INTRODUCTION

*Culex pipiens* Linnaeus (Diptera: Culicidae) is an omnipresent mosquito originated in Africa and distributed through human activity to tropical and temperate climate zones worldwide (Sayed et al., 2018; El-Mehdawy et al., 2021). It is a major vector of various pathogens causing some serious and deadly diseases to humans and animals, such as the West Nile (W.N.) encephalitis, Rift Valley Fever (RVF), St. Louis, Japanese, Venezuelan and eastern equine encephalitis (Amraoui et al., 2012; Abouzied, 2017; Kassem et al., 2018; Baz et al., 2022). For Egypt, *C. pipiens* is the primary vector of the nematode *Wuchereria bancrofti*, the causative agent of lymphatic filariasis, as well as RVF and W.N. viruses (Elhawary et al., 2021; El-Mehdawy et al., 2022). Another important Culicine, *Culex antennatus* Becker, is also a primary vector of the viruses (RVFV), (WNV), Sindbis (SINV), Acado (ACDV) and Perinet (PERV) (Harbach et al., 1988; Fang et al., 2022; WRBU, 2022).
In addition, *C. pipiens* and *C. antennatus* are pervasive mosquito species in most urban, suburban and rural areas of Egypt (Harbach et al., 1988). One of the best ways to control dangerous and epidemic diseases is vector control, and sometimes it is the only way to control diseases with no effective cure, such as the West Nile fever and Dengue fever (Hassan et al., 2014). Developing a good knowledge of vectors’ biology, ecology, distribution, and bionomics are crucial for establishing effective control programs.

Historically, the identification of mosquito species depended on physical traits although these qualities may be difficult to understand without specific taxonomic knowledge, and the distinction between species is further hampered if similar characteristics are destroyed (Verna & Munstermann, 2011). For these reasons, a clear, trustworthy, and user-friendly identification solution is in constant demand (Laurito et al., 2013). Recently, DNA barcoding has been reliable for identifying several species including mosquitoes (Wang et al., 2012). Furthermore, molecular assays are crucial to discriminate between *C. pipiens* and *C. antennatus* (Shahhosseini et al., 2019).

Based on the aforementioned points, the present study focused on providing an updated information about the seasonal abundance of both *C. pipiens* and *C. antennatus* in En Nazla region, Ibshway, Fayoum Governorate, Egypt and study the relationship between different physico-chemical parameters of breeding water and the abundance of both mosquito species which are responsible for previously mentioned diseases.

### MATERIALS AND METHODS

#### 1. Study area and collection of mosquitos' larvae

Larvae of *Culex pipiens* and *Culex antennatus* were collected from four localities of En Nazla region (29°18'58.3" N, 30°39'08.8" E), Fayoum Governorate, Egypt by netting during winter, spring, summer and autumn 2021 (Fig. 1). Collected larvae were put in a white pan filled with clean water. For larval identification, ethanol (70%) was used to kill larvae. Larvae of both *C. pipiens* and *C. antennatus* were morphologically identified according to the key of Harbach (1985). Identified larvae were isolated for molecular identification.

![Fig. 1. The study area; En Nazla region, Ibshway, Fayoum Governorate, Egypt.](image-url)
2. Physico-chemical parameters of breeding water

Water temperature and total dissolved solids were determined using ADwa (AD31) apparatus (Waterproof Conductivity-TDS-Temp Pocket Testers with the replaceable electrode). Potential hydrogen (pH) was determined using ADwa (AD11) apparatus (Waterproof pH-TEMP Pocket Tester with the replaceable electrode). Meanwhile, the dissolved oxygen (D.O.) was estimated using the method of Labasque et al. (2004).

3. Genetic identification of mosquito larvae

3.1. Extraction of DNA from mosquito larvae

Using a scalpel under sterile conditions, the mosquito larvae were cut into small pieces and placed in 1.5 L Eppendorf tubes. The PureLink® Genomic DNA kits were used to extract the mosquito DNA (Invitrogen, Waltham, Massachusetts, USA). Briefly, each sample was mixed with tissue lysis buffer (between 180 and 250µL depending on the size of the larvae), treated with proteinase K (10µL for each 180µL of tissue lysis buffer), and incubated for 4 hours at 56°C. The supernatant was then transferred to a new tube in accordance with the manufacturer's (Invitrogen, Waltham, Massachusetts, USA) instructions. The lysate received 200µl of ethanol and 200µl of Lysis/Binding Buffer before being vortexed. The mixture was then put into a spin column and centrifuged for one minute at 10,000xg. Following two times of washings with wash buffers, DNA was eluted in 50µl of elution buffer and kept at -20°C until use.

3.2. Polymerase chain reaction (PCR)

To identify mosquito species, specific primers amplifying the cytochrome oxidase C.O. subunit I (COI) of mosquito’ mitochondrial DNA LCO1490:5′-GGTCAACAAATCATAAAGATATTGG-3′ as a forward primer and HCO2198:5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ as a reverse primer were used (Folmer et al., 1994). The PCR amplification was performed in a final reaction volume 2X (50 µl), containing 25µl of 2X master mix solution (i-Taq, iNtRON, Seongnam, Korea), 0.2µM (2µl) of each primer, 4µl of template DNA, and 0.2mg/ml of BSA and 14.5µl of nuclease-free water. The thermal cycling program of ticks consisted of an initial denaturation at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 1min, annealing at 46°C for 1min and extension at 72°C for 1min. A final extension was carried out for 10min at 72°C. PCR amplicon was run on a 1% agarose gel, stained with ethidium bromide to check the quality and yield of the PCR product using the transilluminator (U.V. transilluminator, Spectroline, Westbury, USA).

3.3. Sequence analysis

The PCR products were purified using a Macrogen reagent (Seoul, Korea). Single-strand DNA sequencing was performed, after which nucleotide sequences of mosquito larvae COI were aligned.

3.4. Bioinformatics

The obtained sequences were assembled using Chromas Pro 1.5 beta (Technelysium Pty., Tewantin, QLD, Australia). The newly COI sequences Culex pipiens (accession number: MK052921.1) and Culex antennatus (accession number: OP714215.1) were compared to those available in GenBank, using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Muscle alignment was used to align the sequences using MEGA 11.0 software. Kimura's two-parameter as used to calculate
the sequence divergences (K2P) (Kimura, 1980). To illustrate the patterns of species divergence, N.J. trees use the Tamura 3-parameter method (Tamura, 1992). Bootstrapping was performed in MEGA 11.0 (Kumar et al., 2004) with 1000 replications. Visualization enhancement was done using ITOI software (Letunic & Bork, 2021). The minimum spanning network for haplotype divergence was evaluated using inPHAP v.1.1, HapFlow v.1.1.2, and PopArt v.3.0.

4. Statistics

Data were analyzed using variance analysis (ANOVA) according to Bailey (1981). Data were coded and entered using the statistical package SPSS V.22. In addition, data were statistically described in terms of mean, median, standard deviation and standard error for quantitative variables, in addition to frequency for categorical variables. A probability value (P-value) less than 0.05 was considered statistically significant. The correlation coefficient between parameters was done in all the surveyed stations during the study period using MINITAB V.14 computer program. Data visualization becomes available using R-studio V.4.1.3.

RESULTS

1. The abundance of C. pipiens and C. antennatus larvae

As shown in Table (1) and Figs. (2, 3), a significant variation (P< 0.05) was detected between both investigated species in their calculated abundance throughout four seasons except for winter, where there was no significant variation (P> 0.05) in the abundance recorded for both species. While, summer showed the highest abundance for both C. pipiens and C. antennatus (about 683 and 400 larvae), superior to what was recorded in other seasons with a significant margin (P< 0.05). In the same manner, winter was significantly variant (P<0.05), compared to other seasons due to the low abundance recorded.

Table 1. Seasonal abundance of C. pipiens and C. antennatus larvae in En Nazla region, Ibshway, Fayoum Governorate, Egypt

<table>
<thead>
<tr>
<th>Mosquito Species</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clex pipiens</td>
<td>112±15</td>
<td>286±10</td>
<td>683±49</td>
<td>243±16</td>
</tr>
<tr>
<td>Culex antennatus</td>
<td>109±8</td>
<td>163±12</td>
<td>400±16</td>
<td>186±10</td>
</tr>
</tbody>
</table>
Seasonal Abundance and Molecular Identification of Aquatic Larvae of *Culex pipiens* and *Culex antennatus*

**Fig. 2.** Gradient column chart representing the average abundance of *Culex pipiens* and *Culex antennatus* larvae.

**Fig. 3.** Sidak simultaneous 95% C showing differences of the mean for (a) Winter, (b) Spring, (c) Summer, and (d) Autumn.

In addition, the heatmap map represented a comparison abundance between both investigated species throughout the study. As the color grade turns red, the abundance increases; at the same time, the color grade gets blue as the abundance of *C. pipiens* and *C. antennatus* declines (Fig. 4).

**Fig. 4.** Heatmap representing the abundance of seasons for both investigated species.

In addition, the highest correlation between seasons for *C. pipiens* calculated abundance was found between Winter and Autumn (0.83), while the lowest was recorded between winter and summer (0.22). Only one weak negative correlation was recorded between spring and summer (-0.34). Similarly, the correlogram revealed a powerful negative correlation between Spring and Autumn (-0.71) (Figure 5).
Fig. 5. correlogram of seasons abundance representing correlation relationship for (a) *Clex pipiens* and (b) *Culex antennatus*

2. **Physico-chemical parameters of breeding water:**

Temperature tends to follow semi bell shape trend; all season’s temperatures were significantly varied (P< 0.05), except between spring and autumn, there was no significant variation (P> 0.05). The same trend was also observed for TDS, except the only significant variation (P< 0.05) was recorded between winter and summer. The pH values follow an uneven trend with a high peak recorded in autumn, but no significant differentiation (P> 0.05) was recorded among all seasons in their calculated pH value. Finally, a reverse semi-bell shape trend was observed for D.O., with only significant variation (P< 0.05) observed between winter and summer (Table 2 and Figures 6 & 7).

Table 2. Physico-chemical parameters of *C. pipiens* and *C. antennatus* larval breeding water in En Nazla region, Ibshway, Fayoum Governorate, Egypt.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>19.65±0.87</td>
<td>26±0.46</td>
<td>28.03±0.19</td>
<td>25.30±0.31</td>
</tr>
<tr>
<td>pH</td>
<td>7.40±0.25</td>
<td>7.58±0.19</td>
<td>7.45±0.17</td>
<td>7.63±0.15</td>
</tr>
<tr>
<td>TDS</td>
<td>984±8.51</td>
<td>1000±8.97</td>
<td>1016.75±6.18</td>
<td>1000±5.52</td>
</tr>
<tr>
<td>D.O.</td>
<td>3.28±0.15</td>
<td>3.03±0.14</td>
<td>2.83±0.15</td>
<td>3.05±0.05</td>
</tr>
</tbody>
</table>


The environmental-abundance relationship was studied using pairwise regression, as shown in Figures (8 a & b); TDS is considered the most reliable factor that mosquito abundance depends on, followed by pH value for *C. pipiens* and temperature for *C. antennatus*. The model summary for both species was presented with $R^2 = 99.96$ and $99.93\%$, respectively. The regression equation to predict *C. pipiens* is illustrated as $C. pipiens$ abundance $= -12780 - 726.8 \text{ pH} + 18.567 \text{ TDS}$. Meanwhile, the regression equation to predict *C. antennatus* is illustrated as: $C. antennatus$ abundance $= -17813 - 39.53 \text{ Temp} + 19.002 \text{ TDS}$. 
Fig. 6. (a) box plot and (b) Gradient line chart of analyzed physical parameters.
Sum: Summer; Autu: Autumn.

Fig. 7. Sidak simultaneous 95% CIs differences of the mean for (a) Temp, (b) pH, (c) TDS, and (d) DO

Fig. 8. Pareto chart of standardized effects for (a) Clex pipiens and (b) Culex antennatus

Also, the correlogram revealed the most correlated physical parameters throughout the four seasons of the study, showing that in winter, the highest correlated parameters were
TDS and temperature. The temperature was positively correlated with pH (0.60 and 0.73, respectively) during autumn and spring. Counter, pH was powerfully negatively correlated with temperature during summer (-0.98), while TDS was the highest negatively correlated with a value of -1.0 and -0.99 with D.O. during spring and winter, respectively, where pH recorded the highest negatively correlated value (-0.85) with D.O. (Figs. 9 a, b, c & d).

![Correlogram of seasons physical parameters](image)

**Fig. 9.** Correlogram of seasons physical parameters representing their correlation relationship for (a) Winter, (b) Spring, (c) Summer, and (d) Autumn

3. **Genetic identification of collected mosquito species:**

The PCR amplification of mosquito larvae's DNA revealed positive amplification using primers that amplify the COI region. The amplicon size of both mosquito species was ~610 pb (Figure 10).

![Agarose gel electrophoresis](image)

**Fig. 10.** Agarose gel electrophoresis (2 %agarose, stained with ethidium bromide): analysis of PCR amplification for detection of *Cx pipiens* and *Cx antennatus*. M: 100 bp DNA marker; N: negative control; 1&2) (*C. pipiens*, 3) *C. antennatus* 610 bp.

The Neighbor-Joining method inferred the evolutionary history (*Saitou and Nei, 1987*). The optimal tree is shown (Figure 11). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown as branches color graded according to bootstrap value (*Felsenstein 1985*). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method (*Tamura 1992*) and are in the units of the number of base substitutions per site. This analysis involved 23 nucleotide sequences (21 donor sequences were evaluated from NCBI, including one sequence that acted as an outgroup (KR152335.1). Codon positions included were 1st + 2nd + 3rd +Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 428 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (*Tamura et al., 2021*), followed by visualization enhancement using ITOI software (*Letunic and Bork, 2021*).
Also, *C. pipiens* MK052921.1 (nucleotide sequences from the current study) showed a clear relationship (Figure 11) with species identified from Portugal (*C. pipiens* LC102133.1 and *C. pipiens* LC102132.1) and Kenya (*C. pipiens* KU187083.1) with an evolutionary distance of approx. 0.001164. Moreover, *C. antennatus* OP714215.1 (nucleotide sequences from the current study) displayed a close relationship to a species identified from Madagascar (*C. antennatus* MK033248.1) and Kenya (*C. antennatus* KU187037.1, KU187050.1, KU187048.1, and KU187038.1) with an evolutionary distance of approx. 0.00734.

![Fig. 11. Neighbor-Joining phylogenetic evolutionary tree.](image)

In addition, the minimum haplotype spanning network represented the haplotype and geographical location of the 23 nucleotide sequences evaluated in the present work. Revealing the haplotype diversity and change throughout geographical differences. Nucleotide diversity shows a pi-value of 0.096; the number of segregating sites was 80, while 53 parsimony-informative sites were observed. The Tajima's D statistic was calculated to be -2.6005 with $P(D >= -2.6005) = 0.999968$ (Figure 12).
DISCUSSION

As shown from obtained results, *Culex pipiens* and *Culex antennatus* reported a prevalence in Fayoum Governorate, Egypt, along different seasons; however, *C. pipiens* was more abundant than *C. antennatus* in the study area. The prevalence of the Culicine mosquito in Egypt was recorded by Kenawy *et al.* (2013), Hassan *et al.* (2014), Elhawary *et al.* (2021), and El-Mehdawy *et al.* (2021 & 2022). Summer recorded the highest abundance for mosquito species, superior to that recorded in other seasons, while winter was found to be the lowest abundance. Such results can be attributed to the increase in water temperature during summer (about 28.03°C, respectively) than winter (about 19.65°C, respectively). In agreement with the recorded results, White (1974) recorded that the temperature is an important factor affecting the development and growth of different mosquito larvae, Kenawy *et al.* (2013) reported a temperature range of 17-30°C for *C. pipiens* in Cairo Governorate and Elhawary *et al.*, (2020), who recorded a temperature ranged between 21 and 32°C for *C. pipiens*, and Culiesta longiareolata in different breeding sites in Egypt. Also, water breeding habitats in the study area were slightly alkaline, preferable to mosquito larvae (Pelizza *et al.*, 2007 & Oyewole *et al.*, 2009). The alkalinity of breeding water may be due to the nature of these habitats.

In addition, obtained results revealed that TDS is the most reliable factor affecting both mosquito species' abundance. The highest TDS (1016.75 ppm) was recorded in summer, and the lowest (984 ppm) was recorded in winter. In agreement with Williams (2001), we can say that increasing TDS values in summer than in other seasons can be due to Anthropogenic activities during summer. In 1995, Chavasse *et al.* reported that culicine mosquitoes prefer breeding sites with turbid water (polluted environments such as septic tanks and blocked drains) where they can successfully breed. This explains the highest
abundance of *C. pipiens* and *C. antennatus* during summer, which have the highest TDS values compared with other seasons, indicating the tolerance of both species in highly turbid water. The TDS content recorded a powerful negative correlation with D.O. during different seasons. These results are consistent with the previously recorded by Sehgal & Pillai (1970) and Tadesse et al. (2011).

On the other hand, COI sequences obtained from *C. pipiens* in the present study were found to be identical by 100.0% to sequences from *C. pipiens* recorded in Portugal (Mixão et al., 2016) in the GenBank, while sequences obtained from *C. antennatus* were identical 100.0% to sequences related to *C. antennatus* from Madagascar (Jeffries et al., 2018). Also, *C. pipiens* had a greater variation in COI than *C. antennatus*, which is consistent with (Werblow et al., 2013 & 2014). However, we found no indication of additional sub-structuring or taxonomic differentiation within *C. pipiens*, despite earlier research indicating morphological variation within the species (Fedorova and Shaikewich, 2007). Currently, biotypes pippins and antennatus are considered separate monophyletic evolutionary units experiencing incipient ecological speciation; therefore, they may be distinct phylogenetic entities (Fonseca et al., 2004 and Yurchenko et al., 2020). Different mating practices of the two biotypes were regarded as the first cause of sympatric speciation (Gomes et al., 2009). The limited level of hybridization is unidirectional, with male-mediated introgression from the biotypes of both species dominating (Gomes et al., 2009). The identical sequences may be caused by the similarity in the environment and climatic conditions found in Madagascar and Portugal to those in Egypt.

**CONCLUSION:**

It is deduced from the obtained data that *Culex pipiens* and *C. antennatus* had a remarkable abundance in Fayoum Governorate, Egypt, throughout different seasons of the year, depending on the nature of breeding habitats. Hence, its role in disease transmission in Fayoum Governorate needs more investigations.

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Seasonal Abundance and Molecular Identification of Aquatic Larvae of Culex pipiens and Culex antennatus


Walter Reed Biosystematics Unit (2022): Culex antennatus species page. Walter Reed Biosystematics Unit Website. http://wrbu.si.edu/vectorspecies/mosquitoes/antennatus


