# Time-lapse tracing of biological events in an endophytic schizophoran fly, *Atherigona soccata* Rondani (Diptera: Muscidae)

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***Atherigona soccata* Rondani known as shoot fly is an endophytic pest of cultivated sorghum in the Old World. In our study, we demonstrate all critical biological events of the fly on a real-time basis using time-lapse imaging, including the endophytic larval and puparial stages. The most critical biological events in *A. soccata* are egg-hatching, first instar larva, pupariation, eclosion and post-eclosion phases. All these stages are critical because of their time-boundedness from initiation to completion. Any kind of lapse in terms of time delay in completion of an event prevented the insect from advancing to the next phase of life and eventually proved fatal. After hatching from egg, first instar larva took a mean time of 34 min to enter the plant through the gap between leaf sheath and growing central shoot. From this moment of larval entry into the plant until total extrication of adult fly during eclosion, *A. soccata* passed its life endophytically. After larval entry, it took a mean time of 33.7 h for the plant to show first visible symptoms of withering of central growing leaf whorl. From larval hatching to first takeoff of adult fly required an average of 309.6 h (12.9 days). The critical periods in the life cycle of *A. soccata* are less resilient to changes and therefore more vulnerable to external influences. Such phases in the life history can be targeted for managing *A. soccata* in sorghum.**

**Keywords:** *Atherigona soccata*, endophytic pest, sorghum, time-lapse imaging.

SHOOT fly, *Atherigona soccata* is an insect pest of major concern to the cultivation of sorghum in Asia and Africa[1,2]. In India, the species was described as *indica* by Malloch[3] in 1923 and subsequently in 1965, Hennig[4] proposed that all African, Indian and Mediterranean populations from sorghum belonged to a single species called *varia* ssp. *soccata* Rondani. Pont[5] convincingly demonstrated *soccata* as a good single species rather than a subspecies and synonymized all the other names such as *Atherigona indica*, *A. varia* and *A. varia soccata* which were in use in the Indian literature until then. Thereafter, the species has been referred to as *A. soccata* which was originally described by Rondani[6] in 1871. All the adult flies that emerged in our study have been preserved, identified as *A. soccata* Rondani and maintained at the Directorate of Sorghum Research, Hyderabad.

In India, it has been nearly a century now since the establishment of pest status of *A. soccata*[7]. Since then several workers have studied biology of *A. soccata*, whose focus mainly remained on overall life history of the fly[8–13] without any ingress into its critical stages and duration. In the present study, we have demonstrated the unfolding of all critical biological events of the fly, hitherto unknown, with minute by minute real-time tracing. Our findings would help in understading vulnerable stages and their duration in shoot fly as well as in other schizophoran (flies with ptilinal suture) flies of agricultural and medical importance. Female lays eggs singly on the under surface of the leaf selectively on sorghum seedlings that are less than 30 days old[14]. Egg hatches within 48–72 h (ref. 8–13). Larva after hatching enters into the shoot and survives inside by feeding rotten tissues of inner shoot. Pupate inside the shoot and adult emerges from puparium to complete the whole life cycle in 14–28 days[8–15]. The process of extrication of adult fly from the puparium is called eclosion. A group of brachyceran flies (having short antennae) possesses a circular weakened line at the anterior top of the puparium which opens up as a lid during adult eclosion. This group of flies is called cyclorrhaphan flies. Within this group, certain flies in the pupal stage bear an extensive membranous organ on the cephalic aspect of the head known as ptilinum[16]. Ptilinum helps the fly in opening up of puparium during eclosion. Such kind of flies are classified as Schizophora (split-bearers), because they bear a conspicuous ptilinal suture on the frontal area of the head, that is between compound eyes, after the complete retraction of ptilinum. The fly after being completely extricated from the puparium undergoes a series of preparatory activities called post-eclosion events[17] and upon successful completion of such activities the fly becomes a normal adult.

*A. soccata* maintains spatial consistancy in its behaviour, plant injury and biological cycle throughout its area of distribution[15]. However, there are changes which occur across seasons, especially in the duration of different biological stages. Nevertheless, some of the critical phases such as pupariation, eclosion and post-eclosion phases are consistently time-bound across seasons. In this communication, we focus on these time-bound phases and their duration by tracking through time-lapse imaging.

The study was conducted over a two-year period (2010–12) at the Directorate of Sorghum Research, Hyderabad. All the experiments were conducted on genotype DJ-6514, which is highly susceptible to shoot fly. Life-cycle phases, viz. larval hatching from egg, first instar larval entry into plant shoot, appearance of first

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symptom in plant, first instar, pupariation, eclosion and post-eclosion events (wing expansion, tanning, grooming and flight)[17,18] were selected for the study. Sorghum plant samples with various stages of shoot fly infestation, viz. freshly laid egg, initial to advanced deadheart symptom were collected from the field on daily basis. Based on our regular observation of these samples and the literature[8–15], we standardized the timing and approximate duration of individual critical biological events. After arriving at timing and length of each event, the sorghum seedlings grown in plastic cups (6 cm diameter) were exposed to mated female flies for egg-laying[19]. The infested plants were fixed appropriately under Leica EZ4HD stereozoom microscope fitted with Canon DSLR (EOS1000D) camera connected to a laptop computer. Timing, duration and frequency were set in the computer based on previously standardized approximate data. For recording endophytic events, the plant parts were split open during the expected timings and recorded. These time-lapse recordings necessitated several samples due to many failed recordings owing to several practical reasons, mainly insect moving out of focus and/or mortality caused by procrastination. Only those recordings of the whole single event from initiation till the end were considered for the data matrix and further calculations. Such data matrix was arranged so as to arrive at datasets spreading across two years. Data were processed for analysing duration of different stages and mortality details.

Egg period lasted for $60.8 \pm 1.9$ h (range 43–74, $N = 153$). After hatching from egg, first instar larva entered the plant through the gap between leaf sheath and growing central shoot in a time-period of $34.1 \pm 1.3$ min (range 23–47, $N = 28$). Those newly hatched larvae which were wandering beyond the maximum time range failed to reach the destination. Such larvae died due to desiccation or on falling off from the leaf. Ambient humidity significantly affects embryo development in the egg, larval hatch and survival[19], and therefore longer exposure to the ambient weather prevailing on leaf or plant surface caused larval death due to desiccation.

Moisture on the leaf surface also plays an important role in survival and movement of larvae after hatching[20–22]. Therefore, in our study we sprayed a thin film of water on the leaf surface about the time the egg was expected to hatching[19]. In the field, hatching almost always takes place very early in the morning when the plant surface is moist with dew[19]. Larvae spending more time on the plant surface run the risk of losing leaf surface wetness as well as getting exposed to the vagaries of air humidity changes. Thus, the duration from larval hatching to reaching the entry point became crucial because significant per cent mortality occurred at that phase of life (Table 1). The egg (Figure 1 *a*) is laid on under surface of the leaf and always positioned upright. Larva hatching from the egg (Figure 1 *b*) took a U turn and crawled down (Figure 1 *c*, *d*). After some distance it turned towards leaf margin and crossed over onto the upper surface of the leaf blade (Figure 1 *e–h*). Upon reaching the upper leaf surface, the larva crawled down towards leaf base and entered into plant shoot between leaf sheath and growing shoot whorl (Figure 1 *i–j*). From this moment of larval entry into the plant until total extrication of adult fly during eclosion, *A. soccata* passed its life endophytically.

In all our observations ($N = 28$), the larva tended to make only downward movements from hatching until reaching the central plant shoot. Our observations on the larval behaviour after its entry into the plant shoot were made by slightly removing the leaf sheath. After larval entry into the plant, it travelled down between leaf sheath and growing shoot whorl by randomly probing for soft tissue area. Upon spotting soft tissue area over growing shoot whorl, the larva bore the plant tissue with its cephalopharyngeal apparatus (Figure 1 *k*). This provides the larva entry into the central shoot (Figure 1 *l*, *n*). The larva moved further down by probing for more soft tissue. On reaching an area of soft plant tissue the larva started smashing the tissue around it and in that process the central growing leaf whorl got detached (Figure 1 *n*), which caused the plant to show first visible symptoms of withering of central growing shoot (Figure 1 *o*). At this point of time the first instar larva (Figure 1 *m*) molted into second instar larva (Figure 2 *a*). The molting started with first instar larva showing an unusual vibrational movement and in less than 10 s the second instar larva released itself from the older skin (Figure 1 *p*). Appearance of first symptom after larval entry into plant was noticed in a mean time period of $33.7 \pm 0.7$ h (range 27–39, $N = 20$). The duration of first instar larva was $35.1 \pm 0.6$ h (range 30–39, $N = 28$). From second instar to fully matured last instar larval stage it took $127 \pm 1.2$ h (range 120–138, $N = 46$) and fed on inner rotten shoot tissues (Figure 2 *b*). By that time the plant showed the typical dried-up central growing shoot known as deadheart (Figure 2 *c*).

Fully mature larva measured $7.0 \pm 0.1$ mm (range 6–8, $N = 46$) in length (Figure 2 *d*) and prepared itself for pupariation by moving up through the rotten central shoot column and settled a location where the future adult fly would face little obstruction during eclosion. The process of pupariation took place by alternative longitudinal contraction and relaxation of segments. Simultaneously, tanning progressed from posterior end to anterior segments (Figure 2 *e*). The pupariation process ended with formation of puparium of $4.0 \pm 0.08$ mm (range 3.3–4.6, $N = 50$) long (Figure 2 *f*) in a time-frame of $8.1 \pm 0.4$ h (range 6–11, $N = 30$). The pupal duration was an average of $124 \pm 1.2$ h (range 115–133, $N = 116$) during June–October. Eclosion was effected first with a thrust force exerted on the anterior top portion of puparium by ptilinum. Thus, the ptilinal force opened up the puparial lid facilitating the subimago to gradually extricate itself from the puparium (Figure 2 *g*). During the process it was observed that a rhythmic contraction and relaxation was

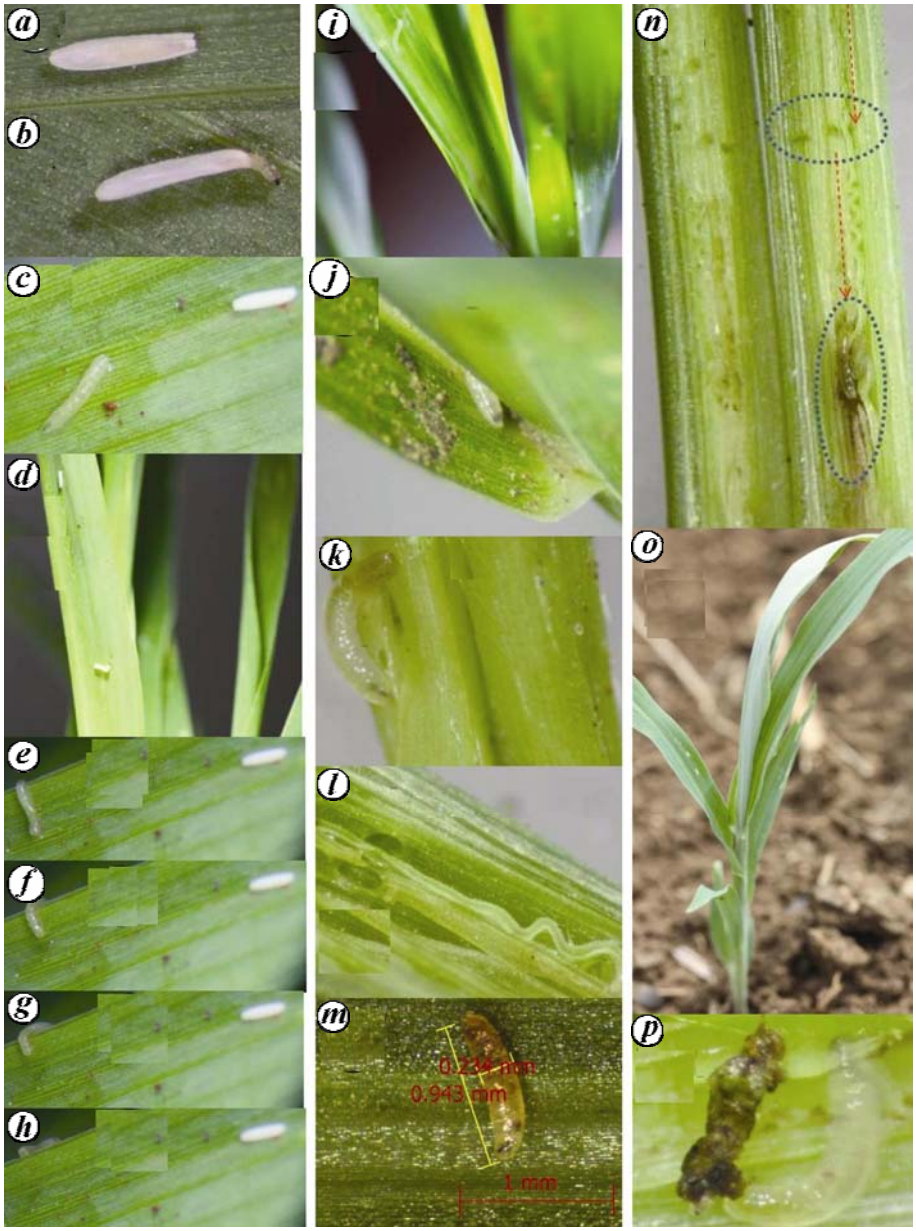


**Figure 1.** Larval hatching and first instar larva entry into plant shoot. ***a***, Egg on lower surface of leaf. ***b***, Larva hatches out from egg. ***c***, Hatched first instar larva crawls down on lower surface of leaf. ***d***, Larva marches towards leaf margin. ***e***–***h***, Larva crosses leaf margin and gets onto upper surface of leaf. ***i***, Larva crawls down towards leaf base. ***j***, Larva enters the plant shoot between leaf sheath and growing shoot whorl. ***k***, After entering between leaf sheaths, larva thrusts itself into central shoot through soft tissue area (outer leaf sheath removed for photography). ***l***, Circular entry hole made by larva. ***m***, First instar larva (0.9 × 0.2 mm). ***n***, Larval entry route inside plant shoot shown with markings on longitudinally split-open shoot and larva cuts off base of innermost growing shoot. ***o***, Plant showing withering of central leaf whorl. ***p***, First instar larva molts into second instar larva (molted skin is seen as pale brown mass).

underway in the ptilinum. Some studies have pointed out that three pairs of large thoracic muscles are involved solely with eclosion[23] and a series of peristaltic waves of muscular contraction and relaxation[24] slowly extricated the fly out of puparium. The eclosion process lasted 9.8 ± 0.4 h (range 5–15, $N = 31$). During eclosion, in case of any obstruction over the ptilinum for a considerably long time, the retraction of ptilinum failed and the fully extricated fly still possessed fully inflated ptilinum (Figure 2 *h*). Such flies undertook no post-eclosion activities and eventually died. The fully extricated flies with fully inflated ptilinum is a clear indication that the ptilinal retraction plays no role in the extrication process and it only helps in opening up the puparial lid. In general, eclosion in insects occurs very early in the morning[25,26] and *A. soccata* also started eclosion in the early morning

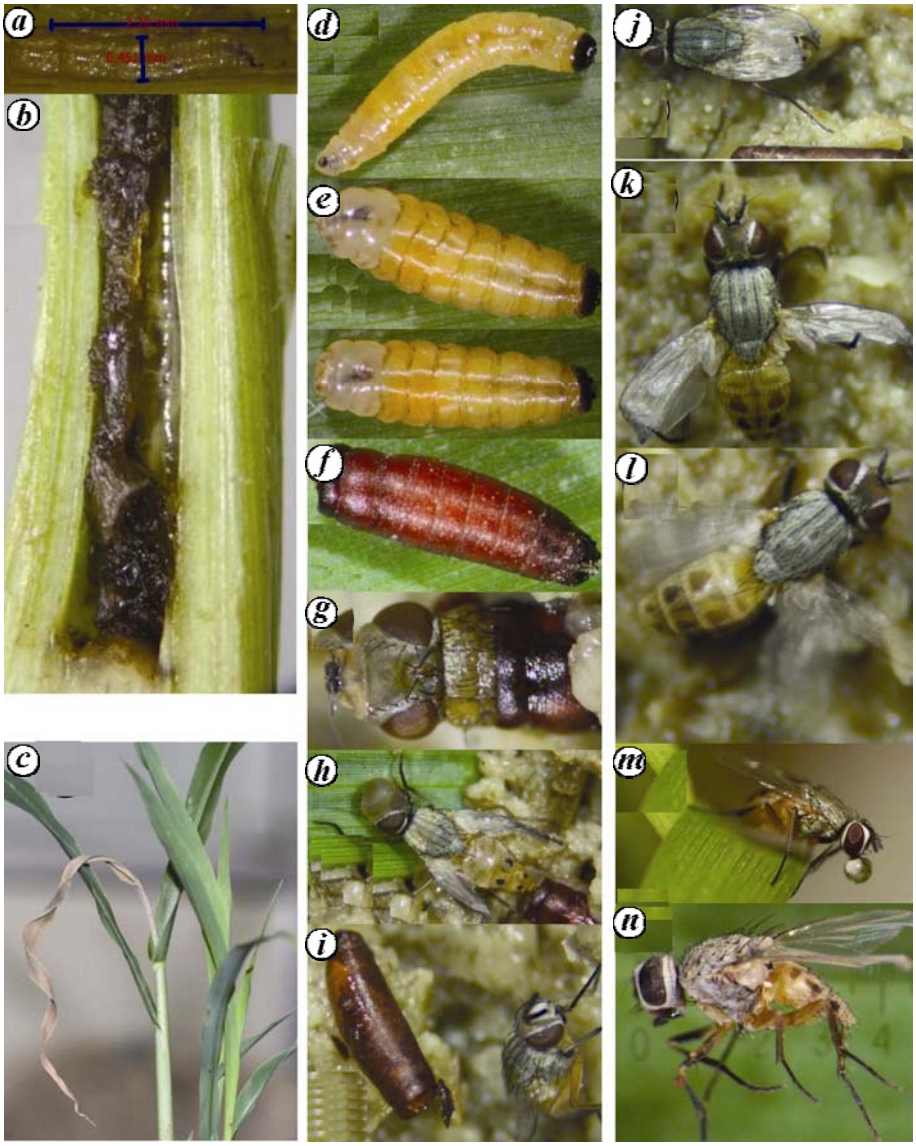

**Figure 2.** Larval feeding, pupariation, eclosion and post-eclosion phases. ***a***, Second instar larva (2 × 0.4 mm). ***b***, Second and subsequent instar larval feeding and rotting plant tissues. ***c***, Plant showing dried-up central leaf whorl known as deadheart symptom. ***d***, Fully grown last larval instar (7 mm), minutes before onset of pupariation process. ***e***, Tanning of larva during pupariation (constriction and relaxation of anterior segments shown). ***f***, Puparium (4 mm). ***g***, Eclosion of fly from puparium (with normal retraction of ptilinum). ***h***, Adult fly fully extricated from puparium (abnormal eclosion with ptilinum not retracted). ***i***, Post-eclosion wing-expansion phase. ***j***, Post-eclosion tanning phase. ***k***, Post-eclosion grooming phase. ***l***, Adult fly tries to take off for its first flight post eclosion. Fully developed (***m***) female (5 mm) and (***n***) male (4 mm) flies.

hours. Post-eclosion phase started after the adult fly extricated from puparium. Post-eclosion phase of *A. soccata* involved a series of well-defined activities, viz. wing expansion, tanning, grooming and flight. Wing expansion (Figure 2 *i*) took place with the pumping of haemolymph into the wing veins[27] and it started 26.1 ± 0.6 min (range 22–31, $N = 28$) after eclosion. Tanning (Figure 2 *j*) of body, including legs was initiated at 48.9 ± 0.6 min (range 44–53, $N = 28$) after eclosion. Grooming was also a remarkable event marked by rubbing of whole body with legs (Figure 2 *k*) and it started 69.8 ± 0.7 min (range 64–76, $N = 28$) after eclosion. After the preceding activities were finished, the fly started practising flight activity by beating its wings in high frequency (Figure 2 *l*). The flight activity started at 136 ± 2.6 min (range 121–163, $N = 28$) after eclosion. Net time length of all the critical biological events, viz. hatch to entry, symptom after entry, first instar, pupariation, eclosion and different post-eclosion phases is depicted in Figure 3. Male (Figure 2 *m*) and female (Figure 2 *n*) adult flies were easily distinguishable with size and colouration. Female has grey head and thorax with pale yellow abdomen, while male had darker body colouration. Males are slender and are 4 ± 0.0 mm (range 3.8–4.3, $N = 28$) in length, whereas females have bulkier

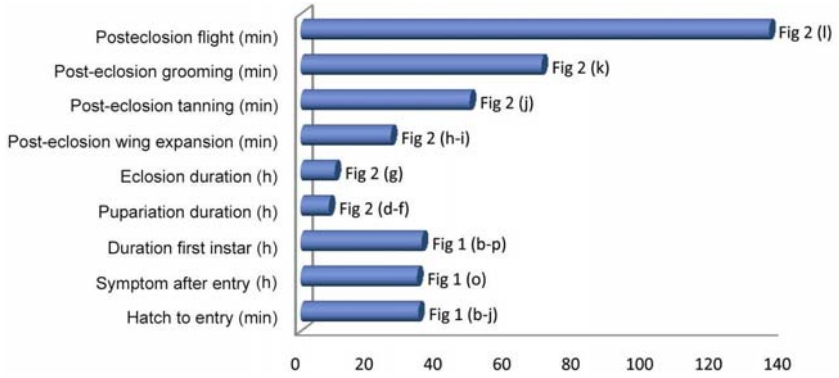


**Figure 3.** Net time length of different biological events of *Atherigona soccata* marked with corresponding time-lapse images of Figures 1 and 2.

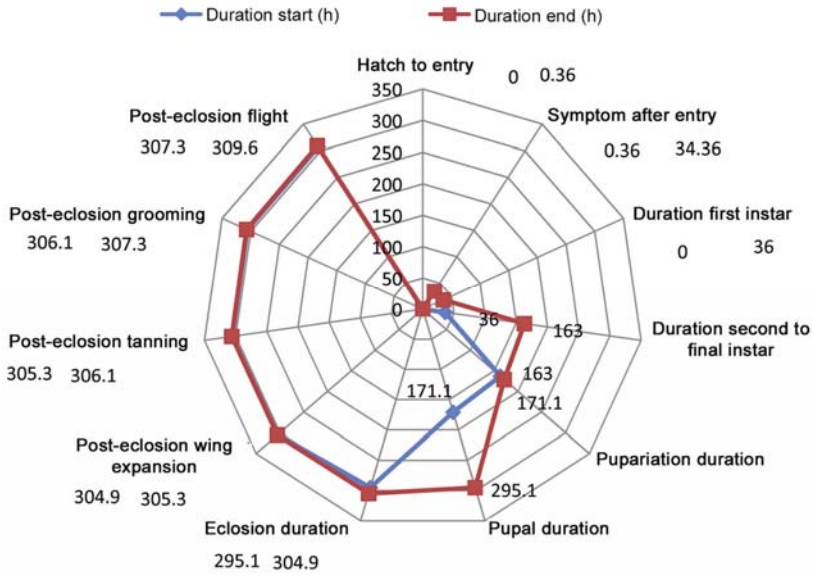


**Figure 4.** Life cycle of *A. soccata* from larval hatching to adult flight with start and end duration of different biological events depicted in sequence (calculated from time-lapse imaging).

abdomen and are 5 ± 0.0 mm (range 4.7–5.2, $N = 28$) in length. The whole process of post-eclosion activities lasted 4.7 ± 0.0 h (range 4.4–5.1, $N = 28$). Eclosion and post-eclosion events were the most critical phases in which the highest percentage of mortality was observed. From larval-hatching to adult required an average of 309.6 ± 1.7 h (range 290.3–318.1; $N = 20$; Figure 4).

Any kind of lapse in terms of time delay in completion of the events, viz. egg-hatching to entry of first instar, pupariation, eclosion and post-eclosion activities prevented the insect from advancing to the next phase of life and eventually proved fatal. We analysed the mortality factors at different stages of life history using one-way ANOVA (Tukey HSD; SPSS 11.5 for windows) at different stages (Table 1). At egg stage, egg parasitization by *Trichogrammatoidea simmondsi* was the major significant cause of mortality with 18% ($F = 785.33$; d$f = 4, 24$; $P = 0.000$). After egg-hatching, prolongation in duration of events, viz. first instar larval entry into leaf sheath ($F = 245.34$; d$f = 4, 24$; $P = 0.000$), pupariation ($F = 63.92$; d$f = 4, 24$; $P = 0.000$), eclosion ($F = 33.42$; d$f = 4, 24$; $P = 0.000$) and post eclosion ($F = 30.46$; d$f = 4, 24$; $P = 0.000$) significantly contributed to the total mortality with 23%, 19%, 29% and 20% mortality respectively. In case of first instar larva, prolonged wandering on plant surface exposed the larva to desiccation which caused mortality. In the processes of pupariation and eclosion there was a gradual decrease in the muscle contraction movement, beyond the normal time limit, which ended up in cessation of muscular contraction and death. During post eclosion, failed or protracted ptilinal retraction caused delay in wing expansion and tanning. Such a delay severely affected the normal development of adults and ultimately led to mortality. During second and subsequent larval stages, parasitization mainly by *Neotrichoporoides nyemitawus* resulted in significantly higher ($F = 74.54$; d$f = 4, 24$; $P = 0.000$) mortality (21%). In puparium, there was a significant increase ($F = 56.48$; d$f = 4, 24$; $P = 0.000$) in mortality by 13% due to parasitization by *Spalangia endius*. These results indicate that

**Table 1.** Mortality during critical phases of *Atherigona soccata*

| Phase | Time duration range (h) | Percentage of mortality due to: Time duration < Lower limit of range | Time duration > Upper limit of range | Parasitization | Unknown cause | Total percentage of mortality |
|---|---|---|---|---|---|---|
| Egg-hatching | 43–74 | 0 | 3.5 | 18 | 2 | 23.5 |
| First instar larva | 30–39 | 0 | 23 | 0 | 5 | 28 |
| Second to final instar larvae | 120–138 | 0 | 0 | 21 | 3.3 | 24.3 |
| Pupariation | 3.3–4.6 | 0 | 19 | 0 | 5 | 24 |
| Puparium | 115–133 | 0 | 3 | 13 | 1.1 | 17.1 |
| Eclosion | 5.1–15 | 0.5 | 29 | 0 | 2.5 | 32 |
| Post eclosion | 4.4–5.1 | 1 | 20 | 0 | 5 | 26 |

*A. soccata* was vulnerable to prolongation of time duration beyond the maximum limit and suffered maximum mortality during hatching to entry, first instar, pupariation, eclosion and post-eclosion periods. Therefore, these periods are critical in the life history of the fly.

From our results, we conclude that the critical periods in the life cycle of *A. soccata* are less resilient to changes and therefore vulnerable to external influences. The fly suffered significant levels of mortality during the critical periods due to prolongation in duration of an event. These can be selected as vulnerable stages for effectively influencing mortality of specific life-history events critical for inflicting economic damage to the crop. Thus, this study brings out such phases in the life history, which can be targeted for managing *A. soccata* in sorghum. The period from egg-hatching to larval entry into plant is of more practical value than other stages as targeting the pest at this particular stage would prevent the fly from entering the plant shoot and initiating the damage. Management interventions at other critical stages, viz. pupariation, eclosion and post-eclosion phases could control the adult flies before they become reproductively active. Therefore, these stages offer a second level of opportunity for managing the shoot fly by way of reducing population pressure in the subsequent generations in sorghum ecosystem.


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# Diversity, representativeness and biotic pressure on plant species along alpine timberline of western Arunachal Pradesh in the Eastern Himalaya, India

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**The timberline being an ecotone area is considered to be among the most prominent vegetation zones in high-mountain areas. However, the timberline in the Eastern Himalaya has not been studied adequately though it supports rich vegetation and is considered one of the most important hotspots of biodiversity. More humid conditions in the Eastern Himalayan timberline compared to Western Himalaya provide a more conducive environment for tree growth and as a result the upper limit of timberline in this sector goes up to 4570 m, which is much higher in comparison to timberline limit of 3600 m in the Western Himalayan region. We recorded a total of 122 plant species from timberline zone belonging to 29 families with 56 species being common to areas above and below the timberline zone. It is interesting to note that 77% of the total recorded plants was found distributed within the Himalayan region, while 28% was confined to Eastern Himalayan region only. Eleven species required significant conservation measures. Collection of firewood by herders and unregulated grazing by local communities were found to be the most common threats in the study sites. Considering most of these areas are under traditional control of local communities, pro-community approach for conservation of biodiversity of these areas has also been discussed.**

**Keywords:** Biodiversity hotspot, ecotone, endemic, grazing.

THE timberline which represents transition from forest to treeless alpine areas is a distinct boundary within the altitudinal zonation of vegetation, which also forms one of the most fundamental ecological boundary[1]. The extent of timberline varies from region to region as it is an oscillating zone and not a sharp line. Such areas are considered the cradle of temperate and alpine floral elements that are highly diverse in vegetation composition. These areas are considered sensitive to biotic pressure that can bring substantial changes in alpine and temperate vegetation[2]. In India, the extent of timberline in the Eastern Himalaya encompasses a large area in West Kameng and Tawang districts in Arunachal Pradesh and is mostly distributed along the area between 3900 and 4200 m altitude[3]. The alpine and temperate regions in the Eastern Himalaya are considered among the richest vegetation zones in the world[4]. More humid conditions in the Eastern Himalaya compared to the Western Himalaya provide a more conducive environment for tree growth and as a result the upper limit of tree vegetation in this sector goes up to 4570 m compared to 3600 m in the Western Himalaya[5]. The region contains a globally significant array of unique flora and fauna with high proportion of endemism[6–10]. The state of Arunachal Pradesh, due to varied climatic conditions and altitude, is also known for its rich vegetation with unique ecosystem ranging from tropical belt to the snow-clad alpine mountains[9]. Although there have been several studies of general vegetation types of Arunachal Pradesh[5,11–13], documentation of plant diversity from the timberline area is still lacking from the region. The timberline is among the most sensitive ecotone[14]; unfortunately it is under tremendous biotic pressure for diverse needs[2]. There is a need to analyse some basic information related to distribution and diversity of plants, their representativeness, and broad significance of species so that suitable conservation measures could be taken for protecting the timberline area. In this communication, an attempt has been made to document the plants collected from the area in and around the timberline zone of West Kameng and Tawang districts.

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