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Reproductive Biology of the Predatory Water Bug, *Diplonychus annulatum* (Fabricius) (Hemiptera: Belostomatidae) on *Anopheles* Mosquito as Prey Under Laboratory conditions

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ABSTRACT

The biology of the predatory water bug, *Diplonychus annulatum* (Fabricius) (Hemiptera: Belostomatidae), was studied under laboratory conditions using Anopheles (Diptera: Culicidae) wrigglers as prey. *D. annulatum* exhibits paurometabolous type of development passing through egg, nymph, and adult stages. An adult female lay about 6-14 egg batches with an average of 55.95±2.36 eggs per batch. Incubation period ranges from 8-11 days with a mean of 9.28±0.06 days and 79.63 % hatchability. The nymph passed through five instars before reaching the adult stage. The mean durations of the different instars were: 4.62±0.16, 4.22±0.15, 5.24±0.24, 8.06±0.58, and 15.86±0.61 days, respectively. The developmental period was 28-54 days ($\bar{x}$=38.14±0.85 days). Longevity of females ranged from 110-283 days ($\bar{x}$=196.76±12.53 days) while that of males was from 115-282 days ($\bar{x}$=212.38±14.04 days). *D. rusticus* was found to be a voracious predator of Anopheles in the laboratory. The mean consumption rates for the respective instars were 10.0±0.35, 17.22±0.31, 36.48±0.73, 98.18±2.89, and 171.88±4.23 individual wrigglers per day.

1. Introduction

The mosquito *Anopheles* (L.) (Diptera: Culicidae) is a major vector of malaria. Mosquitoes such as *Anopheles* exploit a variety of aquatic habitats and containers such as ponds, tanks, tree holes, tires, mud pots, drums, disposed plastic cups, etc. as developmental or breeding sites, many of which are inaccessible for larvicide control by chemical means. In addition, continuous application of pesticides to regulate mosquito populations bears adverse impact due to development of resistant strains and death of non-target species (Chandra *et al.*, 2008). Biological control is the use of parasitoid, predator, pathogen, antagonist, or competitor populations to suppress a pest population, making it less abundant and thus less damaging than it would otherwise be (Van Driesche & Bellows, 1996).

Among the predators of *Anopheles*, hemipterans under the family Belostomatidae are less studied

(Ohba, 2019). Belostomatid bugs or water bugs of the genus *Diplonychus* Laporte, like *D. rusticus* (Fabricius) and *D. annulatum* are known to prey upon mosquito wrigglers (Sivagnaname, 2009; Aditya *et al*., 2004; Saha *et al*., 2007; Ranjini & Kakkassery, 2019) and prefer them under different prey abundance levels and availability (Saha *et al*., 2010 & 2013). They possess many desirable attributes as good biological control agents such as high reproductive potential, competitive ability, searching efficiency, rapid colonization, host specificity, adaptability, and synchronization with available hosts (Sivagnaname, 2000).

2. Aim and Objectives

The water bugs may be used as biological agent against all disease spreading mosquitos as safe and secured because pesticides can give rise to resistant coleopterans. However, there is limited information on the biological characteristics of *D. annulatum* on *Anopheles*. Thus, thorough understanding of their biology and predator-prey interactions is essential in knowing its potential as biological control agent against the malarial mosquito.

3. Materials and Method

The study was conducted at the laboratory of PG Department of Zoology, Jai Prakash University, Chapra (Bihar) mainly from June 2024 to April 2025. The experimental water bugs were collected from Daha river on different sites with abundant lettuce plants in research and different stages of *D. annulatum* were placed inside rectangular plastic rearing trays (33 x 23 x 10 cm) with water lettuce including water taken from the River sites and brought to the laboratory. Males with eggs on their backs were individually placed in rectangular rearing trays. Adult males without eggs and females were combined in rectangular rearing trays separately from the nymphs.

The mass rearing of mosquitoes based upon the method of Garcia (2009), starting with an initial population hatching from eggs and subsequent rearing of wrigglers was maintained as a ready stock of sufficient third and fourth instar larvae. The mosquito larvae served as food for the water bugs during the study period.

3.1 Life History Determination

The water bugs were mass reared in the laboratory for the biological study (Figure 1).

Fig. 1 Laboratory set-up of the life-history of *Diplonychus annulatum*

There ten males with eggs on their backs were used as starting population. They were placed separately in 10 different plastic rearing trays (120 mm diam., 37 mm high) with tap water at ambient room temperature. Larvae of *Anopheles* were used as prey for male water bugs. Initially, five nymphs that hatched at the same time from each egg cluster were combined and reared in a plastic cup (120 mm diam. and 37 mm high). Observations were based on 10 egg clusters or a total of 50 nymphs. The newly hatched nymphs are the first instar bugs. The first and second nymphal instars were fed with 20-30 smaller wrigglers whereas later stages were given 50-100 third instar wrigglers. The number of wrigglers provided to each nymphal instar was *ad libitum*. The nymphs were observed daily for molting. After the first molt (second instar), each nymph was placed individually in a separate rearing cup with aged tap water and wrigglers. Water in the cup was replaced when needed and the number and duration of each instar were recorded until the predator reached the adult stage. The number of prey wrigglers eaten by each instar of water bug in 24 h was estimated by subtracting the number of alive wrigglers from the total number of individuals offered. The sizes of the different stages of *D. annulatum* were determined by placing each of them one by one in a small Petri dish with a ruler securely placed at the bottom. Five representative nymphs that hatch from one egg cluster were placed in a plastic container (120 mm diam., 37 mm high). Measurements were based on 10 egg clusters or a total of 50 water bugs. The appearance of the different nymphal instars was described and their behavior was observed.

3.2 Sex Ratio Determination

Upon reaching the adult stage, the sex of the predator was determined and the ratio between male and female was established. Observations were based on the 50 nymphs previously observed for life history study. Likewise, the appearance of and differences between the male and female were described.

3.3 Fecundity and Percent Hatchability

The sex of the adults that emerged from the last instar nymphs was recorded. Twenty pairs of adult water bugs that were reared from the initial 50 nymphs were placed in a plastic container with water, small twigs and water lettuce, and were fed with third to fourth instar mosquito larvae. The number of days from the time the adults were paired to the time when the female laid her first egg cluster was recorded as the pre-oviposition period. Once the female laid her eggs on the back of the males, the female adult was temporarily separated from the male and reared in separate plastic cup using third instar wrigglers. Meanwhile, the separated male with eggs on its back was fed with mosquito wrigglers until the eggs hatched into nymphs. The incubation period was recorded. Egg hatchability was calculated using the formula:

% Egg hatchability=Total no. of hatched eggs/Total no. of laid eggs×100

Each brooded male was returned to its mating pair as soon as egg hatching was completed. The duration of second laying from re-introduction of the male was recorded. Alternate mating and separation was done until females stopped laying eggs. The total number of eggs laid by a female and the interval between egg laying was recorded. At the same time, the nymphs were maintained in separate containers.

Adult longevity Adult longevity was based on 40 surviving individuals (20 pairs) which were paired out of the 50 randomly selected water bugs. Males and females were allowed to mate and reproduce until death.

4. Results and Discussion

4.1 Life History of D. annulatum

Like all hemipterans, *D. annulatum*, underwent paurometabolous type of development, passing through the egg, nymph, and adult stages. The durations of these different developmental stages, with diet consisting only of live mosquito larvae, is presented in Table 1.

Table 1 Life-history of water bug on *mosquito* wrigglers under laboratory conditions

Life stages	n	Range	Mean± SD*
Pre-oviposition period	10	6-31	25.4±2.27
Egg incubation period (days)	17	8-11	9.28±0.06
Fecundity(eggs)		19-88	55.95± 2.36
Percent hatchability	17	28-97	70.78
Nymphal duration (days)			
First instar	50	4-8	4.62±0.16
Second instar	50	3-10	4.22±0.15
Third instar	50	3-10	5.24±0.24
Fourth instar	50	6-19	8.06±0.58
Fifth instar	50	9-29	15.86± 0.61
Total nymphal period	50	28-54	38.14± 0.85
Adult longevity			
Male	20	115-282	210.65± 14.65
Female	20	110-283	199.1±12.80

The incubation period ranged from 8-11 (mean: 9.28±0.06) days. Sivagnaname (2000) observed slightly shorter incubation period (8.9 days) when *D. annulatum* was fed with limited number of *Anopheles* wrigglers than when fed *ad libitum* (9.4 days). *D. annulatum* underwent five nymphal instars. Newly hatched first instar nymphs are transparent but after a few minutes, their bodies turn light green to brown. The second nymphal instar had the shortest developmental period, 3-10 (mean: 4.22±0.15) days, followed by the first instar, 4-8 (mean: 4.62±0.16) days. Third instar was 3-10 (mean: 5.24±0.24) days. On the other hand, the fourth nymphal instar lasted 6-19 (mean: 8.06 ± 4.10) days. In contrast, the fifth instar had the longest duration of 9-29 (mean: 15.86±0.61) days.

4.2 Total Nymphal Development

The total nymphal development ranged from 28-54 (mean: 38.14±0.85) days. Longer nymphal period (41-63 days) was obtained when *D. annulatum* was fed with mosquito larvae and pupae and shorter (24-48 days) when young *R. rubiginosa* snails were added to the diet (Reyes *et al*., 1970). Therefore, the incorporation of snails in their diet could be considered in the development of a mass rearing technique for *D. annulatum* in the laboratory.

4.3 Pre-oviposition period

The pre-oviposition period (or the time from emergence of the adult female to when it laid its first egg or batch of eggs) ranged from 6-31 (mean: 21.53±3.40) days.

4.4 Fecundity

After almost 10 months of captive breeding, some females were still alive and within 285 days after emergence, they laid 6-14 egg batches with an average of 55.95±2.36 eggs per egg batch. Reyes *et al.* (1970) indicated that *D. annulatum* laid 60-100 eggs under natural conditions but only 50-100 when fed with the snail *R. rubiginosa*. The relatively high fecundity of *D. annulatum* makes this predator a promising biological control agent for field releases against *Anopheles*. Newly laid eggs are oval and light green, but brownish apically. Hatchability was 27.96-97.40% (Table 2).

The low hatchability (27.96%) was due to the removal of eggs by the male, which eventually did not hatch. However, Reyes *et al.* (1970), found that egg hatchability in *D. annulatum* can range from 76.2-100% when fed with the snail, *R. rubiginosa*.

4.5 Sex Ratio

The sex ratio of *D. annulatum* adults emerging from nymphs observed for development is 1.94 males for every female. This is similar to that obtained by Sivagnaname (2000). There are more males than females which seems a good proportion because the males are the ones that brood the eggs until hatching. In this study, there was an instance when gravid female lay eggs in the water since its male counterpart is still brood the eggs previously laid by the female. Brooding of eggs by the male ensures egg survival. Therefore, more males would mean more individuals that will brood eggs, and higher survival. Sex ratio determination is important in the development of mass rearing techniques and in life history studies.

4.6 Adult longevity

Longevity of females ranged from 110-283 (mean: 199.1±12.80) days while that of males ranged from 115-282 (mean: 210.65 ± 14.65) days. The very long life span of adult *D. annulatum* is a very desirable attribute since it will contribute further to the reduction of *Anopheles* population in the field for about 280 days.

Table 2 Reproductive potential and egg hatchability of *Diplonychus annulatum*

Pair Number	Hatched eggs/ Batches	Mean hatched eggs/batch (a)	Mean hatched eggs (b)	Hatchability % (b/a)×100
1	13	61.85	32.20	52.06
2	10	61.30	31.88	52.00
3	12	59.20	54.20	91.55
4	12	59.19	43.60	73.66
5	12	34.69	12.20	35.16
6	14	68.66	61.02	88.87
7	6	60.69	38.80	63.93
8	10	61.42	38.09	62.01
9	14	51.59	45.20	87.61
10	9	38.46	10.95	28.47
11	10	51.40	27.08	52.68
12	9	75.46	60.94	80.75
13	13	51.48	37.28	72.41
14	13	54.17	49.04	90.52
15	10	51.80	51.12	98.68
Mean	11.13	56.09	39.57	68.69

4.7 Description of the Life Stages

4.7.1 Eggs

The eggs of *D. annulatum* are always laid on the back of the male. Newly-laid eggs are round to oval and light green with brownish apical portion. The female oviposits her eggs on its male partner's back from 12 - 24 h, usually in clusters or in batches. All nymphs and adults are the typical belostomatids, i.e., aquatic bugs with dorso-ventrally flattened bodies that are broadly oval and tapering caudally. For this species, the general body color is from beige to brown except in the neonate nymphs. They have raptorial forelegs, and natatorial mid- and hind-legs. The tarsi are one-segmented tarsi, with two pairs of claws. In addition, the rostrum is short, reaching only half of the fore-coxa and the eyes are conspicuous, but the antennae are not, i.e., they are inconspicuous and somewhat concealed beneath the eyes.

4.7.2 First nymphal instar

Newly hatched nymphs are slightly transparent (Figure 2). However, after a few seconds, the head turns light brown while the middle portion of the abdomen becomes light green. After 24 h, the whole body turns beige to light brown. The body measures 3.0-4.0 mm (mean: 3.06±0.02) long and 1.5-2.0 mm (mean: 1.97±0.02) wide.

4.7.3 Second nymphal instar

The second instar nymph is almost similar to the first instar (Figure 2), but bigger, i.e., 3.0-5.0 (mean: 4.91±0.05) mm long and 2.0-3.0 (mean: 2.79±0.05) mm wide. The eyes are somewhat reddish ventrally but black dorsally. Coxae of all legs have few setae or hairs. Tibiae of middle and hind legs are flat and fringed with hairs.

4.7.4 Third nymphal instar

The third nymphal instar (Figure 2) ranges from 6.0-7.0 (mean: 6.79±0.05) mm long and 3.0-5.0 (mean: 3.96±0.05) mm wide. Pronutum appears somewhat fused with the head and the meso- with the metathorax. Setae on the coxae and tibiae are distinct.

4.7.5 Fourth nymphal instar

The fourth nymphal instar (Figure 2), is quite similar to the third but is larger, being 8.5-10.0 (mean: 9.42±0.07) mm long and 5.5-6.0 (mean: 5.37±0.05) mm wide.

4.7.6 Fifth nymphal instar

The fifth instar (Figure 2) is, again, similar with the preceding instars in appearance, shape, and color, only larger. The body length ranges from 12.0-13.0 (mean: 12.45±0.06) mm and the width, 7.0-8.0 (mean: 7.53±0.06) mm.

The pronutum and the head as well as the meso- and metathoraces appear more fused. Tibial setae are more distinct. Prior to transformation into adult, the yellow to orange wing pads of the fifth instar become more noticeable. This indicates that in about 24 h the exuviae of the fifth instar nymph will be shed off and subsequently the adult will emerge. As mentioned above, the different nymphal instars of *D. annulatum* show similarities in appearance, shape, and color, except for the size. As the nymph matures, the body increases from 4.0-13.0 mm in length and from 2.0-8.0 mm in width.

4.7.7 Adult

The body of adult *D. annulatum* is more obviously oval than those of the nymphs, and being dorso-ventrally flattened, the predator may be mistaken for a cockroach to an untrained eye. It is 15.0-17.0 (mean: 15.96±0.08) mm long and 8.5-10.0 (mean: 9.03±0.04) mm wide (Table 3).

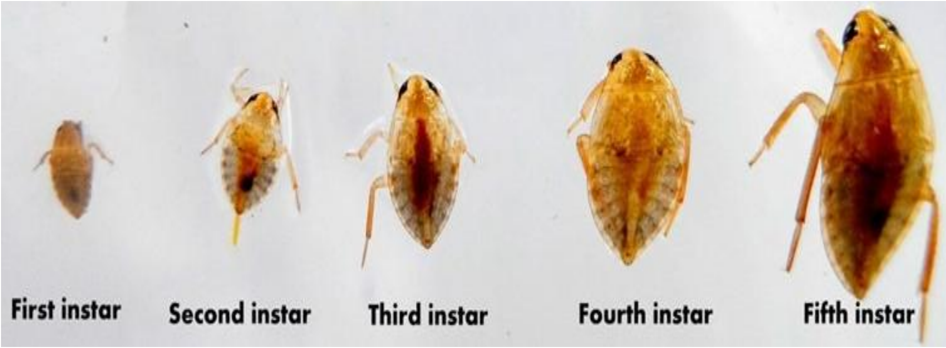

Fig. 2 Different nymphal instars of *Diplonychus annulatum*

Table 3 Measurement of the different life-stages of *Diplonychus annulatum*

Stages	Length range (mm)	Mean±SD (mm)	Width range (mm)	Mean±SD (mm)
Nymphal instars				
First	3.5-4.5	3.08±0.003	1.7-2.2	1.99±0.02
Second	3.5-5.5	4.93±0.005	2.2-3.2	2.81±0.05
Third	6.5-7.5	6.81±0.005	3.2-5.2	3.98±0.05
Fourth	8.5-10.5	9.44±0.007	5.7-6.2	5.39±0.05
Fifth	12.5-13.5	12.47±0.06	7.2-8.2	7.55±0.06
Adult	15.5-17.5	15.98±0.08	8.7-10.2	9.05±0.04
Male (n=50)	15.5-17.5	16.05±0.17	8.7-10.2	9.12±0.12
Female (n=50)	15.5-17.5	16.02±0.14	9.2-9.7	9.09±0.05

The head has a triangular anteoculus but lacks ocelli. The pronotum is divided into two lobes. The membrane of the hemelytra has reticulate veins (Cassis, 2010). *D. annulatum* is an efficient predator because of its raptorial forelegs, with which it captures and holds prey. Likewise, the middle and hind legs are flattened and fringed with hairs which enable it to move underwater as it forages for prey or continue swimming while holding and consuming its prey. Recognition of both sexes of *D. annulatum* is quite important in life history studies and in the development of mass rearing techniques. The last segment of the abdomen or the caudal end of the male has tufts of hairs while that of the female has none. There is not much difference in size between the male and the female.

4.8 Behavior studies

4.8.1 Mating behavior

When male and female water bugs are paired for the first time, they try to avoid each other and keep moving around the container. After a few minutes, they stay together in one location and start mating. Both of them frequently stay on the piece of twig introduced inside the container. Mating starts when the female positions itself on top of the male. In the field, the male water bug creates ripples on the water in order to attract a mate and once the female arrives, she will attempt to lay eggs on the back of the male. However, the male will prevent the female from laying eggs until he has mated repeatedly.

Mating usually lasts for 20-30 minutes as observed in the laboratory. Curtis (2011) reported that alternate egg laying and mating in *Diplonychus* species may take more than 24 hours until the male's back is covered with eggs. Gravid females appeared to have enlarged abdomens that turn dark green when eggs are about to be laid. After the female had laid her eggs on the back of the male, the male was separated from the female in order to prevent frequent disturbance. Brooding of eggs entails great effort on the part of the male.

Egg hatching depends on the activity of the male. Alternate wetting and drying of eggs ensure egg hatching. The male stays closer to the water surface with the eggs for air exposure. He rhythmically brushes the eggs with his hind-legs to circulate water and keep the eggs aerated and clean (Curtis, 2011). In addition, the male ensures that eggs are protected against possible predators. The male was returned to its mating pair as soon as the eggs on its back had hatched. Moreover, there were some cases when females laid their eggs on the piece of twig inside the container even in the presence of males and sometimes when males were absent, eggs were just laid in the water. However, those eggs did not hatch, suggesting that male brooding is necessary for egg survival.

4.8.2 Hatching

An egg is about to hatch when its shape resembles a cylinder which is broad and rounded at the free end while narrow and pointed at the attached end. Prior to hatching, the free end swells dorsally. According to Smith (1974), a pressure from within eventually causes a rupture in the chorion around the cephalic cap and then, the cap is lifted by the head as the nymphal thorax emerges.

4.8.3 Molting

Molting starts at the apex of the head where ecdysial line is normally found. Nymphs are about to molt when their body looks like it is about to burst out. During molting, the nymphs appear motionless or immobile. The head and prothorax comes first which gives the nymph a hunchback appearance. Molting would last for about 10-15 minutes and usually they molt early in the morning or toward late evening or midnight. Newly molted nymphs are transparent with some light green portions but, a few minutes after feeding, their color changes from flesh to brown. After molting, exuviae were just left floating/sinking in the water.

4.8.4 Feeding behavior and predatory consumption of *D. annulatum*

In literature, water bugs are known to feed on frogs, tadpoles, fish fry or other small fish, snails, dragonfly naiads, chironomid larvae, mosquito larvae, and other aquatic insects. Laboratory studies confirmed that *D. annulatum* is a voracious predator of mosquito larvae. In this study, *D. annulatum* was fed with *Anopheles*; feeding as well as its consumption rate were monitored and recorded. Both nymphs and adults of *D. annulatum* are voracious feeders of wrigglers of *Anopheles.* In the laboratory, as soon as the prey was introduced in the container, water bugs moved all over the container which seems like being disturbed by the presence of the wrigglers. However, after few minutes, they will stop and wait for prey to pass by. Water bugs catch their prey using their raptorial forelegs then suck-dry the prey's body contents through its piercing-sucking mouthparts. It will insert its proboscis in any part of the wrigglers. *D. annulatum* are known to inject their preys with toxins causing prey paralysis and tissue necrosis. Their salivary secretions are known to contain proteases and amylases (Cassis, 2010).

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