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THE ANAL PLATES OF LARVAL *HYDRELLIA PAKISTANAE* (DIPTERA: EPHYDRIDAE)

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Abstract

The objective of this research was to describe the anal plates of the larval stages of the ephydrid fly, *Hydrellia pakistanae* Deonier, a biological control agent of hydrilla (*Hydrilla verticillata* (L.f.) Royle). This was accomplished using light microscopy coupled with a specialized staining technique; and scanning electron microscopy (SEM). All 3 instars of *H. pakistanae* were collected from hydrilla cultured in outdoor concrete-lined ponds. To ascribe a function to the anal plates, a modified staining technique was employed that uses a 5% silver nitrate solution, which, upon ionization, binds with chloride ions suggesting an osmoregulatory function. Under light microscopy the two anal plates appeared smooth, flat crescent moon-shaped structures that encircled the anus of the larva. SEM revealed the anal plates to be relatively smooth with an undulating surface pattern possibly an artifact of the high vacuum conditions associated with the SEM. This may therefore indicate a difference in cuticle thickness for the anal plates since the overall body cuticle did not exhibit the same surface changes. A distinct delineation in the form of a thin raised line of cuticle between the anal plates and the outer body cuticle could be discerned from both light microscopy and SEM. The anal plates vary in size from 200 μm to 750 μm in perimeter and 1800 μm^2 to 14000 μm^2 in area depending on instar. A highly positive correlation in anal plate size in relation to larval instar was also detected; i.e., larger anal plates were associated with later instars. The anal plates darkened rapidly after immersion in a weak silver nitrate solution with subsequent exposure to light indicating that they may play an important role in maintaining internal ionic equilibrium.

Key Words: *Hydrellia pakistanae*, anal plates, anal organ, osmoregulation, silver nitrate, epithelia cells

RESUMEN

El objetivo de esta investigación fue describir los platos anales de estadios larvales de la mosca ephydrida, *Hydrellia pakistanae* Deonier, un agente de control biológico de hydrilla (*Hydrilla verticillata* (L.f.) Royle). Se utilizó microscopia de luz con una técnica especializada de tinción y microscopia de barrido electrónico (SEM). Los 3 instares larvales de *H. pakistanae* fueron colectados de hydrilla cultivada en estanques de concreto al aire libre. La técnica de tinción modificada utiliza una solución de nitrato de plata al 5%, la cual, al ser ionizada, se une con iones de cloro, lo cual sugiere funciones de osmorregulación. Usando microscopia de luz los dos platos anales aparecen como estructuras lisas y planas en forma de luna creciente rodeando al ano. Utilizando SEM se observan relativamente lisos con un patrón ondulado en la superficie, posiblemente un artefacto de las condiciones de vacío de SEM. Esto puede indicar que los platos anales tienen un grosor diferente al resto de la cutícula corporal pues esta no exhibió estos cambios. Una distintiva línea delgada, elevada entre los platos anales y la cutícula corporal exterior, fue observada con microscopia de luz y SEM. El tamaño de los platos anales varió de 200 μm a 750 μm de perímetro y 1800 μm^2 a 14000 μm^2 de área dependiendo del instar. Una alta correlación positiva entre el tamaño de los platos anales y el instar larval fue observada; i.e. los platos anales más grandes se observaron en los estadios larvales más avanzados. Los platos anales oscurecieron rápidamente después de ser inmersos en la solución débil de nitrato de plata y su subsecuente exposición a la luz, lo que indica que estos pueden jugar un papel importante en el mantenimiento del equilibrio iónico interno.

Hydrellia pakistanae Deonier (Diptera: Ephydridae), is a host specific biocontrol agent introduced in the USA in 1987 for the management of *Hydrilla verticillata* (L.f.) (Royle) (Center et al. 1997). *Hydrellia pakistanae* is native to Indonesia and has been collected from Pakistan, northern China, and southern India (Deonier 1978). The aquatic larvae damage the plant by mining the

leaves during its three larval stadia (Deonier 1971; Freedman et al. 2001). Deonier (1978) indicated that many species in this genus are important herbivores of various species of aquatic plants especially in eutrophic ecosystems. He also indicated that large populations of *Hydrellia* larvae can impact the biomass of their target host plant, thus influencing littoral plant com-

munities (Deonier 1971). Such is the case for *H. pakistanae*, where numerous studies have shown that sustained feeding can reduce hydrilla photosynthesis thereby impacting biomass, fragment viability, and tuber production causing shifts in plant assemblages toward increased native plant abundance and diversity (Grodowitz 2000; Freedman et al. 2001; Owens et al. 2008).

Hydrilla is an extremely aggressive submersed freshwater plant that forms thick mats of biomass at the water surface in a variety of freshwater habitats (Langeland 1990). It causes severe ecological and economical damage to aquatic ecosystems by impeding water flow, displacing native plant species, and disrupting boat traffic (Godfrey et al. 1996; Haller 1978). Hydrilla is currently found on every continent except Antarctica, and has become a major problem in waterways around the world (Pieterse 1981). The true origin of hydrilla is still unknown. However, Cook and Löönd (1982) believed the origin could be traced to the subtropical regions of Asia, where hydrilla is still considered a problem (Pieterse 1981).

The larvae of *H. pakistanae* are entirely aquatic and, as such, must have evolved specialized structures and/or physiological mechanisms to allow maintenance of internal osmotic conditions when exposed to a highly dilute external medium. However, osmoregulation in this species has not been examined in great detail. To aid in maintaining internal osmotic equilibrium, other species of aquatic insects have evolved specialized internal and external structures (Bradley 1985; Phillips 1981) including chloride epithelia, chloride cells, anal papillae and anal organs (Komnick 1977).

Deonier (1971) noted the presence of 2 plates surrounding the anus in the larva of *Hydrellia* spp. and identified these structures as anal plates. However, Deonier (1970) neither described the morphology of the anal plates in detail, nor attempted to delineate a function. Information on the anal plates or anal organs in other aquatic dipteran larvae is minimal, but several authors have indicated an osmoregulatory or ion transport function based on specialized staining techniques, electron microscopy, and physiological studies (Stoffolano 1970; Schwantes 1989; Schwantes and Seibold 1989; Reeves 2008). This paper describes the anal plates of *Hydrellia pakistanae* in detail using both light and scanning electron microscopy; and based on these observations suggests a possible osmoregulatory function.

MATERIALS AND METHODS

Photography Techniques (*Hydrellia pakistanae*)

In December 2008, all 3 larval instars of *H. pakistanae* were collected from hydrilla cul-

tured in outdoor concrete-lined ponds at the U.S. Army Engineer Research and Development Center (ERDC), Vicksburg, Mississippi. The anal plates of 30 larvae (10 of each instar) were examined using light microscopy at magnifications ranging from 7 X to 80 X. Specimens examined were preserved in 70% ethanol after digital images had been created for subsequent analysis.

Images and measurements were determined for each instar using a dissecting microscope (Nikon® SM21550, Melville, New York). Micrographs were captured using a digital camera (Accu-Scope®, model 122CU, Commack, New York) in conjunction with the software program Micrometrics (Micrometrics® Commack, New York) (<http://www.micrometrics.net/>), which allowed capture of high quality digital images, and the ability to make detailed measurements of the perimeter and surface area of the anal plates.

The scanning electron microscope (SEM) used in this study was a low-pressure SEM (Nova NanoSEM 630, FEI Co.), which eliminates the need for sample preparation, or application of a conductive coating. The Nova NanoSEM 630 has a field-emission gun (FEG) with a tungsten filament. The imaging conditions employed an accelerating voltage of 25 to 30 KeV and 1.69 mA with resolution of 1.6 nm at 1 kV. Imaging of samples used both a TLD (through the lens detector) and a vCD (variable contrast detector) detector. Images of these samples were collected over a period of 30 seconds, and stored as 2 MB tiff files.

Soft-bodied larva, especially dipterans, are extremely difficult to observe via SEM using typical preparation methods without the induction of shrinkage and deformation, which can greatly obscure important features. For SEM, *H. pakistanae* larvae were observed alive, which was made possible by use of the low vacuum associated with the chamber of the Nova NanoSEM 630. For viewing, *H. pakistanae* larvae were affixed to a specimen stub using a piece of double-sided carbon tape, which covered the top of a specimen stub. Use of living specimens under low vacuum permitted observation of external structures with only limited artifacts induced due to typical fixation methods, dehydration, critical point drying, and subsequent metal coating.

Staining Technique

In an attempt to ascribe a function to the anal plates, a modified staining technique was used as described by Stoffolano (1970) and Schwantes (1989). The technique uses a 5% silver nitrate solution in which ionized silver binds with chloride ions to form silver chloride; the latter subsequently darkens upon exposure to light (Komnick 1977; Bradley T. J. 1985). Hence, if a structure dark-

ens after staining it presumably contains higher concentrations of chloride ions and thus indicates a possible osmoregulatory function. The validity of this staining technique for providing evidence for an osmoregulatory function was established by Schwantes & Seibold (1989). Such a staining technique for the detection of ion transport has also been applied to other invertebrates with excellent results (Barra et al. 1983; Kikuchi & Shi-raishi 1997).

Larvae of each instar were rinsed in deionized water and then placed in a 5% solution of silver nitrate for 5-10 s. The larvae were then rinsed for 60 s in deionized water and subsequently placed on a glass slide in a small quantity of 5% magnesium sulfate solution (25 °C). The magnesium sulfate was used to slightly relax the larvae by interrupting muscle activity thereby assuring exposure of the anal plates. The slide was placed on the microscope stage and exposed to a bright light source. After 5 to 10 min, the ventrally located anal plates were examined for evidence of darkening.

Statistics

Correlation analyses on anal organ size throughout the larval stages were accomplished using Statistica (Statistica Version 9.0, 8/11/2009, StatSoft, Tulsa, Oklahoma). Test of significance was conducted at $P = 0.05$ unless otherwise noted, while separation of means was accomplished using the Neuman-Keuls post-hoc test. A log transformation was used when the means were proportional to the variances.

RESULTS

Anal Plate Description

The 2 anal plates of *H. pakistanae* larva are normally transparent to semi-transparent and located on the ventral surface encircling the anus on the 10th larval segment. The anal plates are roughly crescent shaped and appear symmetrical on either side of the anus (Fig. 1b-d). The

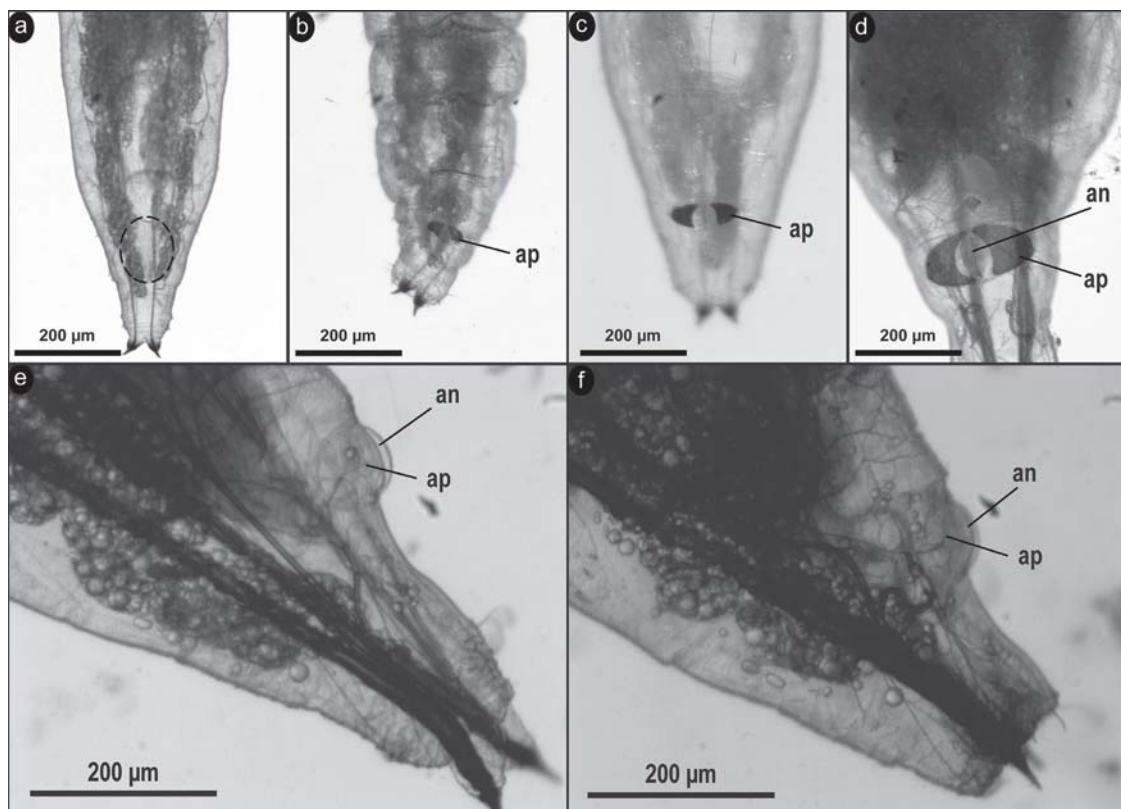


Fig. 1. Ventral and lateral views of the anal organ of *Hydrellia pakistanae*. ap = anal plate, an = anus. (a) Ventral view of the unstained anal plates of a first instar with dashed circle around anal plate location. Note the plates and anus are essentially transparent and difficult to discern. (b) First instar with silver nitrate stained anal organs, (c) Second instar with very distinct crescent-moon shaped anal plates, (d) Third instar larva with slightly transparent anal plates. (e) Lateral view of the anal plates of a third instar, not stained. (f) Lateral view of the anal plates of a third instar with a light silver nitrate stain.

distinct crescent shapes can be seen in the third instar with the first and second instars having less definition but still maintaining a roughly crescent shape (Fig. 1d). Under light microscopy the plates appear smooth, and lie flat or slightly raised above the body (Fig. 1e, 1f). The anal plates are delineated by a distinct line that is difficult to discern when not stained (Fig. 1A).

Under SEM at lower magnifications the anal plates appear relatively smooth similar to that observed under light microscopy (Fig. 2a). However, at higher magnifications an undulated surface to the anal plates is exhibited (Fig. 2b). Whether this undulating surface appearance is an artifact of the high vacuum conditions associated with the

use of living specimens under low vacuum is not known. If high vacuum is the cause, it may indicate a difference in thickness of the cuticle of the anal plates and that of other areas of the body, since the overall body cuticle did not exhibit the same surface change. As under light microscopy, SEM micrographs reveal a distinct delineation in the form of a raised narrow line between the body cuticle and the anal plates (Fig. 2b). At 12,000 X, the cuticle of the anal plate surface appears to have small holes or pits though more detailed electron microscopy is needed to determine the exact nature of these structures separating them from possible artifacts (Fig. 3).

There are significant differences in the perimeter and surface area of the anal plates with each successive instar (Fig. 4). The anal plates vary in size from about 200 μm to 750 μm in perimeter and 1,800 μm^2 to 14,000 μm^2 in area depending on instar. A significant positive correlation ($P < 0.05$, $r > 0.9$) exists between anal plate perimeter and surface area with larval body width and length (Fig. 5). These correlations may indicate that the functioning of the anal plates remains constant for all instars. It is likely that if later instars had limited use of the anal plates, the anal plate size would remain small relative to overall body width and length.

DISCUSSION

The most comprehensive review to date of the literature on *Hydrellia* spp. is that of Deonier (1971). Our morphological description of the anal

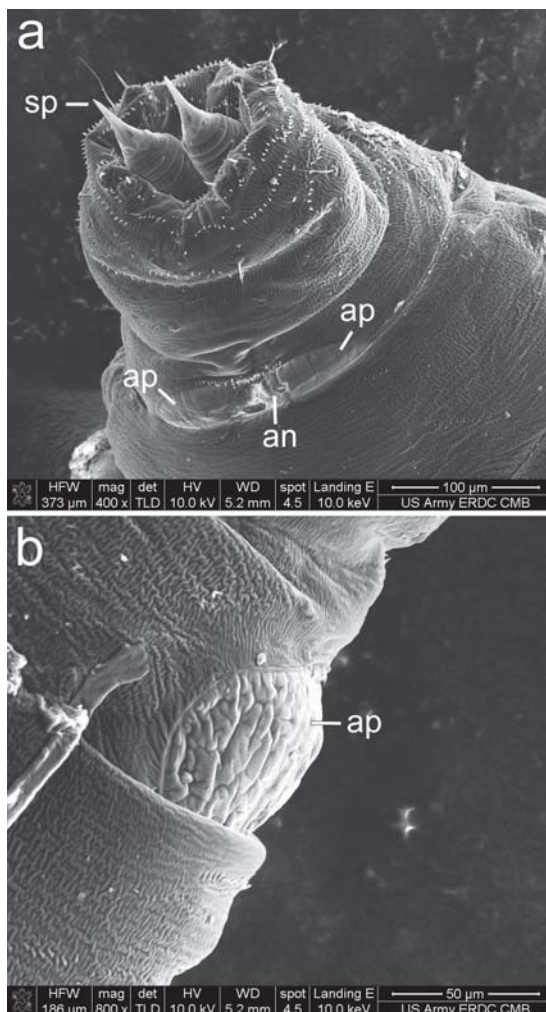


Fig. 2. SEM micrographs of the anal plates of larval *Hydrellia pakistanae*. sp = Spiracles, ap = anal plate, an = anus. (a) Overall morphology of the anal plates (b) Close-up of the anal plates from a lateral view. Note the undulating surface appearance under higher magnification which differs from that of the surrounding cuticle.

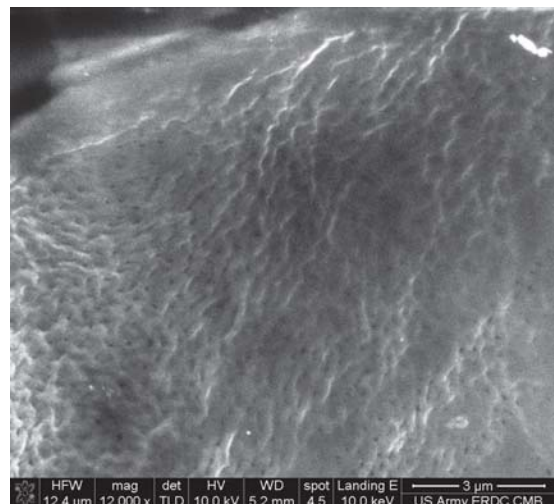


Fig. 3. SEM of the surface of the anal plates of *Hydrellia pakistanae* at 12,000 X. Note the small pits or possible holes scattered throughout the surface. While these do not appear to be artifacts due to specimen preparation, more microscopy work is needed to characterize them.

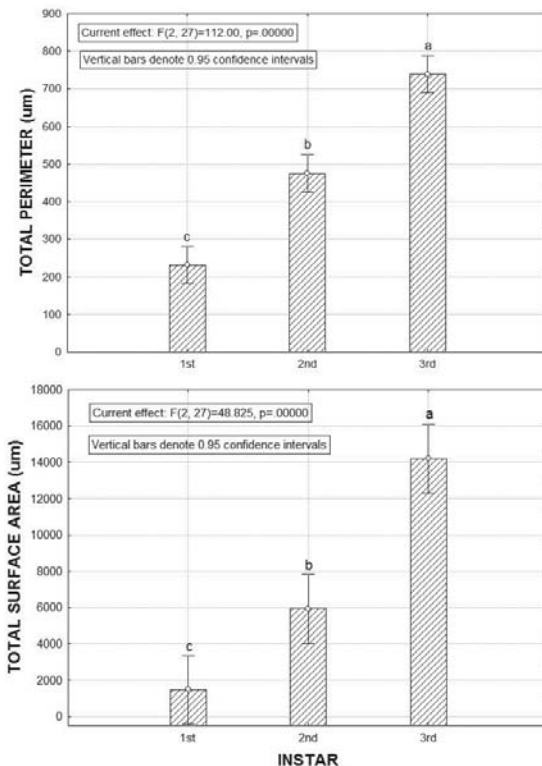


Fig. 4: Total perimeter (μm) and surface area (μm^2) for the anal plates for all three instars. Means with different letters are significantly different at $P = 0.05$.

plates of *H. pakistanae* expands upon Deonier's initial research, where he noted the presence of anal plates. However, he did not describe the morphology in respect to the size and shape of these organs. We observed the same general findings as Deonier, but with more detail. The distinct crescent moon shaped anal plates apparently are characteristic of *H. pakistanae*. However, how does the size and shape of the anal plates of *H. pakistanae* differ from other species of dipteran larva?

Stoffolano (1970) described differences among dipteran species in the size and shape of their anal organs. He suggests the differences between the sizes of the anal organ may reflect larval habitat differences, which, through selection, have favored one size organ over the other; larger anal organs are found predominantly in those species that reside in more dilute freshwater habitats. Further, Stoffolano (1970) described the anal plates or anal organs of *Musca domestica* L. as having lateral arms that are reduced so that the plate is short, squat, and roughly butterfly-shaped. In contrast, *Musca autumnalis* De Geer, a close relative to *M. domestica* has an anal organ with lateral arms that are large and that wrap

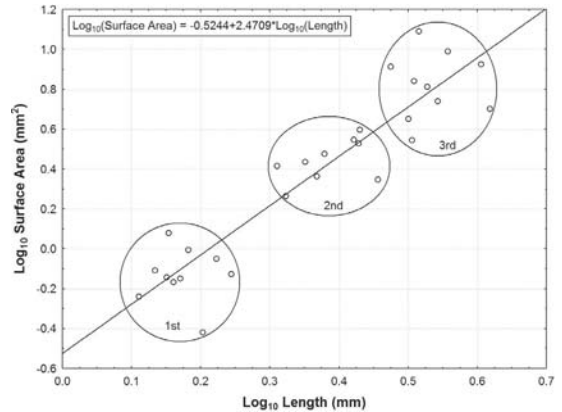


Fig. 5: Graph of larval surface area (\log_{10}) versus larval length (\log_{10}) for all instars combined. Correlation was significant at $P < 0.05$ with r values of 0.95 with a sample size of $n = 30$. Note the distinct groupings for each instar indicating increasing size of the anal organ for each successive instar.

around 3/4th of the circumference of the posterior end of the larva. *Musca autumnalis* resides in fresh manure from cows (*Bos* spp.; Artiodactyla: Bovidae) fed pasture grasses, a larval habitat that is relatively low in dissolved ions (i.e., osmolality typically < 200 mmol/kg); i.e., freshwater (Grodowitz 1985). In contrast, *M. domestica* lives in a wide variety of habitats, but is more successful in those that are higher in dissolved ions; i.e., having a higher osmolality, such as chicken (*Gallus gallus domesticus* (L.); Galliformes: Phasianidae) feces, which are a mixture of feces and urine. Stoffolano (1970) examined figures from Zimmin (1951) of the anal plates of Muscidae and noted that besides differences in anal organ size, there is much variation in surface appearance and shape. For example, the larvae of *M. domestica* have smooth flat plates, while others like *Stomoxys calcitrans* (L) have bulbous or "hemorrhoid-like" surface structures. Based on Stoffolano's work, the relatively small size of the anal plates of *H. pakistanae* is not characteristic for dipteran larvae, like *H. pakistanae*, which thrive in highly dilute freshwater environments. Reasons for this discrepancy are not fully understood, and more research is needed especially using more detailed transmission electron microscopy and other physiological experimentation.

The images *H. pakistanae* larvae using SEM photography documented 2 possible artifacts due to exposure to high vacuum conditions and rapid desiccation. First was an unusual undulating pattern to the entire body of the larvae which was even more pronounced for the anal plate cuticle. The first artifact is most likely due to the vacuum environment of the SEM chamber and subse-

quent desiccation of the specimen. However, the more pronounced 'wrinkling' of the anal plate cuticle may indicate that it is thinner since it has a higher degree of shrinking than the rest of the body cuticle. Stoffolano's (1970) work on dipteran larvae histological sections of the anal plate cuticle showed the plates to be thinner than the rest of the body cuticle. This supports the idea that the higher degree of shrinking seen in *H. pakistanae* anal plate cuticle is a result of a thinner cuticle.

Stoffolano also noted spherical structures underneath the anal plate cuticle of several species of dipteran larvae much like our observations of small pits or holes in *H. pakistanae*. He described these structures as large epidermal cells which contained polytene chromosomes that gave a cobblestoned appearance to the cuticle. More morphological studies of these spherical structures, especially using transmission electron microscopy, may be necessary to further describe their morphology, and to ascribe a function of the small pits.

Many scientists have used the silver nitrate staining method as a reliable tool to ascribe the function of osmoregulation to the anal plates or anal organs of many dipterans (Simmin 1951; Stoffolano 1970; Komnich 1977; Bradley 1985; Schwantes 1989; Schwantes & Seibold 1989; Reeves 2008). The anal plates of *H. pakistanae* stain a dark brown after immersion in a weak solution of silver nitrate and subsequent exposure to high light intensity. This indicates the presence of high concentrations of chloride ions within the anal plate tissues. The presence of high chloride concentrations implies an osmoregulatory function; and similar staining techniques have been used to ascribe osmoregulatory functions to tissues in a wide range of aquatic or terrestrial insects (Komnich 1977; Bradley 1985). Such chloride concentrating epithelium is typically found in freshwater organisms that must have a mechanism to maintain internal osmotic pressures when exposed to an external medium which is highly dilute relative to internal osmotic pressures. Based on Stoffolano's (1970) work, the presence of osmoregulating anal plates in *H. pakistanae* is typical of dipterans residing in a low salinity environment (fresh water) that, therefore, have a need to maintain internal osmotic conditions at a higher level than their surrounding habitat.

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