https://doi.org/10.33307/entomon.v47i4.790
ENTOMON 47(4): 375-382 (2022)
Article No. ent. 47403

Forensic implications of the seasonal changes in the rate of development of the blowfly, *Chrysomya megacephala* (Fabricius) (Diptera, Calliphoridae)

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ABSTRACT: Studies on the development rate of *Chrysomya megacephala* (Fabricius) suggested that the blowfly as a significant candidate for forensic investigations. Under natural ambient conditions development rate of *C. megacephala* in monsoon, winter and summer seasons indicated significant differences among seasons. The larvae began pupation at 92nd h in summer, 157th h in the monsoon season and 191st h in winter. Rapid larval growth in terms of length was observed in summer. During summer, the length of the larvae increased to a maximum of 13.9 mm at 54th h. Time taken for the emergence of the adult fly was 164, 249 and 311h in summer, monsoon and winter seasons respectively. Life table studies were conducted to assess the percentage survival and mortality by recording the survival rate of different development stages. Molecular diagnosis of species was done using COI gene. The analysis included molecular sequences of other samples of the same species from different regions of India. The neighbor-joining method allowed us to identify the species at molecular level with precision and accuracy.

KEYWORDS: Larval growth, pupation, adult emergence, seasonal differences, life table, molecular diagnosis

INTRODUCTION

The blowfly, *Chrysomya megacephala* (Fabricius) (Diptera, Calliphoridae), a synanthropic fly, commonly known as oriental latrine fly inhabiting human settlements and commonly seen on decomposing cadavers, fish, carrion, human feces and sweet materials; indicating its medical, veterinary and forensic significance. Due to their cosmopolitan distribution, ubiquity and abundance, *C. megacephala* is recognized as the one of the most important species of insects in forensic entomology (Badenhorst and Villet, 2018). The larvae feed and grow on soft tissues of living and dead vertebrates especially mammals, birds and fish (Yang and Shiao, 2012). The adult flies were usually attracted to decaying cadavers and reach within a few hours of death of the animal (Zumpt, 1965), and it has been considered as an important fly for the determination of minimum postmortem interval (Wang *et al.,* 2008). Medico legal cases world over have reported the forensic relevance of *C. megacephala* (Gruner *et al*., 2017; Richards and Villet, 2009; Amendt *et al*., 2004; Goff and Flynn, 1991). For the determination of minimum postmortem intervals, age of larvae will be helpful (Gruner *et al*., 2017). Studies focusing the

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development of *C. megacepehala* has been done previously (Subramanian and Mohan, 1980; Bharti *et al.,* 2007; Sukontason *et al.,* 2008; Niederegger *et al.,* 2010; Bala and Singh, 2015; Zhang *et al.,* 2018).

Age grading studies on immature stages of *C. megacephala* at different temperatures in the laboratory has been done at Punjab, India where the fly took 6.3 days for development from egg to adult stage at 30°C (Bharti *et al*., 2007; Bala and Singh, 2015). Estimates of postmortem interval (PMI) based on the known characteristics of the infesting fauna in the natural conditions of the specific geographical location are very important (Sukontason *et al.*, 2008). Niederegger *et al*. (2010) suggested that negligence of fluctuating temperatures in legal cases can lead to distinctly wrong estimates of the PMI. The studies targeted on the time since death assessment has been recognized to be scanty in the present scenario. Studies were undertaken to reveal the seasonal changes in the developmental rate of *C. megacephala* during summer, monsoon and winter season to develop an accurate estimation of PMI.

MATERIALS AND METHODS

Rearing of *C. megacephala:* The adult flies were reared in the outdoor open system rearing facility in Kolangattukara, Choolissery, Thrissur district, Kerala, India (10° 352 34.873 N; 76° 112 22.63 E) during summer, monsoon and winter season. Adult females of *C. megacephala* were trapped and isolated in the rearing cabinet with decomposing pork meat as bait. The insects were reared in triplicate in the rearing cabinets (60 cm × 30 cm ×30 cm). Relative humidity, rainfall and temperature were monitored in May, July, August and December (2019) and January (2020). The average temperature and relative humidity in the respective months were 31.15 ±2.26°C, 27.71± 1.47°C, 26.09±1.35°C and 72.30±10.84, 88.07±4.21, 71.46±17.73 per cent respectively. The average rain fall recorded during July-August months was 1999.7mm. The adult insects were provided with 10 per cent (w/v) sugar solution and 1.5 per cent (v/v) multivitamin syrup solution and water as food and liquid sources (Byrd, 2001; Von Aesch *et al.,* 2003). The decomposing pork meat served as a reflex stimulus for the adult female fly to lay eggs and also served as a food source for the larvae. Wet vermiculite was kept as the bottom layer in the cabinet to assist the migration of third instars for pupation. Few of the blowflies trapped were killed and pinned as dry specimens for morphological identification and few were preserved in ethanol (70%) for molecular identification. The morphological examination was done with LEICA-S8APO stereomicroscope. Six numbers each of eggs, different larval instars and pupae were randomly collected every six hours for further studies.

Observations were done regularly on an hourly basis to detect the presence of eggs. Once the eggs were found, the eggs with the bait were transferred to the larval rearing plastic jars. Wet vermiculite was laid at the bottom of the jar to maintain adequate humidity. The jar was covered with a wet cotton cloth to prevent the entry of other insect parasitoids. Fresh pork meat 50 g was put in to the jar as larval feed. This was continued until the instars reached the non-feeding stage and started pupation. Fresh pupae were collected and transferred to a new rearing jar with moist vermiculite at the bottom and it was kept inside the rearing cabinet for the emergence of the adult fly. Different larval instars were collected for studying their morphology and length parameters. The adult flies were identified using morphological keys provided in the standard literature (Senior-White *et al.,* 1940; Bharti, 2019).

Life table study: Life table studies were conducted to assess the percentage survival and mortality by recording the survival rate of different development stages. Survival studies were undertaken in all seasons in triplicate. In each replicate trials of rearing, survival rate in percentage was calculated for each stage of the life history; egg, 1st, 2nd, 3rd instars, post feeding stage and pupa till the emergence of adult flies. Average number of eggs considered for rearing in each replicate of the triplicate trials in summer, monsoon and winter seasons were 124,121 and 128 respectively. The time of oviposition till the emergence of adult fly

was considered for the study. The time taken for egg hatching was noted. The freshly hatched larvae were transferred to the new larval rearing chamber and 50 g of fresh pork meat was provided as food. Ten larvae were collected every six h and boiled for two minutes at 96-99°C and preserved in alcohol (70 %) for the assessment of length (Adams and Hall, 2003). The time spent in each life stage was recorded. Based on these observations growth curves were plotted. The effect of temperature, relative humidity and rainfall on larval development was also studied.

Molecular characterization was done using Cytochrome oxidase Subunit I (COI) gene. The isolated sequence was submitted in GenBank, NCBI with Accession No: MW 522614. The data were statistically analysed using SPSS 24.0.0. The relation between the temperature and various life stages from 1st, 2nd, 3rd instars and post feeding stages was analysed in one-way analysis of variance.

RESULTS AND DISCUSSION

The adults emerged were identified as of *C. megacephala*. The growth curve was sigmoid during the summer, monsoon and winter seasons (Fig. 1). The time taken for the emergence of the adult fly was long during the winter season (December-January) at an average atmospheric temperature of 26°C (F = 475.7 at df = 28). The maximum length of larvae reached at 138th hour during winter season (F = 120.2 at df = 8). The time taken for the emergence of the adult fly was shorter in the summer season (May) at an average temperature of 31°C (F = 837.0 at df = 14). During summer maximum length of larvae reached at 54th h of development (F = 179.8 at df = 2). The time taken for the development during the monsoon (August-September) (F = 591.9 at df = 23) was found at 28°C, which was slightly longer than that of the summer season. During monsoon season

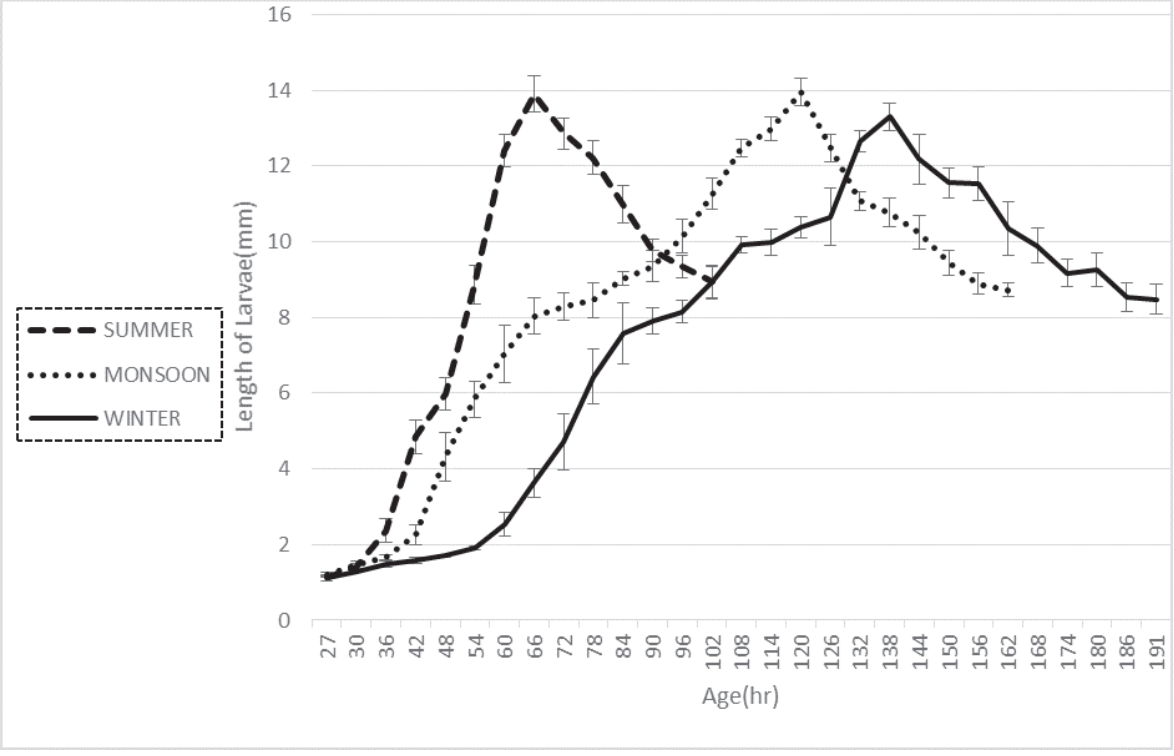


Fig. 1 Developmental rate of *Chrysomya megacephala* from newly hatched larvae until pupation under natural conditions in Kerala, India in Summer, Monsoon and Winter Seasons (black bars indicate mean ± SD)

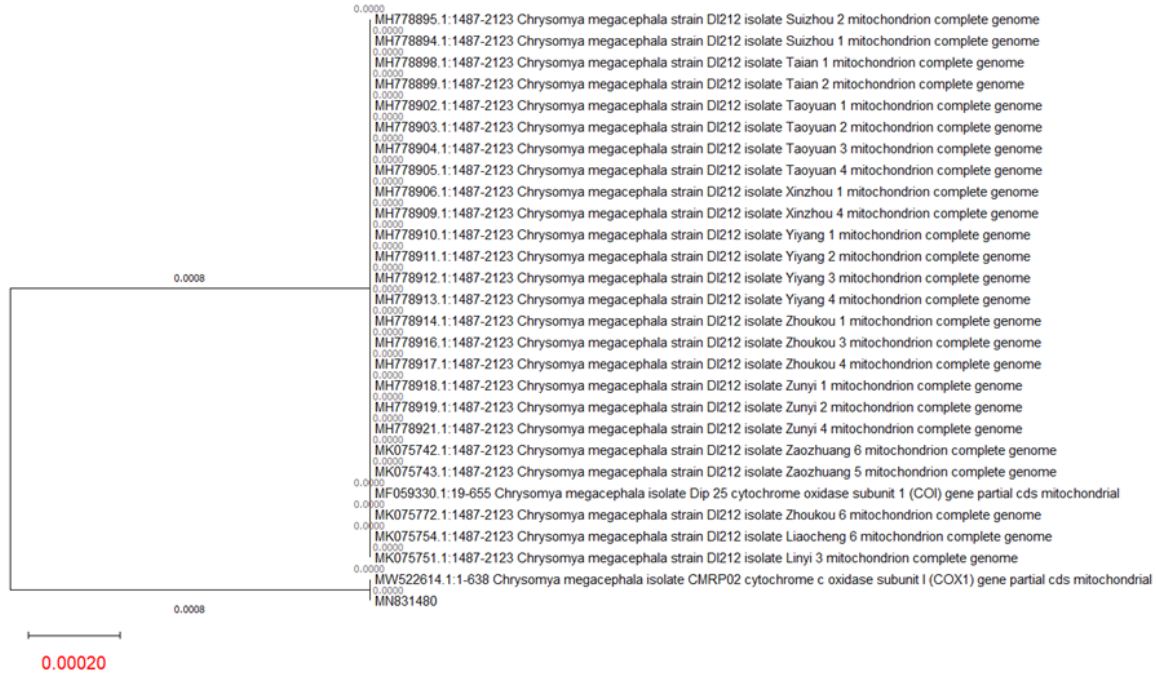
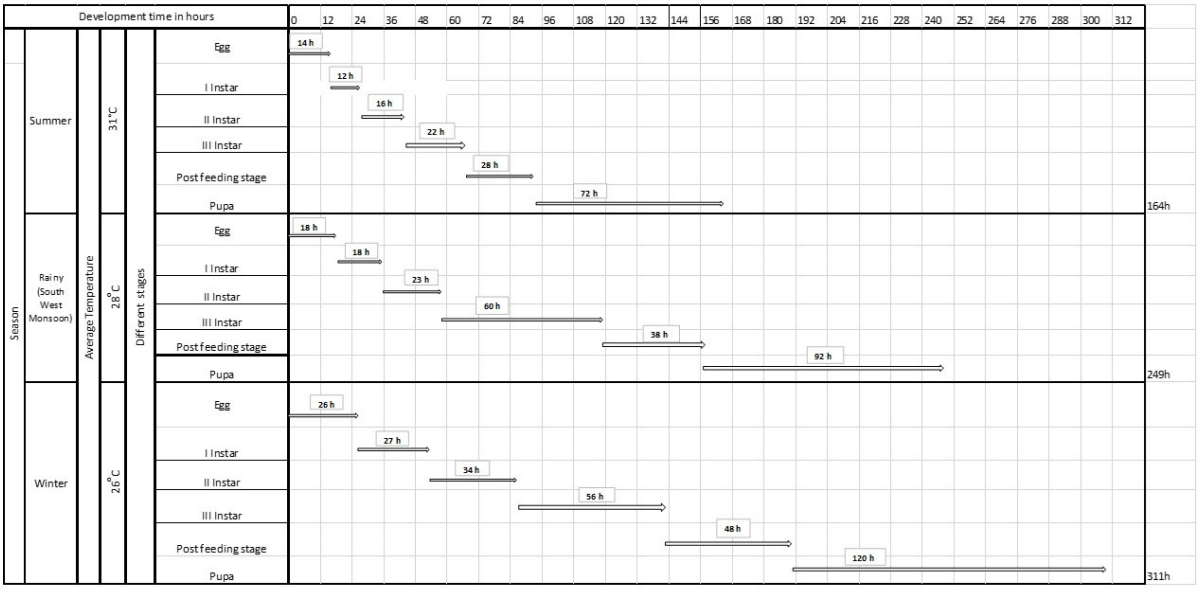


Fig. 2 Evolutionary analysis of *SR1859-COI-F_E03 Chrysomya megacephala* by Maximum Likelihood method

Table 1. Life table showing rate of development of different stages of *C. megacephala* with respect to age in different seasons in Kerala, India (mean value of all replicates)



maximum length of larvae reached at 114th hour (F = 203.715 at df = 9).

As illustrated in the life table, the pupation started at 191st h in winter season, 157th h in monsoon season and 92nd h in summer season. Life table showing rate of development of larval stages of *C. megacephala* with respect to age after egg hatching till commencement of pupation during different seasons is presented. During summer, the development of the larvae was faster with rapid increase in body length. After attaining the maximum size, when the post-feeding stage started, the length of the larvae reduced (Table 1).

Molecular diagnosis of species was done using COI gene. The isolated sequence was submitted in GenBank, NCBI with Accession No: MW522614. It displayed 99.84 per cent identity with sequence of same species collected from China (Accession No. MK075772.1). *C. megacephala* has also been identified using barcoding in northern and southern part of India, with Accession No: AB910390 (Ramraj *et al.*, 2014), KX893351, KX893341, KX893343, KX893342, KX893346, KX893347, KX893344, KX893345 (Bharti and Singh, 2017). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The neighbour-joining method allowed us to identify the species at molecular level with precision and accuracy. The optimal tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 2). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. This analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 638 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.,* 2021)

Life table studies provided the survival rate and mortality rate of each development stage. Survival studies were undertaken in all seasons in triplicate. Maximum hatching was observed during monsoon season (92.18%) and lowest during winter season (85.12%). Similarly 92.18 per cent of first instar larvae became second instar during monsoon season closely followed by summer (89.51%) and the lowest in winter (71.07%). The same trend was followed in the case of third instar larvae with 74.19, 80.46 and 61.15 per cent respectively for summer, monsoon and winter seasons; and reached pupal stage with 61.29, 71.09 and 51.23 per cent respectively. A total of 68, 84 and 47 adult flies emerged from pupae respectively for summer, monsoon and winter seasons. Total survival rate for *C. megacephala* during summer, monsoon and winter was found to be 54.83, 65.62 and 38.84 per cent respectively. The total time taken for the development of was found to be 164, 249 and 311h in summer, monsoon and winter respectively (Table 2).

In forensic investigations, apart from the species identification of the blow fly, the knowledge of the rate of development of the blow fly in the specific geographical location is very crucial in the accurate estimation of Post mortem Interval (PMI). The metabolic rate of the insects increase with the increase in temperature (Anderson, 2000). Byrd and Butler (1997) reported that the results of developmental rate of *C. ruffifacies* conducted at constant temperature could be applied to fluctuating temperature conditions. The developmental rate of *C. dubia* at fluctuating temperatures were similar to that conducted at a mean constant temperature (Dadour *et al.,* 2001). In present study the time taken for pupation during monsoon season (27°C) was 139h which is close to the results (144h at 27°C) obtained on the same species by Wells and Kurahashi (1994) and 132h at 28°C by Sukontason *et al.* (2008). Wells and Kurahashi (1994) also reported that the total time taken for adult emergence was 234h; which is similar to the total time of 249h recorded in the present investigation. The seasonal study on development rate conducted on *C. megacephala* in the present study showed with a shortest period of onset of pupation from newly hatched larvae at summer followed by monsoon season and then the winter. This results are reaffirming the observations made by Smith (1986) in which the higher temperature increased the larval

Table 2. Survival rate of different life stages of *C. megacephala*

Stage	No. of each stage			Survival rate at each stage (%)			mortality rate at each stage (%)		
	Summer	Rainy	Winter	Summer	Rainy	Winter	Summer	Rainy	Winter
Egg	124	128	121	94.35	95.71	85.12	5.65	4.29	14.88
1st Instar	117	122	103	89.51	92.18	71.07	10.49	7.82	28.93
2nd Instar	111	118	86	74.19	80.46	61.15	25.81	19.54	38.85
3rd Instar	92	103	74	61.29	71.09	51.23	38.71	28.91	48.77
Pupa	76	91	62	54.83	65.62	38.84	45.17	34.38	61.16
Adult fly	68	84	47	-	-	-	-	-	-

activity of these fly larvae, while the cold weather was inhibiting the fly activity. Subramanian and Mohan (1980) reported that at 25.5°C pupation time for *C. megacephala* was 150h in comparison to 165h at 26°C in the present study. The results of the present study are in line with studies of Goodbrod and Goff (1990) and Wang *et al.* (2018). While development rate obtained during monsoon season in the present study for *C. megacephala* are rather different from (Bharti *et al*., 2007), but development rate conducted during summer season and winter season was rather similar to their study. In the present study, the time from hatching till pupation was 78h ,139h and 165h at summer, monsoon and winter season at an average temperature of 31°C, 28°C and 26°C respectively, while in the study of Bharti *et al*. (2007), the time taken was 69h, 94h and163h at 30°C, 28°C and 25°C respectively. This might be due to the changes in humidity, rainfall and temperature prevailing in these geographically different areas. Differences in developmental rate under constant temperatures are probably due to genetic variations of common flies with a worldwide distribution (Tourle *et al*., 2009). The changes in the developmental rate of species during different seasons cautions that while performing the assessment of PMI, the investigators should be very careful about the climatic conditions prevailing in the respective study area (Gallagher *et al.,* 2010). This signifies the importance of generating location specific data of forensically important species for accurate assessment of postmortem interval. This is the first report on the developmental rate of this species during different seasons from South India and useful for the PMI assessment of dead bodies under forensic investigations during different seasons in future.

ACKNOWLEDGMENTS

The authors sincerely thank the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala for the DNA sequencing work. The authors also thank the Principal and Management of St. Thomas' College (Autonomous), Thrissur, Kerala, India for providing facility and support for conducting the present research work.

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(Received June 29, 2022; revised ms accepted November 01, 2022; published December 31, 2022)