri dishes (60 × 15 mm) containing 0.3% (w/v) agar slants and allowed to hatch. The larvae were given a diet of *Panagrellus redivivus* Linnaeus nematodes (Carolina Biological Supply Company, Burlington, NC, USA) that were replenished every Monday, Wednesday, and Friday (~2 mg/day). The life history traits recorded were egg stage duration, egg hatch rates, larval survival rates to pupal stage, larval stage duration, pupal stage duration, adult eclosion rates, and sex-ratio of the emerged adults. Each Petri dish had 10 – 20 eggs with four to six dishes per trial. Three independent trials were conducted overall (Table 2). All laboratory experiments were conducted at $26 \pm 1^\circ\text{C}$, 60 – 80% RH, and 14:10 (L:D) h photoperiod cycle.

Table 2
Summary of the larval rearing experiments conducted on *Culicoides insignis*.

Trial (larval dishes [n])	Eggs/dish (n)	Egg stage duration (days)
1 (6)	20	3–4
2 (6)	10	3–4
3 (4)	10–11	3–4

Statistical analysis

For the oviposition experiments, only gravid females were included in the statistical analyses. Variation in the number of eggs produced by gravid females and percentage of eggs retained by ovipositing females across the experiments was analyzed using generalized linear mixed-effects models (GLMM; package *lme4*) with the variation arising from trials and females incorporated as a random-effect under binomial or Poisson distributions. The oviposition preferences exhibited by *C. insignis* in the two-choice assays were analyzed using generalized estimating equations (GEE; package *geepack*) with a logit link function under binomial distribution taking into consideration the percentage of eggs retained by ovipositing females. As each midge was given two choices for oviposition, these dishes were regarded as a cluster and an exchangeable correlation structure was incorporated in the models [25]. Variation in the percentage of egg batch deposited (control + treatment) by females during different experiments was analyzed using generalized linear models (GLM) with a binomial distribution. For the larval rearing experiments, differences in the egg hatch rates, larval survival rates to pupal stage, larval stage duration, pupal stage duration, eclosion rate, and F1 adult sex-ratio between trials were analyzed using GLMM with the variation arising from females as a random-effect under binomial or Poisson distributions. All data were analyzed using R statistical software v.3.6.1 using the packages *MASS*, *car*, *lme4*, and *geepack* [26–30].

Results

Blood-feeding rates on live chicken ranged from 26.7–33.6% ($30.6 \pm 2.0\%$ [overall mean \pm SE]) (Fig. 1A). For the oviposition experiments, a total of 56 *C. insignis* females were blood-fed across the study, which resulted in 41 females that developed eggs, and 34 females that deposited eggs overall (Table 1). The percentage of females that developed eggs varied between experiments from 41.7% (5/12) during mud + cattle manure vs. mud trials to 85.7% (18/21) during DI vs. mud trials (Table 1). Not all gravid females oviposited. Very few gravid females deposited eggs during DI vs. DI trials (40.0%, 4/10) than during the other three experiment trials ($\geq 80.0\%$) (Table 1). The number of eggs produced by gravid females ranged from 2–111 (56.1 ± 4.0 eggs/female [mean \pm SE]) and varied significantly across the study ($\chi^2_3 = 12.0$, $P = 0.0074$). During the double control trials (DI vs. DI), very few eggs were deposited overall with the number of eggs deposited on one dish (6.7 ± 5.6) not being significantly different from that on the other (5.4 ± 4.5) ($\chi^2_1 = 0.9$, $P = 0.3500$) (Fig. 1B). During the DI vs. mud trials, the number of eggs deposited on the substrates with mud (35.3 ± 8.2) was significantly higher (marginally) than on DI substrates (17.7 ± 4.1) ($\chi^2_1 = 3.1$, $P = 0.0780$). During the mud + cattle manure vs. mud trials, the number of eggs deposited on mud + cattle manure substrates (24.6 ± 12.0) was higher than that on mud substrates (11.2 ± 10.7) but was not statistically significant ($\chi^2_1 = 0.7$, $P = 0.4200$). Similarly, during the habitat mud vs. non-habitat mud trials, the number of eggs deposited on habitat mud substrates (35.4 ± 13.9) was higher than that on non-habitat mud substrates (18.0 ± 8.7) but was not statistically significant ($\chi^2_1 = 0.9$, $P = 0.3400$) (Fig. 1B). The percentage of egg batch deposited by females (control + treatment) was the lowest during DI vs. DI trials (31.0%) than during the other three experiment trials ($\geq 75.2\%$) ($\chi^2_3 = 627.0$, $P < 0.0001$) (Fig. 1C). Among the females that oviposited, 55% (19/34) deposited eggs on one dish and the remaining (44.1%, 15/34) deposited eggs on both dishes (Fig. 1D).

The egg stages of *C. insignis* lasted between 3–4 days (Table 2). The egg hatch rates ranged from 55.0–77.7% and differed significantly between the trials ($\chi^2_2 = 8.3$, $P = 0.0157$) (Fig. 2A). Larval survival rates to the pupal stage ranged from 64.5–95.8% ($85.0 \pm 10.3\%$ [overall mean \pm SE]) and differed significantly between the trials ($\chi^2_2 = 24.3$, $P < 0.0001$) (Fig. 2B). Larval stage duration ranged from 15.4–29.0 days (20.3 ± 4.3 days) and differed significantly between trials ($\chi^2_2 = 193.1$, $P < 0.0001$) (Fig. 2C). Pupal stage duration ranged from 2.6–3.2 days (2.9 ± 0.2 days) and did not vary significantly between the trials ($\chi^2_2 = 2.2$, $P = 0.3385$) (Fig. 2D). Adult eclosion rates from the pupal stage were high ($\geq 87.5\%$) and showed no significant differences between the trials ($\chi^2_2 = 0.1$, $P = 0.9648$) (Fig. 3A). Sex-ratios of the F1 adults were male biased $\sim 3:1$ (M:F) and were not significantly different between trials ($\chi^2_2 = 3.2$, $P = 0.2016$) (Fig. 3B).

Discussion

Overall, our study demonstrates useful methods to collect live *C. insignis* midges from the field, blood-feed them under laboratory conditions, collect eggs from gravid females, and rear the larvae till adulthood, and provides valuable insight into the life history traits of *C. insignis*, an important vector of BTV/EHDV in Florida. Although considered a mammal biter, *C. insignis* females showed satisfactory

blood-feeding rates on live chicken in the laboratory. These blood-feeding rates can possibly be increased further by using other laboratory animals (mammals) and/or altering starvation periods or environmental conditions during blood-feeding. In addition, fecundity of *C. insignis* may also be potentially increased by using a mammalian blood source (versus avian blood source used in this study) as host blood meal source can alter fecundity in hematophagous species [31, 32]. However, further studies will be needed to test these hypotheses.

During the oviposition experiments, gravid females deposited a distinctly higher number of eggs on substrates with habitat mud over DI controls, suggesting that mud from the larval habitat provides strong oviposition cues to *C. insignis*. However, the number of eggs deposited on substrates during mud + cattle manure vs. mud trials and habitat mud vs. non-habitat mud trials was not significantly different, suggesting that organically enriched muds other than the habitat mud are also attractive for the oviposition of this species. However, these results should be interpreted cautiously as they could be an artifact of the small sample size of the study (only 12 females oviposited in these two experiments [Table 1]). In addition, whether or to what extent mud from the larval habitat mud of *C. insignis* examined was already enriched with organic matter is currently unknown (cattle had open access to this site on the Archbold ranch). Moreover, if olfactory cues are involved in the oviposition site selection of *C. insignis*, the set-up of the experimental design could have caused errors in the recognition of preferred substrates as the two dishes were placed close to each other in the relatively small sized paper cups. Further studies using Y-shaped olfactometer bioassays could examine whether these results are biologically significant and determine whether these oviposition cues are olfactory/tactile in nature. Further studies are also needed to examine whether other natural sources from the habitat such as vegetation play a role in the oviposition of *C. insignis* (vegetated water bodies often harbor *C. insignis* larvae) [14, 18]. Previously, mud and/or vegetation (*Sphagnum* spp. moss) from the larval habitat were found to strongly influence the oviposition of *C. stellifer* and *Culicoides impunctatus* Goetghebuer under laboratory conditions [22, 33]. Currently, very little is known regarding the oviposition preferences and/or habitat requirements of *C. insignis* and other important *Culicoides* species in North America [14, 18, 22, 23]. Future studies characterizing the larval habitat of *C. insignis*, examining the physicochemical properties of the breeding site, and identifying the key biotic/abiotic factors influencing oviposition site selection of this species in nature are warranted. This information, in the long term, can be potentially exploited to design novel sampling/control strategies targeting gravid females and to manipulate local habitats to discourage the oviposition of *C. insignis*.

The large variation in the number of eggs produced and the percentage of females that developed eggs in the study was not unexpected. This variation may have arisen due to variation in the blood meal size ingested by females (partially-engorged females were also included in the study) and due to variation in the mated status of the females used in the study (midges were field-collected) respectively, patterns that have been observed in mosquitoes [34, 35]. However, variation in the percentage of females that oviposited and variation in the percentage of egg batch deposited by ovipositing females likely represents a differential preference for the available oviposition substrates. For example, very few females deposited a very small percentage of their egg batch during the DI vs. DI trials compared to the other experiment

trials, suggesting avoidance of DI substrates and a preference of habitat mud and other organically enriched muds. Future studies that require oviposition and/or collection of eggs from *C. insignis* in the laboratory may benefit by providing organically enriched substrates to gravid females.

It was interesting that among the 34 females that oviposited across the study, 44% (15/34) demonstrated skip oviposition as they deposited eggs on both the dishes available. Such behavior was documented previously in *C. stellifer* as well but appears to be more common in *C. insignis* than in *C. stellifer* (9% females) [22]. Skip oviposition has been well studied in numerous container-breeding mosquito species [22, 36–38]. It is believed that skip oviposition is advantageous in resource limited habitats (such as artificial containers, plant pitchers, tree holes, or others) as it enhances larval survival by reducing larval densities. However, the role of skip oviposition on the survival of mud breeding species such as *C. insignis* and *C. stellifer* is currently unknown. Further studies will be needed to understand the role of skip oviposition on the ecology/survival and other life history traits of mud breeding *Culicoides* species. Further studies will also be needed to examine whether or to what extent skip oviposition occurs in dung-breeding and tree-hole dwelling *Culicoides* species.

The egg hatch rates of *C. insignis* varied significantly across the study, which was not unexpected. It is likely that fertilization status of the eggs varied possibly due to variation in the age of the field-collected females and/or the mated status of the males these females mated with in the field. Previously, the prior-mated status of males was found to affect egg fertilization rates in tephritid flies and butterflies [39, 40]. The significant variation in larval survival rates and larval stage durations across the study were also not unexpected. It is possible that this variation arose due to, 1) variation in the age/nutritional status of females the eggs were obtained from as parental nutrition can affect larval development traits in insects [41, 42], 2) variation in the number of eggs placed in the larval dishes (ranged from 10–20) as larval densities can affect insect larval development [43–45], and/or 3) age/condition of the nematodes used as midge larval diet as early instar midge larvae may have had difficulties capturing/ingesting adult nematodes.

Overall, the agar/nematode method was convenient and effective for the larval rearing of *C. insignis*. All larval instars could be seen moving through the agar and in/out of the standing water freely. The late instars were frequently observed engulfing nematodes whole while the early instars probably fed on the nematode pieces/carcasses and/or microbial community of the medium. Interestingly, *C. insignis* larvae (late instars) were also observed to feed on dead conspecific larvae, suggesting that *Culicoides* larvae are omnivorous opportunistic feeders. Pupation occurred mainly on the surface of the agar, but the pupae were also found floating in the standing water, albeit with less frequency. Although the larval development of *C. insignis* was successful, sex-ratio of the F1 adults was male-biased, which may not be desirable for potential colony maintenance. The reasons behind this outcome are currently unknown. However, it is likely that the nematode diet used could not satisfy nutritional requirements of the female larvae potentially causing mortality. Previously, female mosquitoes were suggested to require more larval nutrition than males to pupate [46]. Moreover, previous larval rearing studies on *Culicoides* species using the agar/nematode method reported non-distorted sex-ratios in the progeny only for *C. stellifer* and *C.*

circumscriptus Kieffer while the sex-ratios of other species were found to be either male-biased or female-biased [24, 47, 48]. It is likely that the larval nutritional requirements of *Culicoides* midges vary between species. Further studies will be needed, 1) to examine the nutritional requirements of male and female biting midge larvae, and 2) to improve production conditions of *C. insignis* by potentially incorporating nutritional supplements to the nematode diet or by using other larval diets.

Very little is known regarding the mating behavior of *Culicoides* species currently. Many species are believed to be eurygamous (need swarming to mate) while some species are stenogamous (will mate in restricted spaces) [49–51]. Our attempts at inducing swarming/mating in the F1 generation of *C. insignis* by using host cues (octenol), environmental cues (habitat [mud + cattle manure] and dawn/dusk conditions), varying light colors (blue, green, and red), and cage sizes (capillary tubes with terminalia in contact to large 47.5 × 47.5 × 47.5 cm BugDorm cages) were all unsuccessful (non-mating was inferred as F1 females did not deposit viable eggs post blood meal). The reproductive behavior of *C. insignis* has not been reported to date. Further studies will be needed to investigate the mating habits/cues of *C. insignis* in nature, which may offer clues towards providing conditions that encourage captive mating in this species.

Conclusions

Overall, our study provides valuable insight into the oviposition and larval development traits of *C. insignis*, an important vector of BTV/EHDV in Florida. Our study indicates that mud from the larval habitat and other organically enriched substrates provide strong oviposition cues to *C. insignis* gravid females and demonstrates that the agar/nematode method is effective for the larval rearing of *C. insignis*. Further studies are needed, 1) to determine the nature of the oviposition cues (olfactory/tactile), 2) to identify the key biotic/abiotic factors influencing midge oviposition in the field, 3) to resolve the issue of male-biased sex-ratios in the progeny, and 4) to examine the reproductive behavior of *C. insignis* in nature. Collectively, this information may possibly be used to create laboratory conditions that encourage captive mating in this species and, in the long term, may be potentially exploited to design novel sampling/control strategies targeting gravid females and/or to discourage the oviposition of *C. insignis* in important habitats around livestock facilities.

Abbreviations

BTV: bluetongue virus; EHDV: epizootic hemorrhagic disease virus; DI: deionized water; RH: relative humidity; L:D: light:dark; CI: confidence interval

Declarations

Ethics approval and consent to participate

Use of live chicken for midge blood feeding was approved by University of Florida

Institutional Animal Care and Use Committee (IACUC) (Protocol #7682). No human participants were involved.

Consent for publication

Not applicable.

Availability of data and materials

All data collected during this study have been statistically analyzed and published in this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Dinesh Erram: Conceptualization, Methodology, Data Curation, Formal Analysis, Visualization, Validation, Investigation, Resources, Project Administration, Writing – Original Draft Preparation. **Nathan Burkett-Cadena:** Funding Acquisition, Supervision, Writing – Review & Editing.

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Figures

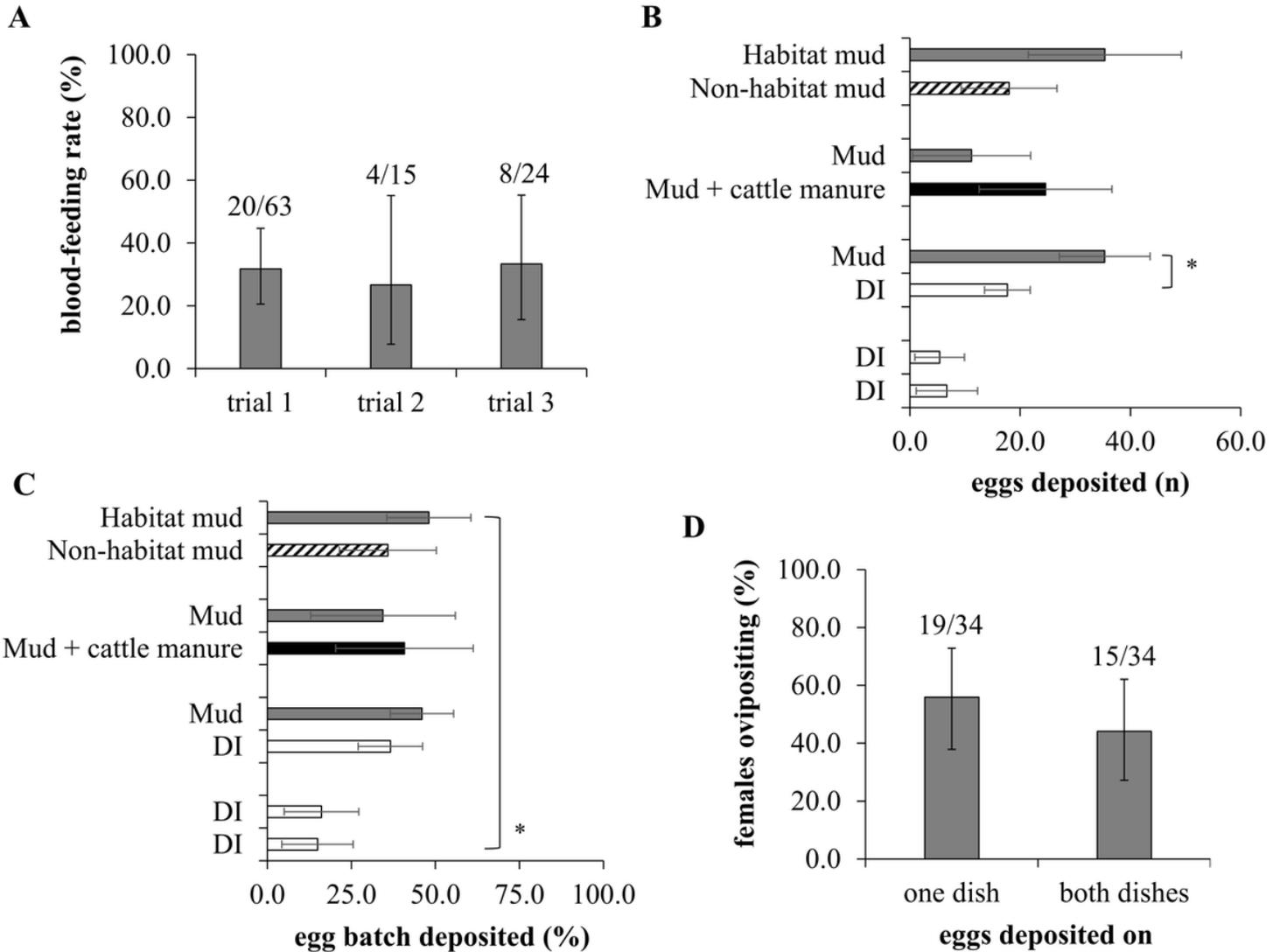


Figure 1

Life history traits of *Culicoides insignis* adult females under laboratory conditions, A) blood-feeding rates on live chicken (\pm 95% CI), B) number of eggs deposited (mean \pm SE), C) percentage of egg batch deposited (mean \pm SE), and D) percentage of females that deposited eggs on one or both dishes (\pm 95% CI). Numbers above bars in A indicate number of midges blood-fed/total number of midges. Asterisk in B indicates significantly higher (marginally [$P = 0.0780$]) number of eggs deposited on mud substrates compared to DI controls, while asterisk in C indicates a significantly lower percentage of eggs deposited during DI vs. DI trials (on both dishes combined) than during other experiment trials. Numbers above bars in D indicate number of females depositing eggs on one or both dishes/total number of females that oviposited.

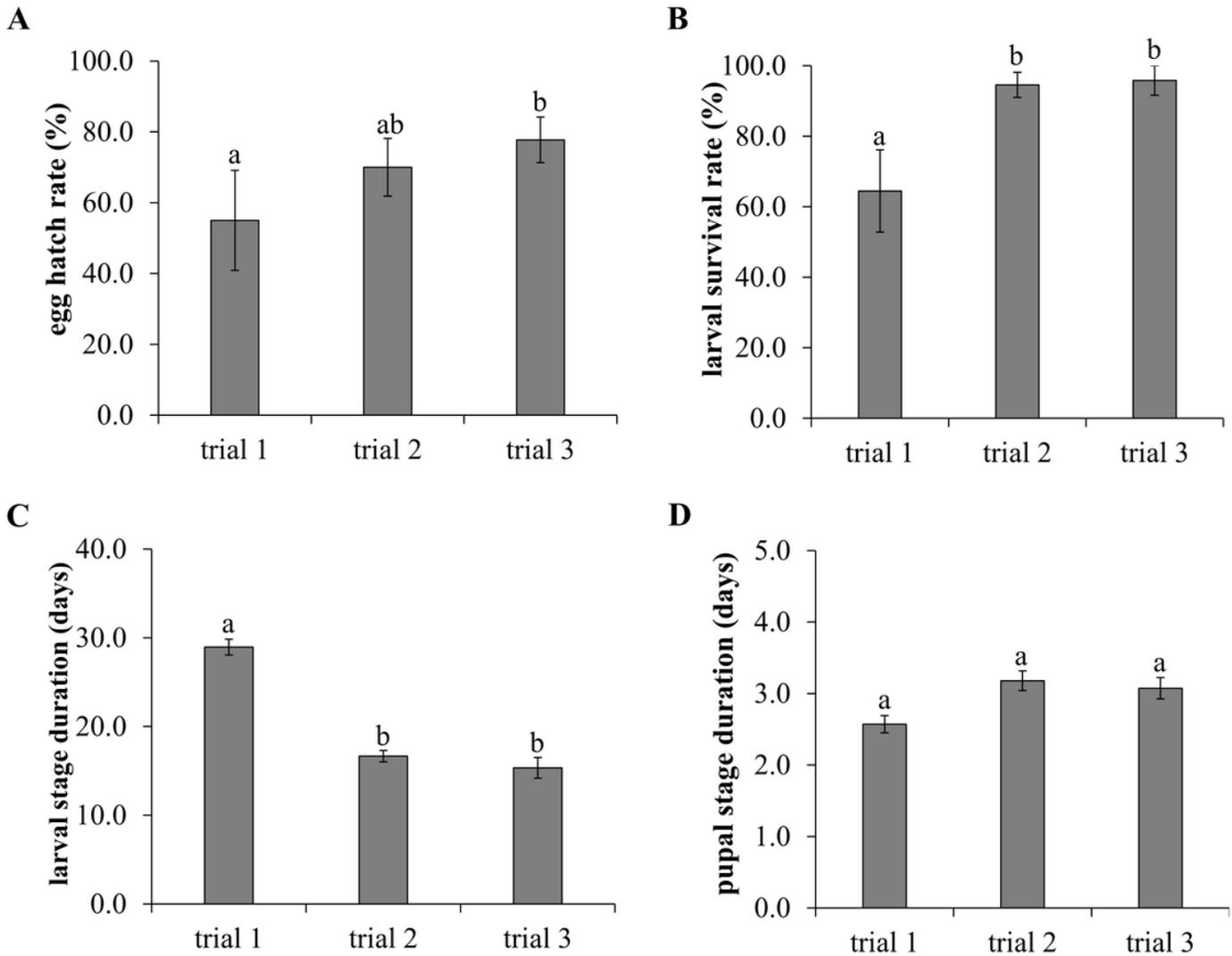


Figure 2

Life history traits of the immature stages of *Culicoides insignis* under laboratory conditions, A) egg hatch rates (mean \pm SE), B) larval survival rates, C) larval stage duration, and D) pupal stage duration. Letters above bars indicate significant differences between trials (P < 0.05).

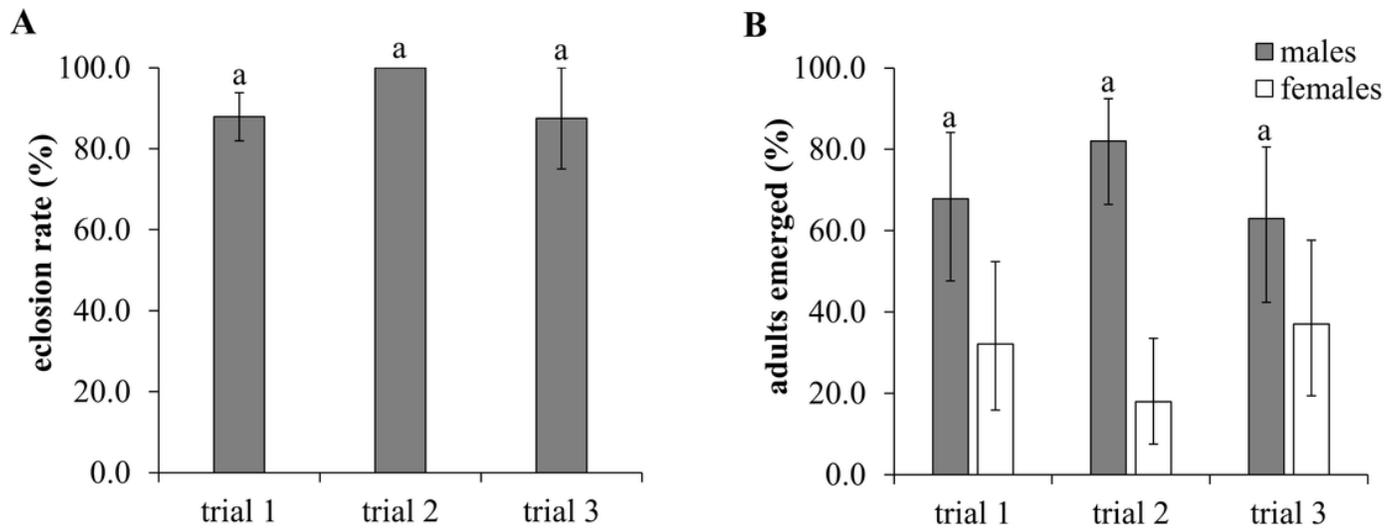


Figure 3

Life history traits of *Culicoides insignis* observed in the study. A) eclosion rates (mean \pm SE), and B) sex-ratios (\pm 95% CI). Letters above bars indicate significant differences between trials ($P < 0.05$).

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