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Effect of substrate salinity and pH on life history traits of the bluetongue virus vector *Culicoides peregrinus*

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Research Paper

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Abstract

Habitat selection of *Culicoides* spp. (Diptera: Ceratopogonidae) is influenced by the physico-chemical factors such as temperature, pH, salinity, moisture, conductivity, organic and inorganic compounds of substrates. These factors determine the life history traits of the vectors. We studied the influence of substrate salinity (0–40 parts per thousand, ppt) and pH (pH 1–13) on oviposition, egg hatching, larval survivability, and adult emergence of *Culicoides peregrinus* Kieffer under laboratory conditions. Most eggs (80.74%) were laid in 0 ppt and 95% in pH 7 but lowered with increased salinity and pH levels. It was observed that the females did not lay eggs in 30 ppt to 40 ppt salinity; pH 1 and pH 13 but interestingly up to 95% of the eggs were retained within the abdomen. Little effect of salinity and pH on egg hatching was observed up to 5 ppt and 10 ppt except at the extreme values of 40 ppt and pH 1, pH 13. Pupation did not occur in rearing plates with high salinities, 30 ppt and 40 ppt, although the few eggs hatched when exposed to such salinity. In low salinity (0 to 2 ppt), occurrence of adult emergence was more and then decreased with increasing salinity. Maximum emergence was seen when the rearing media was alkaline. This study deals with the suitability of breeding substrate of *C. peregrinus* when exposed to salinity and pH ranges. Our study suggests the ambient salinity and pH ranges to be maintained during laboratory rearing of this vector species.

Introduction

Biting midges in the genus *Culicoides* have been implicated as vectors of numerous arboviruses such as epizootic haemorrhagic disease virus, bluetongue virus (BTV) and African horse sickness virus (Purse *et al.*, 2015). *Culicoides peregrinus* Kieffer (Diptera: Ceratopogonidae) has gathered importance due to its high abundance near livestock, and it has been designated as one of the vectors of BTV of livestock, out of seven vector species (Harsha *et al.*, 2020). This species, *C. peregrinus* is distributed throughout the oriental region including India, Sri Lanka, Indonesia, Australia, New Guinea, Taiwan, and Ryukyu Islands (Wirth and Hubert, 1989; Harrup *et al.*, 2016); Japan (Arnaud, 1956; Yanase *et al.*, 2013), Yunnan Province, China (Di *et al.*, 2021). In India, this species was reported from the coastal town of Puri, Odisha by Kieffer (1910) but now it is being spread to various states mainly Andhra Pradesh, Tamil Nadu, Karnataka, Assam, West Bengal, Odisha, and Maharashtra (Sen and Das Gupta, 1959; Reddy and Hafeez, 2008; Prasad *et al.*, 2009; Harrup *et al.*, 2016; Chanda *et al.*, 2019). Habitat of this species was reported from semiaquatic areas like muddy fringe areas of stock ponds, shaded muddy pool margins, muddy areas of paddy field, cattle manure (Edwards, 1922; Buckley, 1938; Das Gupta, 1962; Wirth and Hubert, 1989). Previously Harsha and Mazumdar (2015) successfully reared this species using mud broth with yeast solution in laboratory conditions. This species completed their life cycle from egg to adult emergence in 18–23 days in the laboratory; egg to larva (2–3 days), larva to pupa (12–18 days), pupa to emerged adult (2–3 days) (Harsha and Mazumdar, 2015).

Habitat preference by gravid females may be guided by the preference-performance hypothesis (Jaenike, 1978). Thus, in many insects, maternal habitat selection for oviposition essentially depends upon several factors, determine oviposition preference and offspring performance (Wise and Weinberg, 2002; Gripenberg *et al.*, 2010). Gravid females of mosquitoes have the ability to detect the risk of predation among many factors when making decisions in oviposition site selection (Silberbush and Blaustein, 2011). Oviposition site preference and emergence of *Culicoides* are influenced by different salt concentrations and pH levels viz., in *C. imicola* Kieffer (Venter and Boikanyo, 2008), *C. obsoletus* Meigen (Harrup *et al.*, 2013) and *C. impunctatus* Goetghebuer (Blackwell *et al.*, 1999). Recently, increasing moisture levels and pH showed a potential correlation to the survival of *C. obsoletus* (Harrup *et al.*, 2013), while pH, organic content and moisture along with the proportion of vegetation in the habitat correlated to the presence of *C. impunctatus* (Blackwell *et al.*, 1994). Linley (1986) studied the influence of salinity on oviposition and hatching of *C. variipennis* (Coquillett). Besides, other *Culicoides* species worldwide showed a wide habitat distribution from freshwater to coastal areas like *C. crepuscularis* Malloch, *C. bermudensis* Williams (Williams, 1956). Kline and Wood (1988) reported *C. furens* (Poey) from both salt marsh and freshwater and *C. mississippiensis* Hoffman from salt marsh areas of Florida in low abundance. Magnon and Hagan (1988) isolated *C. melleus* (Coquillet), *C. hollensis* (Melander and Brues), *C. furens* as salt marsh species from Georgia. Immatures of mosquitoes are also influenced by physicochemical parameters like the effect of pH demonstrated by Clark *et al.* (2004) in *Aedes aegypti* (Diptera: Culicidae) and in *Anopheles arabiensis* (Diptera: Culicidae) (Owiti and Christopher, 2017). Ray and Choudhury (1988) examined the soil chemistry characteristics of their habitats and physicochemical characteristics of water and substrate of *C. peliliouensis* Tokunaga, of Hooghly estuary, while the similar investigation was done for *C. variipennis*, *C. sonorensis* Wirth and Jones and *C. occidentalis* Wirth and Jones throughout their geographic ranges (Schmidtmann *et al.*, 2000) and for various *Culicoides* spp. (Uslu and Dik, 2010). Habitat parameters show variations within seasons as well as for species (Schmidtmann *et al.*, 2000; Uslu and Dik, 2010). Changes in ionic concentration of habitat may affect pH and perhaps have a direct relatedness to osmotic consequences for insects (Williams, 1996). For aquatic organisms, pH is an important factor that limits their abundance and distribution (Clark *et al.*, 2007). Multini *et al.* (2021) showed a statistically significant association between mosquito species occurrence and the immature with pH and salinity. An understanding of physicochemical parameters associated with habitat selection is important to elucidate how these factors influence the life history traits of this vector species. The oviposition site selection by this vector species is driven by the availability of suitable habitat and the physicochemical factors of these habitats (Schmidtmann, 2006; Uslu and Dik, 2010).

The main purpose was to examine the tolerance range of *C. peregrinus* towards substrate salinity and pH; and how it affects oviposition, hatching, larval survivability, and adult emergence. This information provides insights into the ephemeral characteristics of the breeding sites and their influence on a few life history traits of the vector *C. peregrinus*.

Materials and methods

Study area, collection of samples and rearing

Adult individuals were trapped by operating UV LED light traps in cattle shed at Gangpur (23°22′ N, 87°90′ E), West Bengal, India; and live engorged females of *C. peregrinus* were segregated and put in small glass vials (5 cm × 1 cm). Afterwards, the engorged females were gently transferred individually to bigger glass vials (7 cm × 2.5 cm) containing oviposition beds. For salinity experiments, 90 engorged females (single individual per vial) were considered for each saline concentration, therefore a total of 540 females (i.e. 90 females × six saline concentrations) were placed in glass vials. Similarly, 60 engorged females (single individual per vial) were considered for each pH level i.e. a total of 420 females (i.e. 60 females × seven pH concentrations) were placed in glass vials. The vials containing females were maintained in the Environmental Test Chamber (model CHM-10S; REMI Elektrotechnik Ltd. Vasai, India) with the temperature set at 26 ± 1 °C, relative humidity 75%, photoperiod 13L:11D and the females were observed for the next 72 hr. for oviposition.

Preparation of oviposition beds with different salinity and pH levels

To observe the effect of salinity and pH on egg laying in no-choice based assay, the oviposition beds were made with a layer of absorbent cotton and moistened filter paper placed over it. Each bed was soaked with different saline concentrations and pH levels. The saline concentrations that were tested as follows, 5 ppt, 10 ppt, 20 ppt, 30 ppt, 40 ppt and distilled water (0 ppt) was treated as control. Different concentrations of saline solutions were prepared by mixing NaCl (Himedia, MB023) in distilled water (Trimble and Wellington, 1979). The salinity levels of the oviposition beds were monitored using a Multiparameter Meter Sension™ 156 portable device. Salinity was also measured as electrical conductivity (EC) in this device such as 5 ppt (12.80 mS cm^{-1}), 10 ppt (25.26 mS cm^{-1}), 20 ppt (44.67 mS cm^{-1}), 30 ppt (65.53 mS cm^{-1}) and 40 ppt (81.58 mS cm^{-1}). As seawater is considered 100% salinity (38.1 ppt) therefore 40 ppt was chosen as higher salinity for our experiment following Nwaefuna *et al.* (2019).

The effect of varied substrate pH on egg laying was looked into. The pH concentration that was taken for testing i.e. pH 1, pH 3, pH 5, pH 9, pH 11, and pH 13 while pH 7 (distilled water) was treated as control. The pH solutions were prepared by adding 1(N) NaOH (HiMedia, MB095) and 1(N) HCl (Merck, CL8C680868) stock solution in distilled water (Ma *et al.*, 2017). The oviposition beds including the filter papers were soaked in solutions with different pH. The pH of the oviposition beds was checked using pH indicator paper, pH 1.0–14.0 (Merck, 61770600011730).

For egg hatching under varied salinity, the number of oviposited eggs from respective salinity ($N = 1150$) was considered; whereas for pH experiments the following number of eggs ($N = 2410$) from respective pH were placed for hatching and kept in an Environmental Test Chamber and were observed up to 72 hr. The paper strips containing the eggs were observed under a stereomicroscope to determine the percentage of eggs that hatched.

Larval rearing and adult emergence under different salt and pH concentrations

Larval survivability was observed by placing oviposited eggs from distilled water (salinity 0 ppt, pH 7) to rearing plates ($n = 35$ per rearing plate) exposed to varied salinity and pH concentrations. The glass vials containing the emergence beds were prepared and soaked in desired ranges of salinity and pH. The hatched larvae in the rearing plates were exposed to various salinities also measured as EC viz., 0 ppt (0.028 mS cm^{-1}), 1 ppt (2.95 mS cm^{-1}), 2 ppt (4.46 mS cm^{-1}), 3 ppt (6.11 mS cm^{-1}), 4 ppt (8.16 mS cm^{-1}), 5 ppt (12.80 mS cm^{-1}), 10 ppt (25.26 mS cm^{-1}), 15 ppt (32.76 mS cm^{-1}), 20 ppt (44.67 mS cm^{-1}), 25 ppt (54.60 mS cm^{-1}), 30 ppt (65.53 mS cm^{-1}) and 40 ppt (81.58 mS cm^{-1}). Mud broth and yeast solution were prepared following Harsha and Mazumdar (2015) with slight modifications by adding distilled water to different ranges of salinity and pH. The salinity of rearing medium was checked by the Multiparameter Meter Sension™ 156 portable device at regular intervals before and at the end of every experiment. Six replicates were done for each saline concentration and pH level.

The other objective was to record the effect of various pH levels of rearing media on the larval survivability of *C. peregrinus*.

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Harsha and Mazumdar (2015) reared these larvae under laboratory rearing conditions and the pH of the rearing media was alkaline (pH 9–11). During this experiment, the effect of acidic condition (pH 3) on larval survivability was also tested. The pH of the mud broth, yeast solution and rearing media was checked with pH indicator paper. The rearing plates were kept in the Environmental Test Chamber with the temperature set at 26 ± 1 °C, relative humidity 75%, photoperiod 13L:11D. Mud broth and yeast solution were added at regular intervals to rearing plates. For each treatment, development period (in day), larval survivability (%), and adult emergence (%) along with the sex of the adults were recorded. Larval survivability was ascertained by counting the number of surviving 4th instar larvae. The formed pupae were carefully removed from the rearing media and placed onto the emergence beds. Adults emerged after 2–3 days, and these were counted and finally preserved in 70% EtOH for further studies.

Statistical analysis

The oviposition data corresponding to different ranges of salt concentrations and pH were analysed by logistic regression (Zar, 1999) following a binomial generalised linear model with logit link (McCullagh and Nelder, 1989; Fox, 2008). A typical expression of the logistic regression (binomial GLM) in the form of: y (response variable) $= 1/\ (1 + \exp\ (-(-a + b_1 \times {}_1 + b_2 \times {}_2 + \ldots + b_n x_n)))$, was used to deduce the relationship between the oviposited eggs (response variable) against the explanatory variable (x; representing different salt concentration or pH level). Similarly, the data on the hatching rate were also subjected to binomial generalised linear model with logit link (McCullagh and Nelder, 1989; Fox, 2008) where y (response variable) was the hatching rate against the explanatory variable (x; representing the different salt concentration or pH). For the response variable, a binary sum form was used and through the application of the maximum likelihood method, the parameters of the model were estimated using *XLSTAT* software, release 10 (Addinsoft, 2010). The parameters of the model (a and b) were tested for significance employing a chi-square method (McCullagh and Nelder, 1989; Fox, 2008). Application of the logistic regression was based on the assumption that the number of oviposited eggs hatched on different salt concentrations and pH follow a binomial (n, p) distribution with observations for each of the explanatory variables (different salt concentrations or pH levels). The probability parameter shown as p, was assumed to be a linear matching of the explanatory variables. The significant contribution of the explanatory variables to the oviposition of *C. peregrinus* was inferred from the logistic regressions. For instance, the number of oviposited eggs and hatched eggs against salinity and pH enabled highlighting the response variable. The application also suffices the assumption of the binomial distribution with the fate of the egg being either hatched or not hatched. The significance of differences in the larval developmental, pupation and adult emergence were analysed by using one-way ANOVA and followed by Tukey's HSD test using *XLSTAT* software, release 10 (Addinsoft, 2010).

Results

Effect of salinity on oviposition, larval survivability and adult emergence

During this experiment 4275 oviposited eggs were considered, of which 80.74% were deposited in 0 ppt and low EC (0.028 mS cm^{-1}), but decreased abruptly to 22.79% in 5 ppt and with higher salinity 4.14% in 20 ppt (44.67 mS cm^{-1}) ; but oviposition did not occur with salt concentration 30 ppt to 40 ppt with high EC i.e. 65.53 mS cm^{-1}, 81.58 mS cm^{-1} respectively (fig. 1a). This is evident from the logistic regression: abundance oviposited eggs = 1 / (1 + exp (-(3.67–2.16*Salinity level))), where the parameters of the equation remained statistically significant (intercept = 3.67 ± 0.069; Wald $\chi 2 = 2829.69$; $P < 0.0001$). When females were treated with 30 ppt and 40 ppt in saline solutions, it was observed that 95% of the abdominal eggs were retained which was confirmed by dissecting the abdomens. The percentage of retained eggs in the abdomen gradually becomes high with more saline with high EC substrate (fig. 2a).

The hatching rate of eggs was highest in 0 ppt (98.84%) and gradually decreased with increasing salinity and EC. At 40 ppt (81.58 mS cm^{-1}), hatching occurred only 3.79% (fig. 3a). However, in 20 ppt (44.67 mS cm^{-1}) and 30 ppt (65.53 mS cm^{-1}), hatching was 85.23%, 57.52% respectively. The logistic regression supported that the number of eggs hatched was dependent on the evidence from the logistic regression: abundance number of hatched eggs = 1 / (1 + exp (-(7.48–1.52*Salinity conc.))), where the parameters of the equation remained statistically significant (intercept = 7.48 ± 0.463; Wald $\chi 2 = 260.83$; $P < 0.0001$). It was observed that the duration of hatching time did not differ.

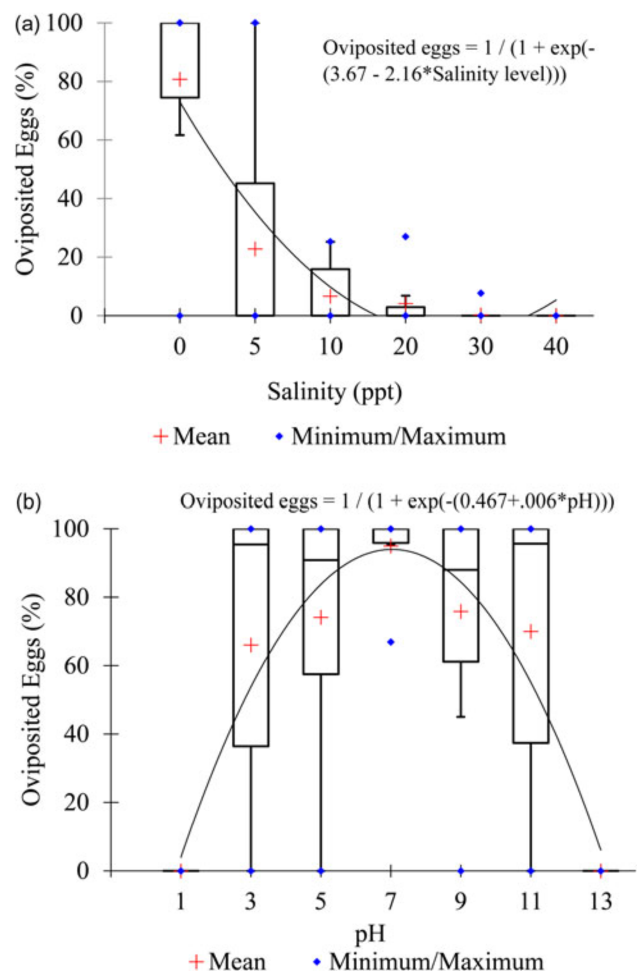

Figure 1. Oviposition response of *C. peregrinus* in different salinity and pH of substrates. (a) Laid eggs (%) on oviposition beds soaked with variable saline concentrations (ppt). (b) Oviposition in different pH levels.

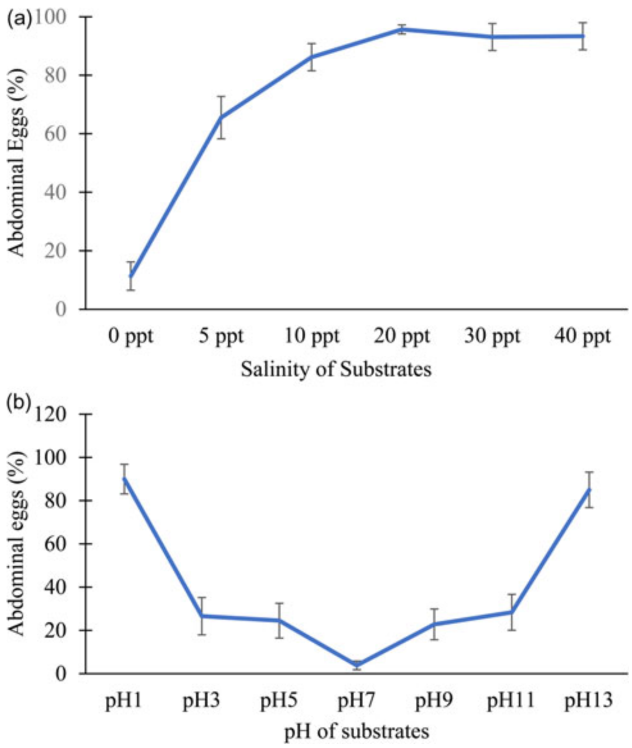

Figure 2. Percentage of retained eggs within the abdomen of *C. peregrinus*. (a) Abdominal eggs (%) across different salinity. (b) Abdominal eggs (%) across various pH levels.

The pupation and adult emergence were highest in distilled water but began to decline with the increasing salt solutions with high EC. Survivability of developmental stages in laboratory conditions varied with salt concentrations that are presented in Table 1. The larval survivability was 79.52% in 0 ppt and reached 7.62% in 25 ppt ($F = 90.95$, df = 9, $P < 0.0001$). The developmental period from egg to adult was about 18–23 days (Harsha and Mazumdar, 2015) however, delayed larval development was observed in 10 ppt to 25 ppt salinity with increasing mortality. Appearances of the first pupa were considered here and were noticed to be longer up to 26–27 days in 25 ppt. As larval mortality in 1st instar was increased in 30 and 40 ppt so the pupal stage was not observed in this high salinity (Supplementary Figure 1a). Pupation recorded 79.52% in 0 ppt, 78.10% in 1 ppt and 76.67% in 2 ppt whereas lower 5.24% in 25 ppt ($F = 80.79$, df = 9, $P <$ 0.0001). In 30 and 40 ppt salinities, no pupation occurred though eggs hatched with protruding larval heads visible but soon succumbed. Adult emergence of both sexes responded in a similar way to salinity and EC. A higher number of adults emerged in the following salinities 0 ppt (79.52%), 1 ppt (76.67%) and 2 ppt (74.76%); then the emergence became lower with higher salinity and EC ($F = 81.20$, df = 9, $P < 0.0001$). The number of emergences decreased considerably in 25 ppt (4.29%) whereas emergence was not observed in 30 ppt and 40 ppt respectively (fig. 4a, 4b). Multiple comparisons (post hoc Tukey tests) revealed significant differences ($P < 0.0001$) in different salinity of rearing plates.

Effect of different pH on oviposition, larval survivability and emergence

The effect of pH range on oviposition, hatching and larval survivability was investigated. In pH 7, 95% of 10,136 eggs were

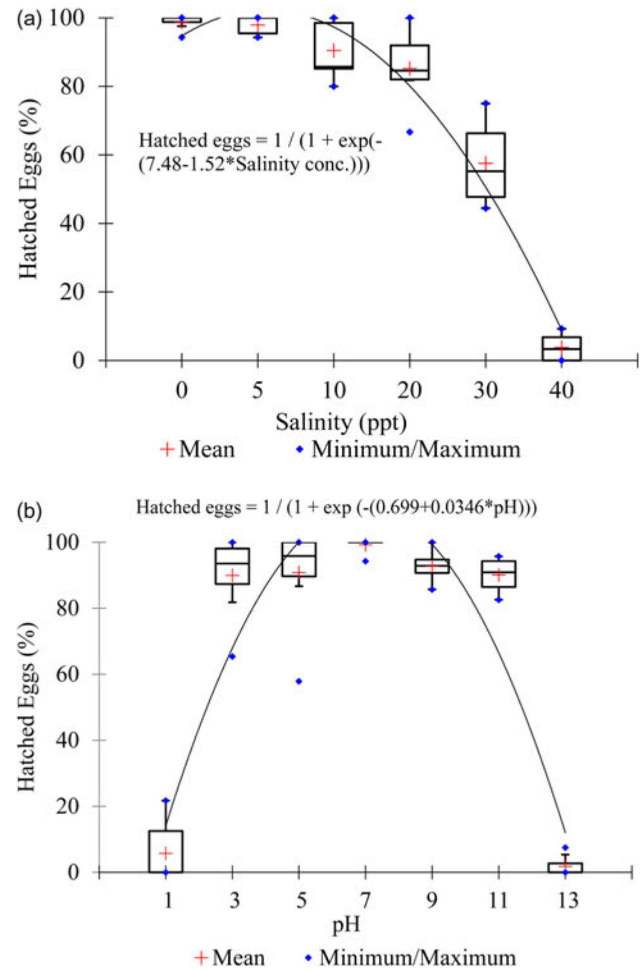

Figure 3. Effect of various saline concentrations and pH levels on hatching of oviposited eggs of *C. peregrinus*. (a) Percentage of egg hatching in different salinity. (b) Percentage of egg hatching in different pH levels.

deposited whereas in pH 3 it was 66.04% and in pH 11 it was 70.04% (fig. 1b). In logistic regression: oviposited eggs = 1 / (1 + exp (-(0.467 + .006*pH))), where the parameters of the equation remained statistically significant (intercept = 0.47 ± 0.04; Wald $\chi 2 = 139.90$; $P < 0.0001$). Gravid females did not oviposit in extreme substrate pH i.e. 1 or 13. It was observed that the number of abdominal eggs was increased with the higher acidic and basic pH value of substrate (fig. 2b). The abdomens of the dead females were dissected out for the validation of their gravid condition; therefore 90% and 85% abdominal eggs were recorded in pH 1 and pH 13 respectively.

The hatching rate of eggs was maximum at pH 7 (100%) among all pH levels tested. However, with the changes in the pH of substrate, the hatching percentage slowly decreased (89.97–90.09%). But in extreme pH levels hatching sharply decreased therefore only 5.78% and 1.82% hatched eggs were observed at pH 1 and pH 13 respectively (fig. 3b). The logistic regression supported that the number of eggs hatched was dependent on the evidence from the logistic regression: abundance number of hatched eggs = 1 / (1 + exp (-(0.699 + 0.034*pH))), where the parameters of the equation remained statistically significant (intercept = 0.70 ± 0.10; Wald $\chi 2 = 46.75$; $P <$ 0.0001). The duration of hatching time did not change under varied pH.

Table 1. Survivability of developmental stages of *C. peregrinus* exposed to various saline concentrations under experimental conditions

Saline concentration (ppt)	No. of eggs in each plate (n)	No. of eggs hatched (Mean ± SE) (%)	No. of larvae (Mean ± SE) (%)	No. of pupae (Mean ± SE) (%)	No. of emergence (Mean ± SE) (%)	No. of emerged males (Mean ± SE) (%)	No. of emerged females (Mean ± SE) (%)
0	35	34.50 ± 0.84 (99%)	27.83 ± 1.02 (80%)	27.83 ± 1.15 (80%)	27.83 ± 1.14 (80%)	13.50 ± 0.97 (39%)	14.33 ± 0.87 (41%)
1	35	33.67 ± 0.84 (96%)	27.83 ± 1.02 (80%)	27.33 ± 1.15 (78%)	26.83 ± 1.14 (77%)	13.17 ± 0.97 (38%)	13.67 ± 0.87 (39%)
2	35	33.17 ± 0.84 (95%)	26.83 ± 1.02 (77%)	26.83 ± 1.15 (77%)	26.17 ± 1.14 (75%)	13.00 ± 0.97 (37%)	13.17 ± 0.87 (38%)
3	35	33.50 ± 0.84 (96%)	20.33 ± 1.02 (58%)	20.33 ± 1.15 (58%)	19.17 ± 1.14 (55%)	9.67 ± 0.97 (28%)	9.50 ± 0.87 (28%)
4	35	33.83 ± 0.84 (97%)	22.00 ± 1.02 (63%)	22.00 ± 1.15 (63%)	21.00 ± 1.14 (60%)	10.33 ± 0.97 (30%)	10.67 ± 0.87 (30%)
5	35	33.67 ± 0.84 (96%)	20.33 ± 1.02 (58%)	19.67 ± 1.15 (56%)	18.67 ± 1.14 (53%)	9.50 ± 0.97 (27%)	9.17 ± 0.87 (26%)
10	35	33.50 ± 0.84 (96%)	14.83 ± 1.02 (42%)	14.50 ± 1.15 (41%)	13.50 ± 1.14 (39%)	6.33 ± 0.97 (18%)	7.17 ± 0.87 (20%)
15	35	32.83 ± 0.84 (94%)	7.17 ± 1.02 (20%)	4.00 ± 1.15 (11%)	3.67 ± 1.14 (10%)	1.50 ± 0.97 (4%)	2.17 ± 0.87 (6%)
20	35	25.33 ± 0.84 (72%)	4.17 ± 1.02 (12%)	3.00 ± 1.15 (9%)	2.83 ± 1.14 (8%)	1.00 ± 0.97 (3%)	1.83 ± 0.87 (5%)
25	35	22.00 ± 0.84 (63%)	2.67 ± 1.02 (8%)	1.83 ± 1.15 (5%)	1.50 ± 1.14 (4%)	0.33 ± 0.97 (1%)	1.17 ± 0.87 (3%)

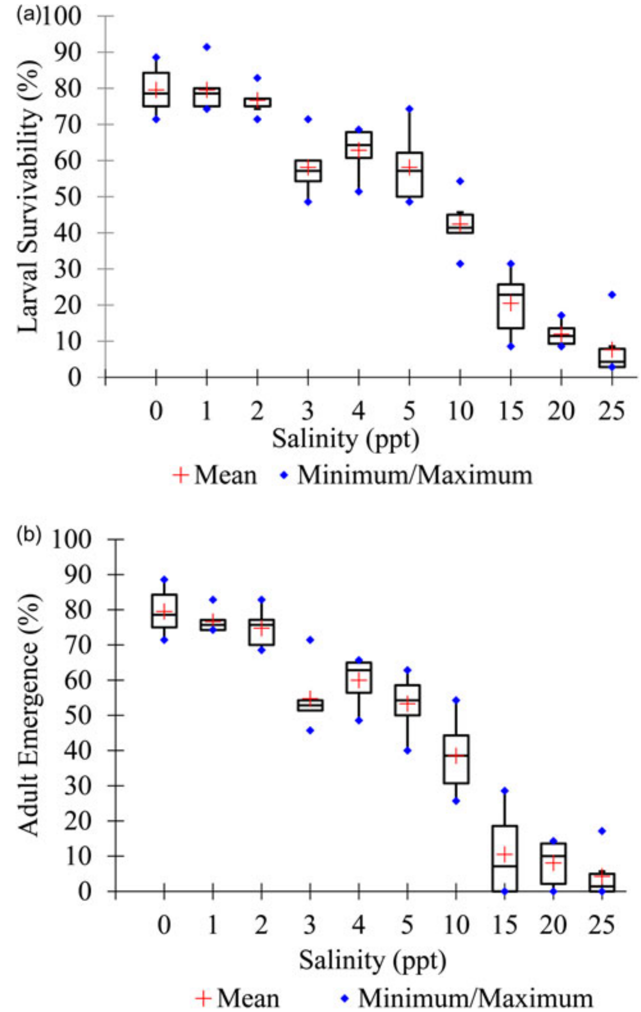

Figure 4. Effect of various saline concentrations on larval survivability and emergence of *C. peregrinus*. (a) Larval survivability. (b) Adult emergence.

Larval survivability and adult emergence were highest in alkaline rearing media (pH 9–11) whereas it declined in acidic rearing media (pH 3) (fig. 5a, 5b). There was a significant variation observed in adult emergence in alkaline rearing media compared to acidic media ($F = 20.03$, df = 1, $P < 0.001$). In highly acidic rearing media delayed larval survivability was recorded. Appearance of pupae was started from 19–27 days whereas in alkaline normal condition, their life cycle duration from egg to adult emergence was reported 18–23 days by Harsha and Mazumdar (2015) (Supplementary Figure 1b). There was no variation observed in the duration of adult emergence.

Discussion

The occurrence of species richness and distribution of *Culicoides* spp. depends on interactions of the physicochemical parameters. The larval ecology of *Culicoides* species is the most neglected area of study of their life cycle. Although various fields collected data have reported on the larval habitats of *Culicoides* in Africa, Europe and India (Braverman *et al.*, 1974; Ray and Choudhury, 1988; Poddar *et al.*, 1992; Blackwell *et al.*, 1994; Harrup *et al.*, 2013; Bakhoum *et al.*, 2016). The precise distribution of *Culicoides* spp. depends on soil physicochemical factors i.e.

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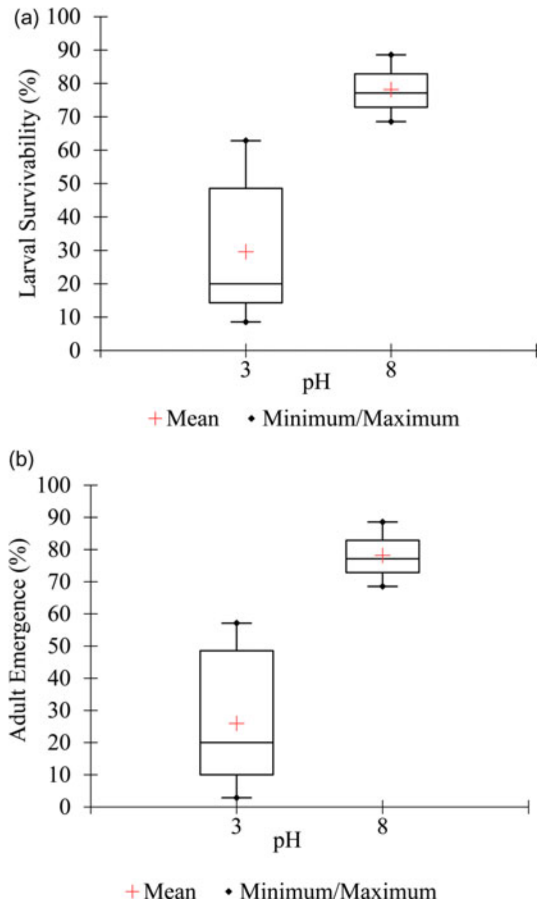

Figure 5. Effect of various pH levels on larval survivability and emergence of *C. peregrinus*. (a) Larval survivability. (b) Adult emergence.

moisture, dissolved oxygen, pH, salinity, organic contents and conductivity (Schmidtmann, 2006; Uslu and Dik, 2010). The occurrence of immatures is not only driven by the availability of larval habitats but the prevailing physicochemical factors of the patches (Roberts and Irving-Bell, 1997). *Culicoides peregrinus* breed mainly in muddy areas mixed with cattle manure (Buckley, 1938). Here we tried to find out the effect of pH and salinity in laboratory conditions on their oviposition, hatching, larval survivability and adult emergence. Our result showed the egg retention phenomenon in high salinity and pH concentration of substrates by *C. peregrinus*.

The gravid females of *C. peregrinus* laid a higher number of eggs in low saline substrate beds (0 ppt) compared to highly saline substrate (fig. 1a). Studies by Venter and Boikanyo (2008) found that *C. imicola* selected salt concentration below 0.06 g 10 ml^{-1} for selecting oviposition sites and Linley (1986) also showed that *C. variipennis sonorensis* (Coquillett) females choose to oviposit in 0‰ rather than 19‰ but it was noticed that no eggs were laid on 34‰. In mosquitoes, substrate parameters influenced oviposition site selection (Multini *et al.*, 2021). *Aedes togoi* mosquito laid a large number of eggs in distilled water and up to 20 g NaCl/L but less numbers of eggs were deposited with increasing salinity between 20 and 40 g NaCl/L. Mosquitoes also showed an oviposition choice between low and high saline substrates (Trimble and Wellington, 1979). Females of *An. aquasalis* preferred to lay eggs in low salinity and avoid laying eggs in high concentration (Woodhill, 1941; O'Gower, 1958; Osborn *et al.*, 2006). Trimble and Wellington (1979) suspected that, *Ae. togoi* is influenced by the osmotic pressure of the water when choosing an oviposition site. Avoiding egg laying in high salinity may be due to a behavioural mechanism by the females. They pointed out the possible reason behind this due to hyperosmotic conditions, where hatched larvae become dehydrated and may not persist long enough to complete development. From the similar observation of this study, we can assume that osmotic pressure may influence gravid females to select oviposition sites. Osmoregulation in Chironomidae (Diptera) larvae reported by the anal papillae (Kefford *et al.*, 2011) and Reeves (2008) also studied osmoregulatory organs like anal papillae, chloride cells in the larvae of *C. sonorensis*. As reported these organs are likely to be involved in osmoregulation to high saline condition in larvae of *C. peregrinus* but it was not studied.

The hatching rate decreased when exposed to high substrate salinity (fig. 3a). Just after hatching the larvae did not survive in 30 and 40 ppt with high EC i.e. 65.53 mS cm^{-1} and 81.58 mS cm^{-1} respectively. Thus, it may be said that high saline concentration and high EC are unfavourable. This also implies that if eggs get deposited in a high saline substrate, larval survivability may decrease. Similarly in Chironomid, only 1.7% of eggs hatched in high saline concentration, corresponding to EC 30 mS cm^{-1} (Kefford *et al.*, 2007). Survivability of *C. peregrinus* larvae gradually decreased when exposed to high salinity. Brei *et al.* (2003) also found that at three to four times the salinity of seawater, all the immatures of *C. molestus* (Skuse) died. They opined that seawater salinity could be an important factor of habitat suitability for *C. molestus* immatures. Clark *et al.* (2004) also found that *Ae. aegypti* mosquito in unfavourable high saline concentration, lower growth rate by extending larval stage duration. Similarly, our study recorded delayed larval development in high salinity and acidic rearing media. The higher saline concentrations of seawater inhibit the survivability and maturation of immatures of *C. molestus*, whereas lower concentrations of natural seawater are more suitable for survivability. A correlation between larval habitat and salinity has been established for several *Culicoides* species (Kardatzke and Rowley, 1971; Lardeux and Ottenwaelder, 1997). Habitat of arid and semi-arid regions consumes large quantities of water from agricultural land which causes secondary salinisation of habitat by irrigation and rising of groundwater labels. These salts from the groundwater and river may be leached into the habitat. The salts are also dissolved during precipitation which enter the surface waters (Cañedo-Argüelles *et al.*, 2013). Habitat salinity ranged from zero to one-time seawater for *C. belkini* Wirth and Arnaud but larvae were generally not obtained from more saline environments (>0.6 times seawater equivalents). This species preferred their habitat with the salinity of 0.15–0.45 times seawater equivalents (Lardeux and Ottenwaelder, 1997). Similarly, Becker (1961) noted that *C. circumscriptus* Kieffer and *C. furens* could thrive in hypersaline water i.e. 1.5 times and 3 times that of seawater respectively. The levels of dissolved salts influence the suitability of aquatic habitats for immature populations of the *C. variipennis* complex (Schmidtmann, 2006). However, Bakhoum *et al.* (2021) noticed the highest abundance of larval habitat of *C. distinctipennis* Austen was in the freshwater lake edges. *Culicoides furens* showed a wider habitat range in both salt marsh and freshwater habitats (Kline and Wood, 1988). Similarly, Osborn *et al.* (2006) found that female *An. aquasalis* preferred to oviposit in freshwater and also higher larval survivability in brackish water which contributes to a coastal distribution. The increased larval mortality in higher salinity of rearing media (fig. 4a) gives an indication that salt concentration may be responsible for disrupting the larval osmotic homoeostasis by gain of ions and loss of water. For this reason, gravid females of *C. peregrinus* probably refused to oviposit in high saline substrates to increase larval survivability.

Besides, substrate pH also played a significant role in the selection of oviposition sites by *C. peregrinus*. We observed a higher number of eggs were laid on neutral pH (fig. 1b). Similar corroboration between salinity and pH with the occurrence of mosquitoes was obtained by Multini *et al.* (2021). Gravid females of *Ae. triseriatus* also showed avoidance of mosquitoes toward oviposition sites at pH 2 and 3 (Siewert *et al.*, 1988). Gopalakrishnan *et al.* (2013) assumed that when the substrate becomes more acidic or alkaline, mosquitoes need some mechanisms that help to survive in this extreme pH condition. It was known that gravid female mosquitoes depend on environmental cues to select oviposition sites and the ability to detect a suitable site is a critical trait in most species (Menach *et al.*, 2005). Consequently, our results indicate that gravid females of *C. peregrinus* could assess the changes in pH levels in selecting oviposition sites and it might possess similar mechanisms to mosquitoes in detecting chemical characteristics of substrates. Oviposition site selection of female mosquitoes and other Diptera insects depends upon several physical and/or chemical cues. These factors tend to constitute a highly complex system of synergistic relationships within the overall process of oviposition (Bentley and Day, 1989; Davis *et al.*, 2013). It has been found that mosquitoes select their oviposition site by olfactory cues evaluated with antennal, labrum, and tarsal receptors as short-range signals for continuously perceiving the quality of the site (Day, 2016). The effort was not made to identify and characterise the ovipositional cues.

Gravid females with a large number of abdominal eggs retained during the experimental substrate conditions were made highly unfavourable i.e. in high saline and extreme pH concentrations (fig. 2a, 2b). Such unfavourable circumstances would have forced gravid females to retain eggs as a survival strategy of immatures. It may also be possible that females would deposit their retained eggs in a suitable substrate. This observation is similar to *Ae. aegypti* that showed retained eggs phenomenon in a repellent substrate (Seenivasagan and Guha, 2015; Seenivasagan *et al.*, 2015). Females showed this egg retention behaviour in support of the preference-performance hypothesis where females perform their best and lay more eggs when they were provided with their preferred substrate which maximises the survivability of immatures (Allgood and Yee, 2017).

Not only salinity but also pH might have some effect on egg hatching. The percentage of hatching was 89.97% and 90.09% in the more acidic to the alkaline substrate (pH 3 and pH 11) whereas the hatching was 100% in the neutral pH substrate (fig. 3b). From this observation of hatching, it could be assumed that a wide range pH compared to substrate salinity may be favourable for egg hatching of *C. peregrinus*. It could be opined that a wide range of substrate pH was probably suitable for larval survivability for that reason eggs hatched.

The acidic pH of rearing media negatively influenced larval survivability as well as adult emergence in *C. peregrinus*. Harrup *et al.* (2013) observed the increasing moisture levels and pH negatively correlated to the emergence of *C. obsoletus*, while pH, organic content and moisture along with the proportion of vegetation in the habitat correlated to the presence of *C. impunctatus* (Blackwell *et al.*, 1999). Erram *et al.* (2019) reported a positive association between habitat selection and habitat pH for *C. haematopotus* Malloch and negatively related for *C. loisae* Jamnback and *C. stellifer* (Coquillett). Larvae of *C. belkini* were found in samples with high pH values (up to 9.7), indicating that alkalinity is not a strictly limiting factor for larval survivability but the limit of tolerance remains undefined. Therefore, *C. belkini* tolerated a broad spectrum of environmental variation and may colonise a wide range of habitats (Lardeux and Ottenwaelder, 1997). Ukubuiwe *et al.* (2020) cited the negative influence of extreme pH on the larval development of *Culex quinquefasciatus* (Diptera: Culicidae) where the highest larval survivorship was noticed in pH 5–8 and lowest in pH 4 and pH 10. Larval development of *Ae. aegypti* mosquito was completed in water ranging from pH 4 to pH 11, but larvae did not survive in water pH 3 or 12. The larvae survived this wide pH range by regulating haemolymph pH. In acidic water, they did not survive because they failed to regulate Na^+ balance rather than to regulate haemolymph pH (Clark *et al.*, 2004). Clements (1963) proposed that in highly acidic water, the digestion of inner layers of old cuticles of mosquito larvae may be altered and inhibit the process of ecdysis subsequently resulting in the death of mosquito larvae. Our result suggests that possibly this inhibition process of ecdysis or failure to ionic balance regulation was also responsible for the mortality of *C. peregrinus* larvae in pH 3 media as we retrieved the dead 4th instar larvae from rearing plates. Although these possible causes have yet remained to be determined. In conclusion, *C. peregrinus* attains high survivorship at pH values ranging from 9 to 11.

The present study revealed a definite correlation between oviposition preference and substrate salinity and pH. The results demonstrate that both salinity and pH of the substrate plays an important role in oviposition site selection for gravid females of *C. peregrinus*. In 0 ppt and neutral pH, females laid the greatest number of eggs. Interestingly, females show egg retention phenomenon in high saline and strongly acidic, basic substrate. Consequently, the result recorded more abdominal eggs retained in gravid females. These parameters also determine hatching, larval survivability and emergence of this vector species. Larval survivability decreased with the increasing salinity of the rearing media and acidic media compared to alkaline media. The range of tolerance shown by *C. peregrinus* is indicative of possible range extension and its preference towards ephemeral microhabitat colonisation. More field-based corroborate studies are required to determine the responsiveness to substrate parameters.

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