ONLINE ISSN 2651-3579

Journal of the Entomological Research Society

Volume: 23 Part: 3 2021

JOURNAL OF THE ENTOMOLOGICAL RESEARCH SOCIETY

Published by the Gazi Entomological Research Society

Subscription information

Published by GERS in single volumes three times (March, July, November) per year. The Journal is distributed to members only. Non-members are able to obtain the journal upon giving a donation to GERS.

Papers in *J. Entomol. Res. Soc.* are indexed and abstracted in Science Citation Index Expanded (SCIE), Zoological Record, Biological Abstracts, BIOSIS Previews, Scopus, Essential Science Indicators, ProQuest, Scimago Journal & Country Rank (SJR), IPIndexing Portal, Resurchify, ORES, Academic Accelerator, CiteFactor, scinapse, Acarindex, Publons, TR Dizin.

Publication date: November 27, 2021

D. Steve Dennis, dipterist, passes away

It is with great sorrow that we record the death of our friend and colleague, D. Steve Dennis, an exceptional entomologist.

After obtaining a degree in Zoology in 1971 at Colorado State University, Steve journeyed to the University of Wyoming, where he attained a degree in Entomology studying robber flies. While working on his PhD thesis, he developed an interest in immature stages of robber flies. His keen mind combined with great patience enabled him to devote the many hours necessary to study these fascinating insects.

Upon graduation, he went to work for the U.S. Environmental Protection Agency monitoring pesticides residues in water. Later, he joined an engineering/construction company. Spending more than thirty years with this company, Steve traveled the world extensively, supporting environmental, engineering, and construction projects in several countries on six continents.

Throughout his life, Steve maintained a keen interest in the behavior of predatory flies. During his working years, Steve continued co-publishing on the behaviors of various species of robber flies. He collaborated with a number of eminent entomologists during his entomological career, including Lloyd Knutson, J.K. Barnes, and R.J. Lavigne.

Steve published over 50 papers. Of special note, he co-published a paper describing the pupae of ten species of Wyoming asilids with R.J. Lavigne, a key to robber fly (Diptera: Asilidae)

subfamilies based on pupal cases, and a review and analysis of information on the biology and morphology of immature stages of robber flies, the latter two with J.K. Barnes and the noted USDA dipterist, Dr. Lloyd Knutson (deceased).

After retiring from environmental engineering and construction, Steve co-authored with J.K. Barnes eight further papers on pupal descriptions between 2011 and 2014. His final years were spent researching robber flies in the Moses Creek Conservation Area near his home in northeastern Florida, USA. In that environment, he spent many happy hours observing a variety of robber fly species upon which he published a series of excellent papers describing their behavior.

Steve was always generous in his praise of the work of his colleagues and will be greatly missed.

Robert LAVIGNE & Jeanne DENNIS

J. Entomol. Res. Soc., 23(3): 187-196, 2021 *Research Article*
Doi: 10.51963/jers.v23i3.1962 *Online ISSN:2651-3579*

A Comparative Study of Gut Enzymes and Nest Materials of Three Mound Building Termites of Eastern India

Satarupa HOTA[1] Sunanda SAHOO[2*]

[1,2]School of Life Sciences, Sambalpur University, JyotiVihar, Burla - 768019, Odisha, INDIA
e-mails: [1]satarupahota369@gmail.com, [2*]drsunanda_sahoo@yahoo.com
ORCID IDs: [1]0000-0001-6761-2181, [2*]0000-0002-1934-8133

ABSTRACT

Termites are found to digest a wide variety of lignocellulosic biomass including wood with high lignin content by the aid of unique cellulolytic and hemicellulolytic microorganisms. A research gap is realized in works associated with termite gut enzyme activities in India. Therefore, the present work is focused on the comparison of termite gut enzyme activities among epigeous, subterranean, arboreal termites and the physicochemical properties of respective mound soils collected from Sambalpur University Campus, Odisha, India. The mound building termites (subterranean and epigeous) showed significantly more cellulase, xylanase and phosphatase enzyme activities than the arboreal termites. The enzyme activities showed efficient correlation with organic carbon, total nitrogen, available potassium and water holding capacity of respective mound materials. This particular study has provided new information regarding the contribution of three distinct termite species towards soil fertility status. Further, a high quantity of nutrients accumulated in subterranean termite mound soils along with high enzyme activities by subterranean species, *O. obesus* in the current study, can be considered a hotspot for microbial concentration, which can be exploited for biofuel production, and mound soil as soil amendment, bioremediation and bio-filtration purpose.

Key words: Termites, termite gut enzymes, termite gut microbes, mound soil, physiochemical properties, microbial concentration.

Hota, S. & Sahoo, S. (2021). A Comparative study of gut enzymes and nest materials of three mound building termites of Eastern India. *Journal of the Entomological Research Society,* 23(3), 187-196.

INTRODUCTION

The termites hold a vital status as a primary consumer and contribute in many ways in tropical ecosystems. They are involved in increasing soil fertility by disintegrating wood in its many forms and thereby enhancing the plant growth. Insects like the termites have the unique ability to digest lignocellulose with high efficiency and often using it as a sole food source. Termites can digest 74-99% lignocelluloses (Ni &Tokuda, 2013). Earlier studies also indicated that novel enzymes with high lignocellulose degradation potential may reside in termite guts. It is well documented that in tropical ecosystems, termites are among the most important soil invertebrate groups for playing an essential role in decomposing processes, nutrient cycling, and changes in soil properties. Cellulose is the basic food requirement for all termites and it is found in all types of plant-based materials. The cellulose is digested by bacteria in the gut of species in the family Termitidae (Slaytor, 2000).

Most termite species eat ground growths, and have an important role in maintaining soil fertility. The plant tissues on which termites feed contain very little protein and therefore have little nitrogen. Gut bacteria are also used by some termites to regulate the nitrogen in the atmosphere. In addition to cellulose, termites are able to degrade other glucose polymers such as starch and glycogen through the action of amylases (Waller & La Fage, 1987).

The symbiotic gut floras of termites are able to degrade huge mass of wood constituents such as cellulose and hemicelluloses (Schäfer et al, 1996). Termites breaks down lignocelluloses by their own enzymes along with those of symbiotic gut microflora (Brune, 2014). The linear polysaccharide β-1, 4-xylan of hemicellulose, a major component of the plant cell wall is degraded into simple xylose by xylanase enzyme (Beg et al, 2001; Lima et al, 2014), cellulases (endoglucanase, exoglucanase, and β-glucosidase), hemicellulases (β-xylosidase, α-l-arabinofuranosidase, and β-d-xylanase), α-amylase, and proteases (trypsin-like, chymotrypsin-like, and keratinase-type) in gut extracts from *Nasutitermes corniger* workers and soldiers.

The termite mound with its extensively accumulated nutrients is considered as a ‘gold mine’ for bacterial clusters (Enagbonma & Babalola, 2019). However, over the years, not much attention has been given to the bacteria present in termite mound soil particularly in India. This is because many studies have focused on approaches to manage termites which they see as a menace to crops and buildings.

The termite gut and its mound soil create well defined ecological environment causing the development of a special group of cellulolytic and hemicellulolytic microorganisms (Varma, Kolli, Paul, Saxena, & Konig, 1994). The mound building termites of the genus *Odontotermes* support rich microbial biomass as reported by several authors. However, there was a paucity of literature on termite gut enzyme activities particularly in Odisha, India. Hence, the present work was aimed to study the gut enzyme activities of three phytogeophagus and mound building termite species namely, *Odontotermes brunneus* (Hagens, 1858) from the roadside area, *Odontotermes obesus* from the subterranean mound of grassland site and

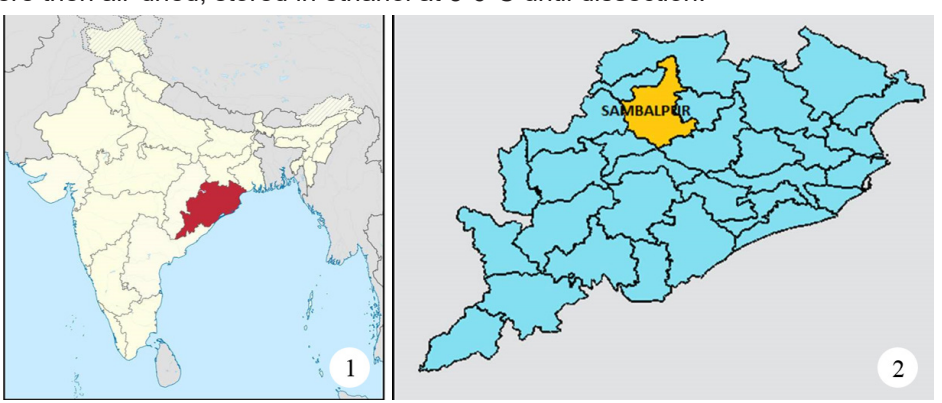
Odontotermes feae (Wasmann, 1896) from the arboreal nest. The study also included physicochemical properties of respective termites mound soil.

The significance of the study lies in the fact that xylophagous termites are capable of degrading lignocellulose by symbiotic gut microorganisms along with the host's indigenous enzymes and it is believed that the termite gut with rich microbial community might probably help to obtain natural yeasts with celluloytic, xylanolytic and ethanologenic traits required for bioethanol production from lignocellulosic biomass. Therefore, the present work was carried out to observe the gut enzyme activities of three native termites as a pioneer study in Western Odisha.

MATERIALS AND METHODS

Termite collection and identification

The termites were collected from six roadside small epigeous mounds, from six underground/ subterranean galleries of grassland sites, and from the galleries on the surface of six mango trees of Sambalpur University Campus (Figs.1-4). They were then sent to ZSI (Zoological Survey of India), Kolkata for species identification. The termites collected from the trap or mounds were washed thoroughly by tap water to remove soil particles and were then washed with distilled water. The clean termites were then air-dried, stored in ethanol at 5-6ºC until dissection.



Figs. 1,2. 1. Political map of India highlighting the state Odisha. 2. Political outline map of the state Odisha and highlighting the district from where the samples were collected. (Sahu, Nayak, & Sahu, 2020; District Administration Sambalpur, 2021).

Termite gut removal

The termites were dissected by following the method adopted by Smith (2007). The termites were removed from freeze, kept on ice; their guts were dissected and removed using surgical blade and forceps, further dipped in Sodium acetate buffer. The gut parts were then manually homogenized in chilled glass motor and pestle with 1ml acetate buffer. The homogenization was done by taking 50 termite gut materials/ ml of acetate buffer from a pool of approximately 500 termites and kept on ice until enzyme extraction was done.

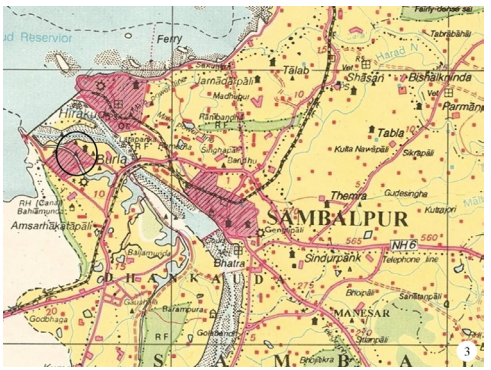


Fig. 3. General reference map of sambalpur district with a black outline of the study sites (Mehta, 1996).

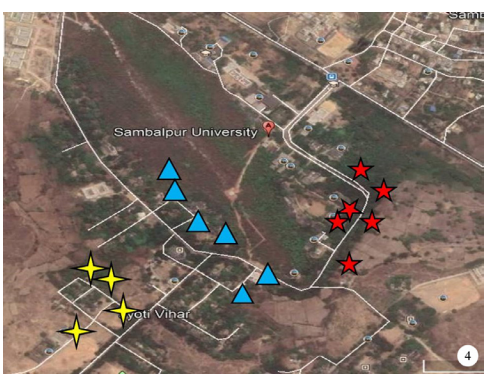


Fig. 4. Study sites:(✦) Subterranean galleries of grassland, (★) From six epigeous mound of road side, (▲) From the galleries on the surface of six mango trees of Sambalpur University Campus (Keyhole, Inc., 2001).

Termites gut enzyme extraction and assay

The homogenate extract containing termite gut contents of each eppendorf tube was then centrifuged at 14,000 rpm at 4ºC for 15 minutes with the help of a cold centrifuge, (REMI C-30). The supernatants were collected, frozen, and kept at - 80ºC until used in enzyme assays. The extracts in sodium acetate buffer were used in assays for cellulase, xylanase, α-amylase, and phosphatase activities. Cellulase and xylanase activity was studied as per Smith (2007).

Cellulase activity

For cellulase assay, the substrate solution used was 0.1% CMC (Carboxy Methyl Cellulose) prepared in sodium acetate buffer (pH 5.5, 0.1 M). The solutions were slightly heated until CMC particles were no longer visible. Then the solution was used as a substrate for enzyme assay. In the sample tubes 1.8ml of CMC solution were taken and 200µl (0.2 ml) of enzyme extract was added. The enzyme-substrate

solutions were then allowed to react for 10 mins at room temperature. Whereas, an equal amount of acetate buffer and enzyme extract was taken in a blank tube i.e. without substrate solution. After the enzyme-substrate reaction, 2 ml of 2, 4 DNSA (2, 4-Dinitro salicylic Acid) was added in all three test tubes. And immediately after the addition of DNSA, the test tubes were then placed in a boiling water bath to induce the oxidation-reduction reaction and the amount of glucose formed was determined spectrophotometrically at 540 nm. The cellulase activity was expressed in terms of µg glucose/ml of enzyme extract/ minute.

Xylanase activity

For xylanase assay, the substrate solution was 0.01% D-Xylose prepared in the same sodium acetate buffer. The solution then slightly heated until Xylan particles were no longer visible. The solution was then used as a substrate for the enzyme assay. Xylanase assays were conducted in three glass test tubes, one as blank and the other two sample tubes. In each tubes 200µl (0.2 ml) of enzyme extract were taken. Then 1.8 ml of xylan solution was added in sample tubes while the blank test tube the same amount of acetate buffer was added. After 10 mins of enzyme-substrate reaction, 2 ml of 2, 4 DNSA was added in all three test tubes. And immediately after the addition of DNSA, the test tubes were then placed in a boiling water bath for 5 minutes to induce the oxidation-reduction and the amount of glucose formed was determined spectrophotometrically at 540 nm. The xylanase activity was expressed in terms of µg glucose/ml of enzyme extract/ minute.

α-Amylase activity

The assay was carried out based on the method described by Bernfeld (1955). Gut extract from termites was incubated at 50°C for 10 min with 400µl of 1% (w/v) soluble starch in 0.1 M sodium acetate pH 5.5 containing 0.02 M $CaCl_2$ and 0.15 M NaCl. The reaction was stopped by adding 500µl of DNSA. Next, the assays were heated at 100°C in boiling water for 6 min and immediately cooled on ice for 15 min. Then, absorbance was measured at 540 nm. The amount of reducing sugars was determined using the calibration curve of glucose. Reaction blanks were performed without starch or without extracts. The amylase activity was expressed in terms of µg glucose/ml of enzyme extract/ minute.

Phosphatase activity

The phosphatase activity was determined according to the method of Tabatabai & Bremner (1969). Gut extracts from termites (0.1 mL) were incubated at 37°C for 1 h in a modified universal buffer (MUB) (12.1 g of Tris-hydrochloric aminomethane, 11.6 g of malic acid, 14.0 g of citric acid, 6.3 g of boric acid in water and 488 ml of 0.1 M NaOH) (pH 5.0) and 0.2 mL of 115 mM p-nitrophenylphosphate (p-PNP). The reaction was stopped by the addition of 0.1 mL of 0.5 M NaOH, and immediate centrifuging for 15 min at 10,000 rpm. The amount of p-nitrophenolreleased from PNP was measured in the supernatant at 405 nm using p-nitrophenol as standard. The enzyme activity was defined as the amount of enzyme required to liberate 1 µg of p-nitrophenol/ ml/ min under assay conditions.

Physicochemical properties of termite mound soil

Soil particle size distribution was determined by the hydrometer method, soil pH was measured by using the pH meter. Organic carbon was determined by Walkley and Black wet oxidation method, total N was determined by the micro Kjeldahl method, Phosphorus content of mound materials was done following the method of Bray & Kurtz (1945), Na and K were determined by flame photometry.

Statistical analysis

Statistical Package for Social Sciences (SPSS, 2010) was used for the statistical test. One way ANOVA followed by LSD was used to determine significant differences between means of the parameters obtained from soils and mound materials. Principal Component Analysis was carried out using MINITAB ®19 to observe the clustering pattern in three species using different gut enzyme activities and respective mound material characteristics.

RESULTS

Enzyme activities

α-amylase activity, cellulase activity, xylanase activity (µg glucose/ml of enzyme extract/ min) and phosphatase activity (p-nitrophenol/ ml/ min) of three different termite guts (*Odontotermes brunneus*, *Odontotermes obesus* and *Odontotermes feae*) has been illustrated in (Table. 1) along with one way ANOVA and LSD values.

Physico-chemical properties of nest soil

The textural composition of respective mounds from which the termites were collected indicated highest clay content of 12.93% in subterranean nest soil of *O. obesus* followed by the clay content of 12.02% in epigeous mound soil of *O brunneus*, and then that of *O. feae* arboreal nest soil (9.64%). The percentage of sand was highest in the arboreal nest soil (89.36%), followed by that of epigeous nest soil 84.98%, and that of subterranean nest soil (80.27%). Water holding capacity (WHC in %) of around 25% was recorded in subterranean nest soil as well as that of epigeous nest soil. The arboreal nests showed the WHC (%) of 21%.The subterranean nest soil showed a pH which was more acidic (5.95 ± 0.736) than that of epigeous (6.858 ± 0.375) and arboreal nest soil (6.317 ± 0.264) and the difference was significant at $p< 0.05$ ($F_{2, 15}= 4.998$).

The highest organic carbon (OC in g%) content of mound material (Table. 2) was found in subterranean termites as compared to epigeous and arboreal nest soil. Similarly total nitrogen (TN), available phosphorus (AP), available potassium (AK), water holding capacity (WHC), and pH showed a significant difference between three types of nest soil.

The LSD tests demonstrate a significant difference in the average value of TN, AP, AK in different termite nest soil. However, Average WHC of *O. bruneus* and *O.obesus* nest soil showed significant difference with that of *O. feae* nest soil, but not among themselves. Similarly, the average pH value of *O. bruneus* and *O. feae* nest soil showed a significant difference with that of *O. obesus*, but not among themselves.

To find out the correlation between the termite enzyme activities, and their respective nest soil multiple correlations were conducted using SPSS 16 (Table. 3). Amylase, xylanase, cellulase, and phosphatase activities of termites showed a significant correlation with the physicochemical properties of mound materials like WHC, OC, TN, AK etc.

Table 1. Gut enzyme activities of different termite species.

Enzyme activities	Termite Species			One way ANOVA (F), Critical value of F = 3.68232	LSD (p≤0.05)
	O. bruneus	*O. obesus*	*O. feae*		
αAmylase	0.945 ± 0.127^{a}	0.62 ± 0.031^{b}	0.608 ± 0.166^{b}	14.670***	0.325
Cellulase	0.433± 0.038^{a}	0.82± 0.061^{b}	0.408± 0.055^{a}	116.881***	0.387
Xylanase	1.083±0.179^{a}	1.443± 0.037^{b}	0.807± 0.115^{bc}	39.198***	0.36000
Phosphatase	8.003± 0.864^{a}	10.143± 0.606^{b}	7.787± 0.749^{a}	18.231***	2.14

***p<0.001, F= values of one way ANOVA between enzyme activities of different termites, values in the same row with different alphabet were significantly different by LSD (p<0.05).

Table 2. Physico-chemical properties of nest soils from *O. bruneus*,*O. obesus*, and *O. feae termites.*

Physico-chemical parameters	Termite Nest Soil			One way ANOVA (F), Critical value of F = 3.68232	LSD (p<0.05)
	*O. bruneus*S	*O. obesus*	*O. feae*		
OC	0.583± 0.01^{a}	1.695± 0.12^{b}	0.583± 0.014^{a}	498.196***	1.11167
TN	1.468± 0.039^{a}	1.84± 0.119^{b}	1.378±0.124^{bc}	34.741***	0.37167
AP	0.693± 0.051^{a}	0.882± 0.025^{b}	0.78± 0.033^{bc}	36.658***	0.18833
AK	342.167±78.062^{a}	1138.667±93.607^{b}	181.33± 5.428^{bc}	317.779***	796.5
WHC	24.295± 3.133^{a}	25.25± 2.518^{a}	20.275± 3.228^{b}	4.721*	4.975
pH	6.858± 0.375^{a}	5.95± 0.736^{b}	6.317± 0.264^{a}	4.998*	0.908

*p<0.05, ***p<0.001, F= values of one way ANOVA between enzyme activities of different termites, values in the same row with different alphabet were significantly different by LSD (p<0.05).

Table 3. Correlation between gut enzymes and soil biochemical characteristics.

	OC	TN	AP	AK	pH	WHC	Amylase	Xylanase	Cellulase	Phosphatase
OC	1	.930**	.841*	.987**	-.839*	.559	.847*	.829*	.754*	.948**
TN	.930**	1	.639	.955**	-.672	.757*	.638	.932**	.808*	.948**
AP	.841*	.639	1	.757*	-.832*	.129	.801*	.402	.604	.819*
AK	.987**	.955**	.757*	1	-.792*	.629	.800*	.884**	.737*	.890**
pH	-.839*	-.672	-.832*	-.792*	1	-.072	-.720	-.580	-.332	-.73
WHC	.559	.757*	.129	.629	-.072	1	.382	.816*	.806*	.055
Amylase	.847*	.638	.801*	.800*	-.720	.382	1	.587	.635	-.057
Xylanase	.829*	.932**	.402	.884**	-.580	.816*	.587	1	.678	.947**
Cellulase	.754*	.808*	.604	.737*	-.332	.806*	.635	.678	1	.876*
Phosphatase	.948**	.948**	.819*	.890**	-.73	.055	-.057	.947**	.876*	1

*Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). (OC: Organic Carbon (g%), TN: Total Nitrogen (g%), AP: Available phosphorus (g%), AK: Available Potassium (g%), WHC: Water Holding Capacity (g%), Amylase, Cellulase, Xylanase activity (µg glucose/ ml gut extract/min) and Phosphatase (µg of p-nitrophenol/ ml gut extract/ min).

CONCLUSIONS AND DISCUSSION

Carbon in plant biomass is the largest reservoir on Earth and the extensive verity of organisms is using this carbon as the main food source. However, most of the animals are unable to assimilate plant biomass, due to the complex architecture of the plant cell wall (Pauly & Keegstra, 2008). The plant cell wall is primarily composed of cellulose, hemicellulose, pectin, and lignin (McNeil, Darvill, Fry, & Albersheim, 1984). Different factors that cause the complexity of termite nests are by virtue of their social organization, feeding habit, morphology and behavior of different casts. Moreover, environmental characteristics attribute to wide range of variations. There are some termite species that create simple nests and some build complex nests which represent a large architecture (Petr, Jiří, František, & Tomáš, 2013). The decomposition of any wood substrate is a result of symbiotic associations between subterranean termites with other microorganisms, especially fungi (Resh & Cardé, 2009).

Termite digestomes are found to be associated with as much as 250 different species of microorganisms (Nadin, 2007). However, not all of them are cellulolytic microorganisms. These microorganisms are specifically degrading specific components of the plant structures resulting in different end products. According to previous reports, termites could potentially decompose lignocelluloses within a day by degrading 74-99% of the cellulose, 65-87% of the hemicellulose, as well as 5-83% of the lignin producing weak acids and simple sugars (König, Fröhlich, & Hertel, 2006).

We observed the highest enzyme activities by subterranean species *O. obesus* associated with fungus garden in comparison to epigeous *O. bruneus* and tree-dwelling *O. feae* species. The highest OC, TN, AP, AK and higher clay%, WHC% in subterranean termite mound soil as compared to the epigeous, arboreal mound are likely to be contributed by the combined action of foraging materials available and the enzyme activities of the termites residing in the mound to convert these materials to available nutrients. Termites that excavate hardwood or soft wet wood (arboreal termites) cut out tunnels and then plaster them with faces that are heavily fibrous with plastic texture. It is reported that they even utilize sand grains for partitioning and cementing that with their faeces to create a thick fabric of 2mm (Lee & Wood, 1971). Menichetti et al (2014) have reported that the grazing activity and nutrient flux into the subterranean mound material is more in comparison to the epigeal part of the mound. This might be the reason for a significantly high enzyme, both cellulase and xylanase activities of subterranean termite in comparison to those collected from epigeal parts of large roadside nests. The salivary glands and the upper gut region secrete amylase enzymes that do not contain microbes as reported by O'Brien &Slaytor (1982). Most termites use soil, together with saliva and faeces, to construct their nests (Wood, 1988), the reason for which we observed the highest amylase activity in *O. bruneus* collected from large epigeous mounds from the roadside.

Principal Component Analysis was carried out considering the data of Amylase, Cellulase, Xylanase, Phosphatase activities of epigeous *Odontotermes brunneus* (A), subterranean *Odontotermes obesus* (B) and arboreal *Odontotermes feae* (C) termite guts and OC, TN, AP, AK, WHC, pH of respective termite nest soil (Fig. 5). Termites

that have similar on the basis of their nest characteristics are clustered together and we obtained 3 clusters of three termite species. The characteristics that have heavily influenced PC1 strongly, differentiates *Odontotermes obesus* (B) from other two species and the characteristics that heavily influenced PC 2 segregated *Odontotermes brunneus* (A) from *Odontotermes feae* (C).

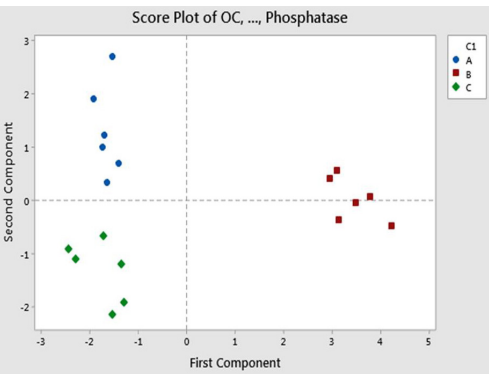


Fig. 5. PCA of enzyme activities in of three termite species and physicochemical properties of respective mound materials: red colour, subterranean species *O. obesus*; blue, epigeous *O. bruneus*; green colour, tree dwelling *O. feae* species.

This study indicated and statistically proved that different builder species cause significant variation in mound structures and physicochemical properties as well. Hence the study will help in the effective usage of suitable termite species and respective mound soil material in an eco-friendly manner to ensure environmental sustainability.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Head, School of Life Sciences, Sambalpur University, Odisha for providing the necessary research facilities.

REFERENCES

Beg, Q.K., Kapoor, M., Mahajan, L., & Hoondal, G.S. (2001). Microbialxylanases and their industrial applications: A review. *Applied Microbiology and Biotechnology*, 56(3-4), 326-338.

Bernfeld, P. (1955). Amylases, alpha and beta. *Methods in Enzymology*, 1, 149-158.

Bray, R.H. & Kurtz, L.T. (1945). Determination of total organic and available forms of phosphorus in soils. *Soil Science*, 59, 39-45.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews Microbiology*, 12, 168-180.

District Administration Sambalpur (2021, October, 17). Coordinates (21.466°N, 83.983°E). Retrieved from https://en.wikipedia.org/wiki/Sambalpur_district#/media/File:India_Odisha_Sambalpur_district.svg.

Enagbonma, B.J. & Babalola, O.O. (2019). Potentials of termite mound soil bacteria in ecosystem engineering for sustainable agriculture. *Annals of Microbiology*, 69, 211-219.

Sahu, C., Nayak, H.N., & Sahu, S.K. (2020). Carbon sequestration potential of trees in an urban area: a case study of sambalpur town in eastern india. *Fresenius Environmental Bulletin*, 29, 8757-8766.

Keyholl, Inc. (2001, October, 17). Map Courtesy: Google Earth. Retrieved from https://earth.google.com/web/search/Sambalpur+University,+Jyoti+Vihar,+Burla,+Odisha/@21.47616833,83.8827404,177.88349303a,3027.4930729d,35y,0h,0t,0r/data=CigiJgokCR6EBmvPejVAEarts_QieTVAGVB8DDPf-FRAIdYuPt7v91RA

König, H., Fröhlich, J., & Hertel, H. (2006). Diversity and lignocellulolytic activities of cultured microorganisms. In König, H. & Varma, A. (Ed.). *Intestinal microorganisms of termites and other invertebrates* (pp. 271-301), *Soil Biology*, Springer, Berlin, Heidelberg.

Lee, K.E. & Wood, T.G. (1971). *Termites and soils*. Academic Press, London, New York.

Lima, T.A., Pontual, E.V., Dornelles, L.P., Amorim, P.K., Araújo, R., Coelho, L.C.B.B., Napoleão, T.H., & Paiva, P.M.G. (2014). Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*. *Comparative Biochemistry and Physiology*, *Part B*, 176, 1-8.

McNeil, M., Darvill, A.G., Fry, S.C., & Albersheim, P. (1984). Structure and function of the primary cell walls of plants. *Annual Review of Biochemistry*, 53, 625-663.

Mehta, S.P. (1996). Survey of India Maps, 1st ed, Government of India.

Menichetti, L., Landi, L., Nannipieri, P., Kätterer, T., Kirchmann, H., & Renella, G. (2014). Chemical properties and biochemical activity of colonized and abandoned litter-feeding termite (*Macrotermes* spp.) mounds in Chromic Cambisol Area on the Borana plateau, Ethiopia. *Pedosphere*, 24, 399-407.

Nadin, E., (2007). For the love of termites. *Engineering Science*, 2, 24-31.

Ni, J. & Tokuda, G. (2013). Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances*, 31(6), 838-50.

O'Brien, R.W. & Slaytor, M. (1982). Role of microorganisms in the metabolism of termites. *Australian Journal of Biological Sciences*, 35, 239-262.

Pauly, M. & Keegstra, K. (2008). Cell-wall carbohydrates and their modification as a resource for biofuels. *The Plant Journal: for Cell and Molecular Biology*, 54, 559-568.

Petr, P., Jiří, B., František, Š., & Tomáš, O. (2013). Investigation of subterranean termites nest material composition, structure and properties. In Yitzhak, M. (Ed.), *Materials science-advanced topics*, IntechOpen, Doi: 10.5772/55145 (pp. 519-549).

Resh, V.H., Cardé, R.T. (2009). *Encyclopedia of insects*, 2nd ed., Academic press, Elsevier, United States.

Schäfer, A., Konra, D.R., Kuhnigk, T., Kämpfer, P., Hertel, H., & König, H. (1996). Hemicellulose-degrading bacteria and yeasts from the termite gut. *Journal of Applied Ecology*, 80, 471-478.

Slaytor, M. (2000). Energy metabolism in the termite and its gut microbiota. In Abe, T., Bignell, D.E., & Higashi, M. (Eds.). *Termites: evolution, sociality, symbioses, ecology* (pp. 307-332). Springer, Dordrecht.

Smith, J.A. (2007). *Cellulolytic and xylanolytic gut enzyme activity patterns in Major Subterranean termite pests*. *Phd Thesis*, University of Florida, Gainesville, Florida.

Tabatabai, M.A. & Bremner, J.M. (1969). Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry*, 1(4), 301-307

Varma, A., Kolli, B.K., Paul, J., Saxena, S., & Konig, H. (1994). Lignocellulose degradation by microorganisms from termite hills and termite guts: A survey on the present state of art. *Federation of European Microbiological Societies Microbiology Reviews*, 15:9-28

Waller, D.A. & La Fage, J.P. (1987). Nutritional ecology of termites. In Slansky, Jr. F. & Rodriguez, J.G. (Eds.). *Nutritional ecology of insects, mites, spiders and related invertebrates*. (pp. 487-532). A Wiley-Interscience Publication, New York.

Wood, T.G. (1988). Termites and the soil environment. *Biology and Fertility of Soils*, 6, 228-236.

Received: October 17, 2020 *Accepted: October 30, 2021*

J. Entomol. Res. Soc., 23(3): 197-212, 2021 *Research Article*
Doi: 10.51963/jers.v23i3.1989 *Online ISSN:2651-3579*

Antifeeding and Insecticidal Activity of *Ailanthus altissima* and *Morus alba* Extracts Against Gipsy Moth (*Lymantria dispar* (L.), Lepidoptera, Lymantridae) Larvae Under Laboratory Conditions

Snežana TANASKOVIĆ[1] Sonja GVOZDENAC[2] Radenka KOLAROV[3]
Vojislava BURSIĆ[4] Bojan KONSTANTINOVIĆ[5] Dejan PRVULOVIĆ[6*]

[1]Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, 32102 Čačak, SERBIA
[2]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, SERBIA
[3,4,5,6]Faculty of Agriculture, University of Novi Sad,
trg Dositeja Obradovića 8, 21000 Novi Sad, SERBIA
e-mails: [1]stanasko@kg.ac.rs, [2]gvozdenacsonja@gmail.com,
[3]kolar.rada@gmail.com, [4]bursicv@polj.uns.ac.rs, [5]bojank@polj.uns.ac.rs,
[6*]dejanp@polj.uns.ac.rs
ORCID IDs: [1]0000-0002-7853-2328, [2]0000-0001-9366-5172, [3]0000-0003-1976-7618,
[4]0000-0001-8331-095X, [5]0000-0001-5071-1386, [6]0000-0002-1880-4934

ABSTRACT

Gypsy moth (*Lymantria dispar* (L.) is one of most important defoliating pests of deciduous trees. Due to increased environmental demands, the use of plant-based preparations is gaining in importance as a control option for this pest in forestry, agriculture and horticulture. The aim of this study was to evaluate antifeeding and insecticidal activity of 0.5, 1 and 2% extracts of *Ailanthus altissima* bark and leaves, and *Morus alba* leaves, against *L. dispar larvae under* laboratory conditions. Antioxidant capacity of plant extracts was determined, as well as the content of phenolic compounds by spectrophotometric and HPLC-DAD methods. Antifeeding and insecticidal effects were tested in a "no-choice" test. The highest content of all bioactive phenolic compounds was in *A. altissima* bark and *M. alba* leaf extracts. The lowest leaf consumption after 24 and 48 h was in *A. altissima* bark (5.03, 9.30%, respectively) and *M. alba* leaf (1.44, 3.22%, respectively) extracts. *A. altissima* bark and *M. alba* leaf extracts expressed strong antifeeding activity. After 24 h, all extracts expressed slight insecticidal effect (2.25-17.50% of mortality). The mortality increased after 48 h in treatments with *A. altissima* bark extract, at all applied concentrations (40.0-57.50%) and *M. alb*a leaves at 1 and 2% concentrations (30.0-62.50%). Our results indicate that extracts of *A. altissima* bark and *M. alba* leaves may act as effective low-cost natural protectants able to control the presence of gypsy moth in ecosystems. Extracts of *A. altissima* bark and *M. alba* leaves expressed strong antifeeding activity and significant insecticidal effect on gypsy moth larvae, at all applied concentrations.

Key words: Lymantria dispar (L.), botanical insecticides, feeding intensity, larval mortality, phenolic compounds.

Tanasković, S., Gvozdenac, S., Kolarov, R., Bursić, V., Konstantinović, B., & Prvulović, D. (2021). Antifeeding and insecticidal activity of *Ailanthus altissima* and *Morus alba* extracts against gipsy moth (*Lymantria dispar* [L.], Lepidoptera, Lymantridae) larvae under laboratory conditions. *Journal of the Entomological Research Society*, 23(3), 197-212.

INTRODUCTION

Gypsy moth *(Lymantria dispar* (L.), Lepidoptera; Lymantridae) is one of the most devastating defoliating pests of deciduous forests, but it is also very harmful in orchards and urban greenery (Kostić, Popović, Brkić & Milanović, 2008; Milanović et al, 2014; Cao et al, 2015). To prevent losses that gypsy moth larvae cause in forestry, agriculture and horticulture, it is necessary to harmonize pest control strategies with the principles of integrated pest management, using selective and less toxic insecticides, mechanical measures and biological insecticides (*Bacillus thuringiensis* var. *kurstaki* or botanical insecticides like azadirachtin) (Kostić et al, 2008; Singh, Cheema & Singh, 2020). Koul (2005) reported growing interest in use of botanical preparations (i.e. plant-based insecticides) for the control of gypsy moth in organic and sustainable agriculture due to increased environmental demands. Botanical insecticides based on secondary plant metabolites represent an important group of biopesticides (Drobnjaković et al, 2018).

Plants are a rich source of compounds that exhibit high biological activity against harmful insects, thus botanical preparations are good potential substitutes for synthetic insecticides in agricultural and forest pest control (Bohinc et al., 2020; Šućur et al, 2015; Hikal, Baeshen & Said-Al Ahl, 2017; Gvozdenac, Šućur, Manojlović, Prvulović & Malenčić, 2018). In recent years, antifeeding effects of various plants and plant extracts have been studied for gypsy moth larvae and several findings confirm the antifeedant or repellent activity of *Pinus taeda* L., *Juniperus virginiana* L., *Acer rubrum* L. (Keena & Richards, 2020), *Ocimum basilicum* L. (Kostić et al, 2008; Popović et al, 2013), *Morus alba* L. and *Aesculus hippocastanum* L. (Gvozdenac, Inđić, Vuković, Grahovac & Tanasković, 2012). Botanical insecticides have many advantages, primarily low toxicity and selectivity towards non-target, and high toxicity to target organisms and also the capacity to avoid the occurrence of insect resistance and biodegradability (Kostić et al, 2008; Krinski, Massaroli & Machado, 2014).

Ailanthus altissima (Mill.) Swingle, commonly known as the “tree of heaven”, is a perennial invasive species, native to China and introduced in Europe at the end of 18th century (Kowarik *&* Säumel, 2007; De Feo, Mancini, Voto, Curini & Digilio, 2009). The extracts and essential oils obtained from different parts of *A. altissima* have been reported to exhibit diverse biological activities, such as antioxidant (Aissani, Jabri, Mabrouk & Sebai, 2018), phytotoxic (Albouchi, Hassen, Casabianca & Hosni, 2013; Caser et al, 2020; Kozuharova, Benbassat, Berkov & Ionkova, 2020), antimicrobial (Albouchi et al, 2013; Aissani et al, 2018; Kozuharova et al, 2020), insecticidal (De Feo, Mancini, Voto, Curini & Digilio, 2009; Pavela, Zabka, Tylova & Kresinova, 2014), and also different pharmacological effects (Rahman, Rasool & Imran, 2019). *Morus alba* L., white mulberry, is native to eastern and central China, India and Japan (Kostić et al, 2013). The leaves of *M. alba* are the main source of food for silkworm, thus white mulberry is cultivated throughout the world, wherever silkworms are or were reared. In European countries it is grown also for fruit production (Sanghi & Mushtaq, 2017). The leaves of *M. alba* are traditionally used in Asian countries as a treatment for coughs, fever, sore and inflamed eyes, sore throats, headaches, dizziness and vertigo. Modern medicine proves the antidiabetic, antioxidant, anticancer, antimicrobial,

hepatoprotective and neuroprotective activity of *M. alba* extracts (Singh et al, 2013; Sanghi & Mushtaq, 2017; Thaipitakwong, Numhom & Aramwit, 2018). However, the reports on potential use of white mulberry extracts in pest management are scarce.

The aim of this study was to evaluate biological activity and assess effects of aqueous extracts of *A. altissima* leaves and bark, and *M. alba* leaves on feeding intensity (antifeeding activity) and mortality of *gypsy moth larvae in* laboratory conditions.

MATERIAL AND METHODS

Plant extracts

For bioassay, extracts of *Ailanthus altissima* (Mill.) Swingle bark and leaves, and *Morus alba* L. leaves were used. Plant material was collected on Suvobor (44.1339° N, 20.2195° E) and Jeljen (44.0007° N, 20.2589° E) mountain (Serbia). The plant species was identified by a botanist, and a voucher specimen has been deposited in the Herbarium (labelled as *A. altissima* - 17708 and *M. alba* - 17709) of Botanical Garden, Belgrade, Serbia.

Plant parts were previously dried at dark and windy place, at 20 °C. Plant material (10.0 g) was extracted with 70% ethanol (100.0 mL) as a solvent. The extraction was carried out using ultrasonic bath at room temperature for 1 h. Extracts were diluted in distilled water to concentrations of 0.5, 1 and 2%, and applied to oak leaf slices (30x30 mm) by soaking method for 5 sec. Leaf slices soaked in distilled water were untreated control. Leaves were air-dried for 20 sec after the extract application.

Chemical analysis of plant extracts

Total phenolic, total tannin and antioxidant capacity

Components of non-enzyme antioxidative system were determined using spectrophotometric methods with UV/VIS spectrophotometer (model Evolution 220, Thermo Fisher Scientific, USA).

Total phenol content (TP) was determined by colorimetric method using Folin-Ciocalteu reagent (Saha et al, 2013). Total tannin content (TT) was determined by the Folin-Ciocalteu procedure, after removal of tannins by their adsorption on insoluble matrix PVPP (polyvinylpolypyrrolidone). Calculated values were subtracted from total phenols content. Results were expressed as micrograms of quercetin equivalents per 1 mL of plant extracts (μg QE/mL).

Free radicals scavenging activity was tested in a DPPH (2,2-diphenyl-1-picrylhydrazyl) acetone solution (Lai & Lim, 2011). Ferric reducing antioxidant power (FRAP) assay was performed according to the method of Valeñtao et al (2002). The scavenging activity of the plant extracts on $ABTS^{+}$ radical (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was measured according to the method of Re et al (1999). The total antioxidant activity (TAA) of plant extracts were evaluated by phosphomolybdenum method as reported by Kalaskar & Surana (2014). The standard curve for antioxidant

capacity (activity) was plotted using ascorbic acid (AsA) solution. Superoxide dismutase (SOD) mimetic activity was assayed according to the method of Mandal, Mitra & Mallick (2008) slightly modified by measuring ability of plant extracts to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. One unit of the SOD mimetic activity was defined as the amount of enzymes required to inhibit reduction of NBT by 50%. The activity was expressed as IU per 1 mL of plant extracts.

HPLC-DAD analysis of phenolic compounds

All solvents used were of chromatography grade and obtained from J.T. Baker (Deventer, Netherlands). The analytical standards (manufactured by Sigma-Aldrich) used in the research are: trans-cinnamic acid (99.0%), 2-hydroxy cinnamic acid (97.0%), caffeic acid (98.0%), p-coumaric acid (98.0%), chlorogenic acid (95.0%), quercetin (98.0%), and kaempferol (97.0%). The stock standard solutions were prepared by dissolving an analytical standard in methanol while the working solution i.e. the mixture of the studied phenol compounds was obtained by mixing and diluting the stock standards with mobile phase resulting in the final mass concentration of 100 μg/mL. The composite mixtures of all phenol compounds at appropriate concentrations were used to spike samples in validation data settings. Acetic acid was of “pure for analysis” grade (J.T. Baker, Deventer, Netherlands).

The chromatographic separation of phenolic compounds was achieved using the Agilent 1100 (Agilent Technologies, USA) HPLC system with a binary pump and diode array detector - DAD. The phenolic acids were separated on a ZORBAX SB-Aq (5 μm particle size: 4.6 x 250 mm, Agilent) column. The extracts were filtered through 0.45 μm syringe filters and directly injected through a 30 μl fixed loop into the column. The mobile phase was acetonitrile with 2.0% acetic acid (solvent A) and Milli-Q water with 2.0% acetic acid (solvent B) in gradient mode, with the flow rate of 1.0 mL/min, equipped with a ZORBAX SB-Aq column. The gradient was as follows: 92% A at 0 min, 80% A at 18 min, 60% A at 25 min, 55% A at 30 min, 35% A at 40 min and 20% A at 42 min. Stop time was 2.5 min.

The detector linearity response was checked by preparing the blank plant extract sample (bark and leaves separately) and after the extraction the residue was diluted in 1.5 mL of the phenol compounds mixture standard in mass concentrations of 10.0, 25.0, 50.0 and 100.0 μg/mL.

The extracts were filtered through 0.45 μm syringe filters and directly injected into the HPLC-DAD. The repeatability of the method was determined by analyzing the sample of the same mass concentration level (10.0 μg/mL) in six replicates and shown as the relative standard deviation - RSD (%). The detection limit (LOD) was defined as the amount of phenolic compounds which produces the signal three times the noise signal. The quantification limit (LOQ) is the amount of phenolic compounds produces a signal ten times the noise signal. The LODs were determined by adding 100 μL of phenols mixture standard to the concentration of 1.0 μg/mL, in 0.5 g of the sample in six replicates and the LOQs was calculated.

Bioassay

Insects collecting and rearing

Egg masses of gypsy moth field population were collected from oak trees (Apatin, northern Serbia) during the winter 2018/19 and kept in a refrigerator (3 ºC) until the beginning of the experiment. Eggs were separated from mass and placed in glass tubes (Ø 1cm, height 16cm), previously filled with water up to ¼ of volume, on a layer of cotton wool (which does not have the contact with water). Tubes were closed with cotton wool cover to prevent the larvae from leaving the tube after hatching. Tubes with eggs were incubated at 22-25 ºC and a normal light regime for 3-5 days. After hatching, L_1 larvae were isolated from the tubes with a soft brush, placed in Petri dishes and fed daily with fresh *Quercus robur* leaves, until the stage L_2/ L_3.

Feeding intensity and insecticidal effects

Feeding intensity of extracts was assessed in a “no-choice” test. Ten larvae (L_2/ L_3 ratio 1:1) per replication were introduced in Petri dishes (Ø14) containing two oak leaf slices (30x30mm) previously treated with an extract, or distilled water in the control variant. “No choice” test was carried out at room temperature (22-25 ºC) and usual light regime (16:8). Feeding intensity, expressed as consumed leaf area (mm^2), was measured after 24 and 48 h and for further analysis the obtained values were transformed in relative values (%).

Antifeeding activity of extracts was assessed using Antifeeding index (AFI) and calculated according to Farrar, Barbour & Kennedy (1989):

$$AFI = (C - T / C + T) \times 100$$

C- Consumed area in the control variant (%); T- consumed area in the treatment (%)

The criterion according to Liu, Goh & Ho (2007), was applied to categorize the plants:

AFI <20% - no antifeeding activity (-)

50%> AFI ≥ 20% - slightly antifeeding activity (+)

70%> AFI ≥ 50% - medium antifeeding activity (++)

AFI ≥ 70% - strong antifeeding activity (+++)

Insecticidal effect was also assessed in the “No-choice” test. Dead and paralyzed larvae (determined by “palpation method”) were counted after 24 and 48 h and the values were transformed into %.

All experiments were set up in four replications.

Statistical analysis

The data on the consumed leaf area (%) were subjected to Two-way analysis of variance (ANOVA) to evaluate the influence of two factors and their interaction. The first factor was the plant species and the second was the concentration of extracts. Duncan`s multiple range test was used to assess the significance of differences

between treatments, only for the factor that had significant influence on feeding intensity and mortality. All tests were performed at the level of significance 95% in software STATISTICA.10.

RESULTS

Chemical composition of plant extracts

Spectrophotometric methods are widely used for a rapid determination of different phe-nolic compounds and antioxidant capacity of plant samples. The content of extracted phenolic and antioxidant compounds differed on the plant species and selected plant part (Table 1). The yield of total phenols in extracts, expressed as quercetin equivalents (QE), varied between 2.74 (M. alba leaves extract) and 163.25 (*A. altisima* bark extract) mg/g of dry plant material. The content of total tannins and antioxidant capacity of extracts of selected plants, measured by five different assays, followed the same pattern as the total phenolic content.

Table 1. Phenolic content and antioxidant capacity of *A. altissima* and *M. alba* extracts.

Parameter	Extract			F value
	A. altissima bark	*A. altissima* leaves	*M. alba* leaves	
TP (mg QE/g)	163.25 ± 3.42 a	17.90 ± 0.55 b	2.74 ± 0.07 d	8324.07**
TT (mg QE/g)	141.51 ± 1.73 a	14.93 ± 0.15 b	1.96 ± 0.03 d	18931.69**
DPPH (mg AsA/g)	41.07 ± 1.66 a	3.76 ± 0.09 b	0.19 ± 0.02 d	7495.9**
FRAP (mg AsA/g)	15.79 ± 2.37 a	5.27 ± 0.36 b	0.19 ± 0.01 c	295.6**
ABTS (mg AsA/g)	84.52 ± 7.47 a	13.19 ± 1.26 b	1.21 ± 0.05 c	354.70**
TAA (mg AsA/g)	16.67 ± 1.47 a	2.64 ± 0.11 b	0.29 ± 0.02 d	340.49**
SOD mimetic (IU/g)	1324.27 ± 126 a	365.55 ± 32.2 b	38.10 ± 3.73 d	469.49**

DPPH - 2,2-diphenyl-1-picrylhydrazyl; FRAP - Ferric reducing antioxidant power; ABTS - 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); TAA - total antioxidant activity; SOD - superoxid dismutase; AsA - ascorbic acid; TP - total phenolics; TT - total tannins; QE- quercetin equivalents

The method for HPLC-DAD analysis of phenolic compounds was evaluated in terms of linearity and repeatability, LOD and LOQ for trans-cinnamic, 2-hydroxy cinnamic, caffeic, p-coumaric and chlorogenic acid, quercetin and kaempferol. The obtained LODs for all investigated phenolic compounds were 0.01 µg/mL with the LOQs of 0.03 µg/mL. The HPLC analysis detected the presence of phenolic compounds in *A. altissima* bark, leaves and *M. alba* leaves (Table 2).

The dominant compound in *A. altissima* bark extract was kaempferol (450.14 µg/g) and 2 hidroxycinnamic acid (434.49 µg/g) and dominant compound in *A. altissima* leaf extract was also kaempferol followed by quercetin and 2 hidroxycinnamic acid. The most prevailing compound in *M. alba* leaves extract was kaempferol, followed by 2 hydroxycinnamic acid, quercetin and caffeic acid.

Table 2. HPLC-DAD detected and quantified phenolic compounds in *A. altissima* and *M. alba* extracts.

Phenolic compounds (µg/g)	Extract		
	A.altisima bark	*A.altisima* leaves	*M.alba* leaves
caffeic acid (hydro cinnamic)	55.91	<LOQ	6.8
trans cinnamic acid	35.94	<LOQ	<LOQ
2 hidroxycinnamic acid	434.49	3.96	12.84
chlorogenic acid	71.83	<LOQ	<LOQ
p-coumaric acid	42.16	<LOQ	<LOQ
Kaempferol	450.14	19.22	32.0
Quercetin	8.86	5.96	6.95

LOQ - Limit of quantification.

Bioassay

Feeding intensity of gypsy moth larvae

Two-way ANOVA results indicate that after 24 h only plant species, and the interaction of plant species x concentration, had statistically significant influence on the feeding intensity (consumed leaf area) of gypsy moth larvae (Table 3). However, after 48 h, both factors and their interaction had significant influence of the feeding intensity of gypsy moth larvae.

Table 3. The influence of different factors and their interaction on feeding intensity of gypsy moth larvae.

Factors (sources of variation)	SS	DF	MS	F value
24 h				
extract	136345.5	5	27269.1	2005.05**
concentration	38.5	2	19.2	1.41 ns
extract x concentration	468.9	10	46.9	3.45**
48 h				
extract	145626.0	5	29125.2	4207.09**
concentration	76.9	2	38.4	5.55**
extract x concentration	394.3	10	39.4	5.70**

ns - non significant differences ($p>0.05$), * - significant differences ($p<0.05$); ** - highly significant differences ($p<0.01$); SS-sum of squares; df-degrees of freedom; MS-median

Feeding intensity of gypsy moth larvae was assessed based on the average value of consumed leaf area (%) (Table 4). After 24 h the lowest average feeding intensity was registered in treatments with extracts of *M. alba* leaves (1.44%) and *A. altissima* bark (5.03%). The increase of concentration significantly reduced the feeding intensity

in treatments with *A. altissima* bark. The difference between the average consumed leaf area was highly significant (F=21604.43**, p<0.05). After 48 h, the lowest feeding intensity was in treatments with *M. alba* leaves (3.22%) and *A. altissima* bark (9.30%) and it was concentration-dependant, namely the feeding intensity decreased with the increase of concentration. The differences between consumed leaf area are highly significant (F=76689.33**, p<0.01).

Table 4. Feeding intensity of gypsy moth larvae.

Extract	% of consumed leaf area			Average (%)	F value
	0.5%	1%	2%		
24 h					
A. altissima (bark)	6.72 ± 0.81 aB	5.12 ± 1.03 aB	3.06 ± 0.44 bB	5.03 c	4.25*
A. altissima (leaves)	91.31 ± 2.11 aA	90.22 ± 1.22 aA	89.10 ± 2.17 aA	90.21 a	2.19ns
M. alba (leaves)	2.04 ± 0.15 aC	1.07± 0.11 aC	1.20 ± 0.09 aB	1.44 d	4.33 ns
Control	90.33 ± 2.41 aA	90.47 ± 1.22 aA	82.11 ± 3.65 aA	87.64 b	0.85 ns
F value	28253.89**	27622.35**	28824.55**	21604.43**	
48 h					
A. altissima (bark)	11.97 ± 4.23 aB	11.27 ± 1.3 aB	4.67 ± 2.02 bB	9.30 b	621.97**
A. altissima (leaves)	96.92 ± 0.67 aA	98.51 ± 2.15 aA	95.87 ± 2.11 aA	97.10 a	4.11ns
M. alba (leaves)	6.55 ± 0.11 aC	1.52 ± 0.16 bC	1.12 ± 0.11 bC	3.22 c	47.60**
Control	97.55 ± 1.34 aA	97.40 ± 1.75 aA	97.11 ± 0.63 aA	97.35 a	1.33ns
F value	79379.91**	28824.96**	96485.43**	76689.33**	

Values with the same lowercase letters are at the same level of significance in columns- between concentrations (α=0.05); Values with the same uppercase letters are at the same level of significance in rows- between plant species (α=0.05); ns - non significant differences (p>0.05), * - significant differences (p<0.05); ** - highly significant differences (p<0.01).

Based on antifeeding activity index (AFI values), after 24 and 48 h, the extracts of *A. altissima* bark and *M. alba* leaves expressed strong antifeeding activity on gypsy moth larvae regardless on the concentration (Table 5). However, the extracts of *A. altissima* leaves did not cause antifeeding effect regardless on the applied concentration (AFI ranged from 0.20 to 3.55 after 24h and 0.32 to 0.87 after 48 h).

The mortality of gypsy moth larvae increased after 48 h in treatments with *A. altissima* bark and leaf extracts, at all applied concentrations (40.0 - 57.50%) as presented in Table 6.

CONCLUSIONS AND DISCUSSION

The content of phenolic compounds in *M. alba* leaf extracts is greatly influenced by a growing region (Radojković, Zeković, Vidović, Kočar & Mašković, 2012; Kim

et al, 2014; Polumackanycz, Sledzinski, Goyke, Wesolowski & Viapiana, 2019), cultivar (Lee & Choi, 2012; Sánchez-Salcedo, Mena, García-Viguera, Hernández & Martínez, 2015; Pothinuch & Tongchitpakdee, 2019), harvest period (Lee & Choi, 2012; Pothinuch & Tongchitpakdee, 2019), heat processing (Lee & Choi, 2012), extraction solvent (Polumackanycz et al, 2019), and processing conditions (Przeor et al, 2020).

Table 5. Antifeeding activity of tested plant extracts on gypsy moth larvae.

Extracts	Antifeeding activity			
	24 h		48 h	
	AFI	activity	AFI	activity
A. altissima (bark) 0.5%	91.34	+++	77.43	+++
A. altissima (bark) 1%	95.05	+++	87.50	+++
A. altissima (bark) 2%	93.52	+++	79.33	+++
A.altissima (leaves) 0.5%	3.55	-	0.32	-
A. altissima (leaves) 1%	2.32	-	0.36	-
A. altissima (leaves) 2%	0.20	-	0.87	-
M. alba (leaves) 0.5%	85.54	+++	92.08	+++
M. alba (leaves) 1%	89.11	+++	95.15	+++
M. alba (leaves) 2%	96.44	+++	93.43	+++

- no antifeeding activity; +++ - strong antifeeding activity.

Table 6. Mortality (%) of gypsy moth larvae in treatments with *A. altissima* and *M. alba* extracts.

Extracts	Mortality (%)			
	24 h	F value	48 h	F value
A. altissima (bark) 0.5%	13.50 ± 0.50 ab	36.76**	40.00 ± 0.00 bc	556.47**
A. altissima (bark) 1%	10.00 ± 1.00 b		43.33 ± 0.51 b	
A. altissima (bark) 2%	16.50 ± 1.03 a		57.50 ± 1.50 a	
A.altissima (leaves) 0.5%	2.25 ± 0.25 d	83.31*	45.00 ± 0.00 b	649.23**
A. altissima (leaves) 1%	3.75 ± 0.20 d		30.00 ± 1.00 c	
A. altissima (leaves) 2%	4.77 ± 0.17 cd		47.50 ± 2.50 b	
M. alba (leaves) 0.5%	6.50 ± 0.50c	129.99**	8.21 ± 0.60 d	10107.71**
M. alba (leaves) 1%	10.00 ± 1.00 b		30.00 ± 0.40 c	
M. alba (leaves) 2%	17.50 ± 0.25 a		62.50 ± 0.50 a	
Control	2.50 ± 0.10 d	/	3.75 ± 0.30 e	/
F value	646.06**	/	2324.11**	/

Values with the same lowercase letters are at the same level of significance in columns- between concentrations and plant species (α=0.05); ns - non significant differences (p>0.05), * - significant differences (p<0.05); ** - highly significant differences (p<0.01)

Various factors affect the content of phenolic compounds and antioxidant activity in *A. altissima* extracts: selected tissue or organ (Luis, Gil, Amaral, Domingues & Duarte, 2012; Aissani et al, 2018), growing conditions (Vidović, Morina, Milić & Veljović Jovanović, 2015), extraction solvent (Luis et al, 2012; Poljuha et al, 2017; Aissani et al, 2018), and processing conditions (Poljuha et al, 2017). According to several authors (Luis et al, 2012; Albouchi et al, 2013; Poljuha et al, 2017; Aissani et al, 2018) leaves and bark of *A. altissima* are rich sources of polyphenolic compounds and possess very strong antioxidant activity. Presented work is in agreement with the results obtained in our study that reveal that the bark extract possessed ten times higher content of total phenolics and total tannins compared to leaf extracts. Bark extract also manifested higher antioxidant capacity from three times (measured by FRAP assay) up to 11 times (measured by DPPH assay) comparing to *A. altissima* leaves extract.

Several authors reported that *M. alba* leaf extracts contain relatively high content of polyphenolic compounds and strong antioxidant activity (Kim *et al*., 2014; Polumackanycz et al, 2019; Lee & Choi, 2012; Sánchez-Salcedo et al, 2015; Przeor et al, 2020). However, due to different extraction procedures and presentation of the results, it is hard to compare the results. Polumackanycz et al (2019) and Przeor et al (2020) obtained results similar to those reported in our study.

Several studies reveal more detailed chemical composition of *A. altissima* bark. Kowarik & Säumel (2007) report that the bark contains oleoresin, resin, some mucilage, ceryl alcohol, ailanthon, 'quassiin', calcium oxalate crystals, and isoquercetin (quercitin 3-glycoside), tannin, phlobaphene, ceryl palmitate, saponin, quassin and neoquassin. Ailanthone was identified as the most effective phytotoxic component and may potentially be used as a broad spectrum herbicide (Lin et al, 1995; Heisey, 1996) and may potentially be used as a broad spectrum herbicide (Heisey & Heisey, 2003). Besides ailanthone, which showed the greatest inhibitory activity, De Feo, Mancini, Voto, Curini & Digilio (2009) isolated ailanthinone, chaparrine, and ailanthinol B (quassinoid derivatives), while Okunade et al (2003) isolated the quassinoids ailanthone and 6-alpha-tigloyloxychaparrinone and revealed antiplasmodial activity of these compounds and Tamura et al (2003) and Tamura, Fukamiya, Okano & Koike (2006) detected new quassinoids: ailantinol E, F, G, and H. From the bark, Hwang et al (2005) isolated five coumarin derivatives.

Aissani et al (2018) identified quinic and syringic acids as dominant component in *A. altissima* leaf extract followed by caffeic and p-coumaric acids. The composition of *A. altissima* leaves differed from that of the bark. In bark extract, all tested phenolic acids (caffeic acid (hydro cinnamic, trans cinnamic acid, 2 hidroxycinnamic acid, chlorogenic acid, p-coumaric acid, kaempferol, quercetin) were detected in different amounts. The content of the majority of phenols investigated was many-fold lower in leaves (kaempferol, quercetin and 2 hydroxycinnamic acid), which is partially in accordance with the reports of other authors. In several studies (Luis et al, 2012; Albouchi et al, 2013; Vidović et al, 2015) quercetin was detected as one of the dominant phenolic compounds in extracts of *A. altissima* leaves, while our results point kaempherol and 2 hidroxycinnamic acid as the dominant components.

The obtained results for the most prevailing compound in *M. alba* leaves (kaempferol, followed by 2 hydroxycinnamic acid, quercetin and caffeic acid) are partially in agreement with previous findings. Flaczyk et al (2013) and Przeor et al (2020) detected chlorogenic and caffeic acids as a dominant components followed by p-coumaric acid, kaempferol and quercetin. Radojković et al (2012) identified ferulic acid, rutin, gallic acid and chlorogenic acid, among other compounds, in *M. alba* samples. Sánchez-Salcedo et al (2015) have reported quercetin derivatives, chlorogenic and caffeoylquinic acid as major components, but also kaempferol derivates.

Flavonoids, quercetin, chlorogenic acid and rutin are the most frequently found phenolic compounds among diverse crop species and represent a base of plants resistance (Rojht, Kosir & Trdan, 2012; Martens, Preuss & Matern, 2010; Hichri et al, 2011). They affect herbivore larval growth and development mainly by feeding inhibition (Treutter, 2006; Page, Sultana, Paszkiewicz, Florance & Smirnoff, 2012). The results of our work is in compliance with these reports. The highest amount of all tested phenolic compounds including quercetin, chlorogenic acid and kaempferol detected by HPLC analysis was in the extract of *A. altissima* bark (8.86, 71.83, and 450.14 µg/g, respectively) which expressed the strongest antifeeding activity against gypsy moth larvae in this work. Onyilagha, Lazorko, Gruber, Soroka & Erlandson (2004) also reported the detrimental effect of kaempferol from *Brasica napus* L. leaves to another lepidopterian pest, *Mamestra configurata* Walker. Beninger et al (2004) found that chlorogenic acid (100 and 1000 ppm) when added in artificial diet significantly reduced growth of *L. dispar* and *Trichloplusia ni* (Hübner) larvae, and according to Simmonds & Stevenson (2001) and Simmonds (2003), the same effects were caused to *Helicoverpa armigera* (Hübner) (50 ppm).

The extract of *M. alba* leaves exhibited strong antifeeding effect, which is in accordance with the results of previous research of Gvozdenac et al (2012). Antifeeding activity can be attributed to kaemferol, 2 hidroxycinnamic and caffeic acid and quercetin detected in the extract. Stamp, Temple, Traugott & Wilkens (1994) proved negative effects of caffeic acid on early stages of *Manduca sexta* (L.), which was also detected as the dominant phenolic compound in leaf extract of *M. alba*. Additionally, according to Pelletier (1996) over 250 different glycosides can be isolated from the leaves of *M. alba*, which are most probably responsible for feeding inhibition.

Previous studies prove that secondary metabolites released from roots and leaves of *A. altissima* have pronounced insecticidal effect (Heisey, 1996; Tsao, Romanchuk, Peterson & Coats 2002), which is in accordance with the results obtained in this study. According to De Feo et al (2009), extract isolated from *A. altissima* plant was successfully used to control *Acyrtosiphon pisum* (Harris). Pavela (2011) reported insecticidal effects and anteefidant activity (FDI 41.6%) of *A. altissima* leaf fractions on 4th instar *Leptinotarsa decemlineta* (Say) larvae and acute and chronic toxicity, antifeedant efficacy and larval growth inhibition of leaf extract on *Spodoptera littoralis* (Bois.) (Pavela et al 2014). *A. altissima* leaf extract expressed oviposition deterrence of *Spodoptera frugiperda* (Smith) in a concentration-dependent manner, as reported

by Wagner & Card (2020). In this work, *A. altissima* bark extract expressed stronger insecticidal activity, compared to leaf extract. This is in compliance with reports of Lu & Wu (2010) who tested essential oil of *A. altissima* bark but on different insect pests. The essential oil exhibited strong contact toxicity to *Sitophilus oryzae* (L.) adults (76.5% mortality after 72 h of exposure), fumigant activity (99,3% and 81.9% mortality within 24 h) for *Oryzaephilus surinamensis* (L.) and *S. oryzae* respectively, and repellency (class IV) for adults of *Tribolium castaneum* (Herbst), *O. surinamensis, S. oryzae* and *Liposcelis paeta* (Pearman & J.V.). However, there is no available research data on insecticidal effects of *A. altissima* bark extracts on lepidopteran larvae. Therefore, this work presents the first report on the bioactivity of *A. altissima* bark extract on gypsy moth larvae, emphasizing its high potential as a moth control agent. Extract of *M. alb*a leaves expressed significant insecticidal effect at 1 and 2% concentrations (30.0 - 62.50% mortality). These results comply with the reports from laboratory feeding tests of *L. dispar* larvae performed by Miller & Hanson (1994). The authors suggested that plants from the genus *Morus* were unsuitable for larval development and even indicated a high mortality of younger larvae and reduced feeding intensity by fifth instars' caterpillars. Also, as mentioned, *M. alba* leaves contain nine glycoside compounds belong to the group of deoxynojirimycin which expressed inhibitory effect on phytophagous (Lepidoptera) larvae of *Spodoptera frugiperda* (Smith) (Pelletier, 1996).

Insect pests represent a growing economic and environmental problem worldwide. Due to their high availability and low toxicity, plant extracts may represent excellent repellents and insecticides to be used in different ecosystems. Our results indicate that extracts of *A. altissima* bark and leaves and *M. alba* leaves may act as an effective low-cost natural protectants able to control the presence of gypsy moth in ecosystems. Extracts of *M. alba* leaves and *A. altissima* bark at all applied concentrations expressed strong antifeeding activity and significant insecticidal effect on gypsy moth larvae.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant numbers: 451-03-68/2020-14/200117 and 451-03-68/2020-14/200032). The authors are thankful to the Dr Duško Brković, Department of Biology, Industrial Microbiology and Plant Protection, University of Kragujevac for the formal identification of the plant material used in study i.e. providing necessary vauchers for used plant material.

REFERENCES

Aissani, N., Jabri, M.-A., Mabrouk, M., & Sebai, H. (2018). Antioxidant potential and antimicrobial activity of *Ailanthus altissima* (Mill.) Swingle extracts against *Pseudomonas aeruginosa*. *Journal of Molecular Biology and Biotechnology*, 3, 3.

Albouchi, F., Hassen, I., Casabianca, H., & Hosni, K. (2013). Phytochemicals, antioxidant, antimicrobial and phytotoxic activities of *Ailanthus altissima* (Mill.) Swingle leaves. *South African Journal of Botany*, 87, 164-174.

Bohinc, T., Horvat, A., Ocvirk, M., Košir, I.J., Rutnik, K., & Trdan S. (2020). The first evidence of the insecticidal potential of plant powders from invasive alien plants against rice weevil under laboratory conditions. *Applied Sciences*, 10(21), 7828.

Beninger, C.W., Abou-Zaid, M.M., Kistner, A.L., Hallett, R.H., Iqbal, M.J., Grodzinski, B., & Hall, J.C. (2004). A flavanone and two phenolic acids from *Chrysanthemum morifolium* with phytotoxic and insect growth regulating activity. *Journal of Chemical Ecology,* 30, 589-606.

Cao, C., Sun, L., Wen, R., Shang, Q., Ma, L., & Wang, Z. (2015). Characterization of the transcriptome of the Asian gypsy moth *Lymantria dispar* identifies numerous transcripts associated with insecticide resistance. *Pesticide Biochemistry and Physiology*, 119, 54-61.

Caser, M., Demasi, S., Caldera, F., Dhakar, N.K., Trotta, F., & Scariot, V. (2020). Activity of *Ailanthus altissima* (Mill.) Swingle extract as a potential bioherbicide for sustainable weed management in horticulture. *Agronomy*, 10, 965.

De Feo, V., Mancini, E., Voto, E., Curini, M., & Digilio, M.C. (2009). Bioassay-oriented isolation of an insecticide from *Ailanthus altissima*. *Journal of Plant Interactions*, 4, 119-123.

Drobnjaković, T., Marčić, D., Prijović, M., Perić, P., Milenković, S., & Bošković, J. (2018). Sublethal effects of NeemAzal- T/S botanical insecticide on Dutch and Serbian populations of *Encarsia formosa* (Hymenoptera: Aphelinidae). *Biocontrol Science and Technology*, 28, 1-19.

Farrar, R.R. Jr., Barbour, J.D., & Kennedy, G.G. (1989). Quantifying food consumption and growth in insects. *Annals of the Entomological Society of America*, 82(5), 593-598.

Flaczyk, E., Kobus-Cisowska, J., Przeor, M., Korczak, J., Remiszewski, M., Korbas, E., & Buchowski, M. (2013). Chemical characterization and antioxidative properties of Polish variety of *Morus alba* L. Leaf aqueous extracts from the laboratory and pilot-scale processes. *Agricultural Sciences*, 4(5b), 141-147.

Gvozdenac, S., Inđić, D., Vuković, S., Grahovac, M., & Tanasković, S. (2012). Antifeeding activity of several plant extracts against *Lymantria dispar* L. (Lepidoptera: Lymantridae) larvae. *Pesticides and Phytomedicine*, 27(4), 305-311.

Gvozdenac, S., Šućur, J., Manojlović, A., Prvulović, D., & Malenčić, Đ. (2018). Influence of winter savory (*Satureja montana*) aqueous extract on mortality of lesser grain borer (*Rhyzopertha dominica). Botanica Serbica*, 42(2), 277-281.

Heisey, R.M. (1996). Identification of an allelopathic compound from *Ailanthus altissima* (Simaroubaceae) and characterization of its herbicidal activity. *American Journal of Botany*, 83(2), 192-200.

Heisey, R.M. & Heisey, T.K. (2003). herbicidal effects under field conditions of *Ailanthus altissima* bark extract, which contains ailanthone. *Plant and Soil*, 256, 85-99.

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., & Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *Journal of Experimental Botany*, 62(8), 2465-2483.

Hikal, W.M., Baeshen, R.S., & Said-Al Ahl, H.A.H. (2017). Botanical insecticide as simple extractives for pest control. *Cogent Biology*, 3, 1404274.

Hwang, S.W., Lee, J.R., Lee, J., Kwon, H.S., Yang, M.S., & Park, K.H. (2005). New coumarins from the *Ailanthus altissima*. *Heterocycles*, 65, 1963-1966.

Kalaskar, M.G. & Surana, S.J. (2014). Free radical scavenging, immunomodulatory activity and chemical composition of *Luffa acutangula* var. Amara (Cucurbitaceae) pericarp. *Journal of the Chilean Chemical Society*, 59(1), 2299-2302.

Keena, M.A. & Richards, J.Y. (2020). Comparision of survival and development of gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae) population from different geographic areas on North American conifers. *Insects*, 11, 260.

Kim, D.-S., Kang, Y.M., Jin, W.Y., Sung, Y.-Y., Choi, G., & Kim, H.K. (2014). Antioxidant activities and polyphenol content of *Morus alba* leaf extracts collected from varying regions. *Biomedical Reports*, 2(5), 675-680.

Kostić, D.A., Dimitrijević, D.S., Mitić, S.S., Mitić, M.N., Stojanović, G.S., & Zivanović, A.V. (2013). A survey on macro- and micro-elements, phenolic compounds, biological activity and use of *Morus* spp. (Moraceae). *Fruits*, 68, 333-347.

Kostić, M., Popović, Z., Brkić, D., & Milanović. S. (2008). Larvicidal and antifeedant activity of some plant-derived compounds to *Lymantria dispar* L. (Lepidoptera, *Lymantriidae*). *Bioresource Technology*, 99(16), 7897-7901.

Koul, O. (2005). *Insect antifeedants*. CRC Press, Bota Racon, FL, USA.

Kowarik, I. & Säumel, I. (2007). Biological flora of Central Europe: *Ailanthus altissima* (Mill.) Swingle. *Perspectives in Plant Ecology, Evolution and Systematics*, 8(4), 207-237.

Kozuharova, E., Benbassat, N., Berkov, S., & Ionkova, I. (2020). *Ailanthus altissima* and *Amorpha fruticosa* - invasive arboreal alien plants as cheap sources of valuable essential oils. *Pharmacia*, 67(2), 71-81.

Krinski, D., Massaroli, A., & Machado, M. (2014). Potencial inseticida de plantas da família Annonaceae. *Revista Brasileira de Fruticultura*, 36, 225-242. https://doi.org/10.1590/S0100-29452014000500027.

Lai, H.Y. & Lim, Y.Y. (2011). Evaluation of antioxidant activities of the methanolic extracts of selected ferns in Malaysia. *International Journal* of *Environmental Science and Development*, 2, 442-447.

Lee, W.J. & Choi, S.W. (2012). Quantitative changes of polyphenolic compounds in mulberry (*Morus alba* L.) leaves in relation to varieties, harvest period, and heat processing. *Preventive Nutrition and Food Science*, 17(4), 280-285.

Lin, L.J., Peiser, G., Ying, B.P., Mathias, K., Karasina, F., Wang, Z., Itatani, J., Green, L., & Hwang, Y.S. (1995). Identification of plant growth inhibitory principles in *Ailanthus altissima* and *Castela tortuosa*. *Journal of Agricultural and Food Chemistry,* 43, 1708-1711.

Liu, Z.L., Goh, S.H., & Ho, S.H. (2007). Screening of Chinese medicinal herbs for bioactivity against *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst*). Journal of Stored Products Research*, 43, 290-296.

Lu, J. & Wu, S. (2010). Bioactivity of essential oil from *Ailanthus altissima* bark against 4 major stored-grain insects. *African Journal of Microbiology Research*, 4(3), 154-157.

Luis, Â., Gil, N., Amaral, M.E., Domingues, F., & Duarte, A.P. (2012). *Ailanthus altissima* (Miller) swingle: A source of bioactive compounds with antioxidant activity. *Bioresources*, 7, 2015-2020.

Mandal, S., Mitra, A., & Mallick, N. (2008). Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum f. sp. lycopersici*. *Physiological and Molecular Plant Pathology*, 72(1-3), 56-61.

Martens, S., Preuss, A., & Matern, U. (2010). Multifunctional flavonoid dioxygenases: flavonol and anthocyanin biosynthesis in *Arabidopsis thaliana* L. *Phytochemistry***,** 71(10), 1040-1049.

Milanović, S., Lazarević, J., Popović, Z., Miletić, Z., Kostić, M., Radulović, Z., Karadžić, D., & Vuleta, A. (2014). Preference and performance of the larvae of *Lymantria dispar* (Lepidoptera: Lymantriidae) on three species of European oaks. *European Journal of Entomology*, 111(3), 371-378.

Miller, J.C. & Hanson, P.E. (1994). Laboratory feeding tests on the development of *L .dispar* larvae with reference to plant taxa and allelochemicals. Agricultural Experiment Station Ir; Oregon State University. Agricultural Experiment Station, Oregon State University, Station Bulletin, 674, 1-63.

Okunade, A.L., Bikoff, R.E., Casper, S.J., Oksman, A., Goldberg, D.E., & Lewis, W.H. (2003). Antiplasmodial activity of extracts and quassinoids isolated from seedlings of *Ailanthus altissima* (Simaroubaceae). *Phytotherapy Research*, 17, 675-677.

Onyilagha, J.C., Lazorko, J., Gruber, M.Y., Soroka, J.J., & Erlandson, M.A. (2004). Effect of flavonoids on feeding preference and development of the crucifer pest *Mamestra configurata* Walker. *Journal of Chemical Ecology*, 30, 109-124.

Page, M., Sultana, N., Paszkiewicz, K., Florance, H., & Smirnoff. N. (2012). The influence of ascorbate on anthocyanin accumulation during high light acclimation in *Arabidopsis thaliana*: further evidence for redox control of anthocyanin synthesis. *Plant, Cell and Environment*, 35, 388-404.

Pavela, R. (2011). Antifeedant activity of plant extracts on *Leptinotarsa decemlineata* Say. and *Spodoptera littoralis* Bois. Larvae. *Industrial Crops Products*, 32, 213-219.

Pavela, R., Zabka, M., Tylova, T., & Kresinova, Z. (2014). Insecticidal activity of compounds from *Ailanthus altissima* against *Spodoptera littoralis* larvae. *Pakistan Journal of Agricultural Sciences*, 51, 101-112.

Pelletier, S. (1996). *Alkaloids: chemical and biological perspectives*, Volume 15, Pergamon, Oxford, UK.

Poljuha, D., Sladonja, B., Šola, I., Dudaš, S., Bilić, J., Rusak, G., Motlhatlego, K.E., & Eloff, J.N. (2017). Phenolic composition of leaf extracts of *Ailanthus altissima* (Simaroubaceae) with antibacterial and antifungal activity equivalent to standard antibiotics. *Natural Product Communications*, 12, 1609-1612.

Polumackanycz, M., Sledzinski, T., Goyke, E., Wesolowski, M., & Viapiana, A. (2019). A comparative study on the phenolic composition and biological activities of *Morus alba* L. commercial samples. *Molecules*, 24, 3082.

Popović, Z., Kostić, M., Stanković, S., Milanović, S., Sivčev, I., Kostić, I., & Kljajić, P. (2013). Ecologically acceptable usage of derivatives of essential oil of sweet basil, *Ocimum basilicum*, as antifeedants against larvae of the gypsy moth, *Lymantria dispar*. *Journal of Insect Science*, 13(1), 1-12.

Pothinuch, P. & Tongchitpakdee, S. (2019). Phenolic analysis for classification of mulberry (*Morus* spp.) leaves according to cultivar and leaf age. *Journal of Food Quality*, 2019, 2807690.

Przeor, M., Flaczyk, E., Kmiecik, D., Buchowski, M.S., Staniek, H., Tomczak-Graczyk, A., Kobus-Cisowska, J., Gramza-Michałowska, A., & Foksowicz-Flaczyk, J. (2020). Functional properties and antioxidant activity of *Morus alba* L. leaves var. Zolwinska Wielkolistna (WML-P) - The effect of controlled conditioning process. *Antioxidants*, 9, 1-15.

Radojković, M.M., Zeković, Z.P., Vidović, S.S., Kočar, D.D., & Mašković, P.Z. (2012). Free radical scavenging activity and total phenolic and flavonoid contents of mulberry (*Morus* spp. L., Moraceae) extracts. *Hemijska Industrija*, 66(4), 547-552.

Rahman, H.M.A., Rasool, M.F., & Imran, I. (2019). Pharmacological studies pertaining to smooth muscle relaxant, platelet aggregation inhibitory and hyposensitive effects of *Ailanthus altissima*. *Evidence-Based Complementary and Alternative Medicine*, 2019:1871696.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26(9-10), 1231-1237.

Saha, A.K., Rahman, R., Shahriar, M., Saha, S.K., Al Azad, N., & Das, D. (2013). Screening of six Ayurvedic medicinal plant extracts for antioxidant and cytotoxic activity. *Journal of Pharmacognosy and Phytochemistry*, 2, 181-188.

Rojht, H., Kosir, I.J., & Trdan, S. (2012). Chemical analysis of three herbal extracts and observation of their activity against adults of *Acanthoscelides obtectus* and *Leptinotarsa decemlineata* using a video tracking system**.** *Journal of Plant Diseases and Protection,* 119, 59-67.

Sánchez-Salcedo, E.M., Mena, P., García-Viguera, C., Hernández, F., & Martínez, J.J. (2015). (Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods*, 18(B), 1039-1046.

Sanghi, S.B. & Mushtaq, S. (2017). Pharmacological activity of *Morus alba* Linn. extracts - A review. *Asian Journal of Pharmaceutical Education and Research*, 6, 10-19.

Simmonds, M.S. (2003). Flavonoid-insect interactions: recent advances in our knowledge. *Phytochemistry*, 64, 21-30.

Simmonds, M.S.J. & Stevenson, P.C. (2001). Effects of isoflavonoids from cicer on larvae of *H. armigera*. *Journal of Chemical Ecology*, 27, 965-977.

Singh, H., Cheema, H.K., & Singh, R. (2020). Field evaluation of horticultural mineral oils and botanicals against bean thrips, *Megalurothrips distalis* (Karny) (Thysanoptera: Thripidae), in summer mung bean. *Egyptian Journal of Biological Pest Control*, 30, 1-8.

Singh, R., Bagachi, A., Semwal, A., Kaur, S., & Bharadway, A. (2013). Traditional uses, phytochemistry and pharmacology of *Morus alba* Linn.: A review. *Journal of Medicinal Plant Research*, 7, 461-469.

Stamp, N.E., Temple, M.P., Traugott, M.S., & Wilkens, R.T. (1994). Temperature-allelochemical interactive effects on performance of *Manduca sexta* caterpillars. *Entomologia Experimentalis et Applicata*, 73, 199-210.

Šućur, J., Popović, A., Petrović, M., Anačkov, G., Malenčić, DJ., & Prvulović, D. (2015). Allelopathic effects and insecticidal activity of *Salvia sclarea* L. *Studia Universitatis Babes-Bolyai Chemia*, 60(1), 253-264.

Tamura, S., Fukamiya, N., Okano, M., & Koike, K. (2006). A new quassinoid, ailantinol H from *Ailanthus altissima*. *Natural Product Research*, 20, 1105-1109.

Tamura, S., Fukamiya, N., Okano, M., Koyama, J., Koike, K., Tokuda, H., Aoi, W., Takayasu, J., Kuchide, M., & Nishino, H. (2003). Three new quassinoids, ailantinol E, F, and G, from *Ailanthus altissima*. *Chemical and Pharmaceutical Bulletin*, 51, 385-389.

Thaipitakwong, T., Numhom, S., & Aramwit, P. (2018). Mulberry leaves and their potential effects against cardiometabolic risks: A review of chemical compositions, biological properties and clinical efficacy. *Pharmaceutical Biology*, 56, 109-118.

Treutter, D. (2006). Significance of flavonoids in plant resistance: A review. *Environmental Chemistry Letters*, 4, 147-157.

Tsao, R., Romanchuk, F.E., Peterson, C.J., & Coats, J.R. (2002). Plant growth regulatory effects and insecticidal activity of the extracts of the tree of heaven (*Ailanthus altissima* L.). *BMC Ecology*, 2, 1-6.

Valeñtao, P., Fernandes, E., Carvalho, F., Andrade, P.B., Seabra, R.M., & Bastos, M.L. (2002). Antioxidative properties of cardoon (*Cynara cardunculus* L.) infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. *Journal of Agricultural and Food Chemistry*, 50, 4989-4993.

Vidović, M., Morina, F., Milić, S., & Veljović Jovanović, S. (2015). An improved HPLC-DAD method for simultaneously measuring phenolics in the leaves of *Tilia platyphyllos* and *Ailanthus altissima*. *Botanica Serbica*, 39(2), 177-186.

Wagner, R.L. & Card, J.A. (2020). *Ailanthus altissima* aqueous extract deters *Spodoptera frugiperda* oviposition. *The Great Lakes Entomologist*, 53(1), 68-72.

Received: December 25, 2020 *Accepted: October 05, 2021*

J. Entomol. Res. Soc., 23(3): 213-224, 2021
Doi: 10.51963/jers.v23i3.2010

Research Article
Online ISSN:2651-3579

Differences Between the Allometric Rules Governing Two *Formica lugubris* Zetterstedt, 1838 (Hymenoptera: Formicidae) Ants Populations in Northern Greece

Angelos TSIKAS[1]* Paraskevi KARANIKOLA[2]

[1,2] Department of Forestry and Management of the Environment and Natural Resources, Laboratory of Forest Protection, Democritus University of Thrace, School of Agricultural and Forestry Sciences, Ath. Pantazidou 193, 68 200 Orestiada, GREECE
e-mails: *atsikas@fmenr.duth.gr, pkaranik@fmenr.duth.gr
ORCID IDs: [1]0000-0001-9754-5135, [2]0000-0001-8370-5126

ABSTRACT

Red wood ant workers, despite being monomorphic, show a wide range in their body size even within the same colony. As overall size grows, some organs grow in a negative allometric relationship with the body. Genetic and environmental factors, like diet and temperature, are considered as key factors that determine differences in body and organ growth. We studied the allometric rules governing the organs in two different populations of red wood ants in two different environments in Northern Greece to identify similarities and differences. These rules, although linear for both populations and negative allometric for most of the traits measured, differ in the two study areas, while the shape of the head found to be governed by different allometric rules between the two populations. The whole shape and size of red wood ants varies greatly even within the same species and we cannot create allometric models for two different areas, even when they belong in the same species. The differences might be a result of differences in the genome and the environmental conditions between the two populations.

Key words: Red wood ants, *Formica lugubris*, morphological traits, allometry, Northern Greece.

Tsikas, A. & Karanikola, P. (2021). Differences between the allometric rules governing two *Formica lugubris* Zetterstedt, 1838 (Hymenoptera: Formicidae) ants populations in northern Greece. *Journal of the Entomological Research Society*, 23(3), 213-224.

INTRODUCTION

Body size is the main phenotypic feature of organisms, as it affects the way they interact with the environment. In nature, adult individuals of the same species may vary widely in body size due to a combination of genetic and environmental factors. This is the result of differential growth: as an individual organism grows in size overall, some organs grow faster or slower than others (Eberhard et al, 1998; Emlen, Warren, Johns, Dworkin, & Lavine, 2012) due to differential allocation of resources to different organs, at different rates (Bonduriansky, Day, & Pitnick, 2003; Kodric-Brown, Sibly, & Brown, 2006; Emlen et al, 2012).

Variations in the size of an organ that accompany variations in body size can be characterized by allometric relationships. The scaling of any quantitative characteristic of an organ can be modelled using the allometric equation,

$y=bx^a$

Where x is the size of the body, Y is the size of the instrument, a is the exponent of scaling and b is the initial growth index (Huxley & Tessier, 1936). A log transformation of the allometric equation produces a simple linear equation, log(y)=log(b)+a log(x) and log-log plots of the size of different traits among individuals of the same species typically reveal linear allometries with an intercept of log(b) and a slope of a, called the 'allometric coefficient' (Huxley & Tessier, 1936). When the organs grow in the same rate with the rest of the body (a = 1) then we have isometry. More commonly, however, the organs have a negative allometric relationship with the body (a < 1), *i.e.* they grow at a relatively slower rate than the body, resulting in larger individuals having relatively smaller organs (Eberhard et al, 1998), while in rare cases, and especially in organs related to sexual choice, the organs may show positive allometric relationships (a > 1), *i.e*. they grow in a faster rate than the body (Gould, 1973; Emlen, 1997). In some cases, however, the equation is not always linear, and may be sigmoidal or intermittent (Emlen & Nijhout, 2000).

In holometabolous insects, the organs do not grow at the same time as the larva, but in the later stages of larval development from extracellular monolayers called imaginal discs (Morata & Lawrence, 1979; Currie, Milner, & Evans, 1988). The differential resource allocation in the imaginal discs is resolved through insulin-like peptides and their receptors, making nutrition the most important factor for size, and consequently for scaling (Shingleton, Das, Vinicius, & Stern, 2005). Hence, the nutritional quality of larval diets impacts the metabolic functioning of imago insects, with diets more optimal for survival resulting in a higher metabolic rate per unit of body mass (Nicholls, Rossi, & Niven, 2021). In addition, because larvae stop feeding before metamorphosis, imaginal disc development takes place under conditions of constant resources in the form of protein and fat storage (Tschinkel, 2013). Therefore, there is competition among imaginal discs for limited resources, and the increase of one is accompanied by the decrease of another (Nijhout & Wheeler, 1996). Other factors that determine differences in the size and scaling rules of insect organs are the genome (Stevenson, Hill, & Bryant, 1995; Bargum, Boomsma, & Sundström, 2004; Shingleton, Estep, Driscoll, & Dworkin, 2009) and temperature (Shingleton et al, 2009;

Nijhout et al, 2014). Therefore, it is expected that allometric rules in holometabolous insects will be different from those that apply to other animals whose organs develop continuously and simultaneously with their overall body.

The peculiarity of the development of imago holometabolous insects has two consequences on allometry (Nijhout & Wheeler, 1996). First, where allomorphic relations are observed between organs and the whole body, they cannot be interpreted as a mere result of relative growth, as the organs develop at different times and under completely different regimes of endocrine and nutritional conditions compared to the body. Second, some rare cases of allometric relationships, such as sigmoid and intermittent allometry observed in some polymorphic ant worker castes (Feener, Lighton, & Bartholomew, 1988; Wheeler, 1991) but also in some beetles (Emlen, 1994; Kawano, 1995), may be related to interaction of imaginal discs with each other during metamorphosis.

Ants are organisms with large intra-specific variation in body size (Kaspari & Weiser, 1999; Geraghty, Dunn, & Sanders, 2007; Hurlbert, Ballantyne, & Powell, 2008). The size variation in ants, because they are holometabolous insects, is determined mostly by the conditions of their growth, and mainly by the trophic regime of the larvae. As food is distributed by the workers to the larvae, a higher ratio of the number of workers to that of the larvae means that the larvae receive more resources (Fedoseeva, 2011). The trophic regime is affected by the abundance of prey, as well as by certain factors that suppress the food of the workers or increase their mortality. One year after clear-cutting, Sorvari & Hakkarainen (2009) found that the workers from *F. aquilonia* nests in the deforested areas were smaller in size than in undisturbed stands.

Red wood ants (*Formica rufa* Group) workers are monomorphic and not divided into different morphological castes, but show a wide range in their body size, and even within the same colony (Perl & Niven, 2016) with workers working outside the nest being smaller than workers on the nest (Véle & Mondlinger, 2019). The purpose of this work was to study the allometric rules governing the organs in two different populations of red wood ants in two different environments, with different soil, climatic and vegetation conditions, and their comparison to identify similarities and differences. Given the monomorphism, we expected to find linear allometric relations between their organs and their body.

MATERIAL AND METHODS

Study area

The study areas are located in Elatia Forest, Drama (41° 29′ N, 24° 18′ E), and in Lailias Forest, Serres (41° 14’ N, 23° 34’ E), both in Northern Greece. The altitude of the study area in Elatia ranges between 1500-1650 meters with a mild to moderately strong slope, while the altitude of the study area in Lailias ranges between 1450-1550 meters with a mild to moderate strong slope. They are both natural managed mixed forests, where Elatia is dominated by spruce (*Picea abies* L.), while Lailias is dominated

by Scots pine (*Pinus sylvestris* L.). The geological formation in Elatia consists mainly of granite, gneiss, and limestone (Zagas, 1990), and in Lailias is dominated by acidic igneous rocks (granites, granodiorites, monzonites) with lake and soil reserves in the lowlands (Flocas, Giles, & Angouridakis, 1983).

Sampling and measurements

We collected manually and randomly from the top of the nest mound individual ants from 30 mounds from Elatia in 2013, and from 30 mounds from Lailias in 2017. Samples were taken in summer in the daytime. All samples were preserved in ethanol in glass vials. The species were identified by the keys of Agosti & Collingwood (1987) and Stockan, Robinson, Trager, Yao, & Seifert (2016). 5 individuals were sampled from these vials; a total of 300 individuals. They were then cut with a scalpel, and the pieces (head, antennae, legs, mesosoma) were arranged on a gridded, numbered card that was covered with double-stick tapes to hold them in place. The head was arranged in a face-on view and the mesosoma in lateral view. The pieces were photographed on the stereoscope OLYMPUS SZX7 with a NIKON D90 camera at 10x magnification and with the help of a scale their morphological characteristics were measured with the program Natsumushi v 1.10.1.

The measured traits were: head width (HW), head length (HL), the length of the scape (SL), the length of the upper jaw (MandL), the length of the mesosoma or Weber's length (WL), and the hind femur length (HFL). The head index (CI = 100 * HW / HL), the scape index (SI = 100 * SL / HW) and the mandible index (MandIndex = 100 * MandL / HL) were then calculated. The explanation of the abbreviations is given in Table 1.

Table 1. Selected morphological traits measured in workers.

Abbreviation	Description	Metric
HW	The maximum width of the head in full face view	mm
HL	The length of the head capsule excluding the mandibles, measured in full face view in a straight line from the mid-point of the anterior clypeal margin to the mid-point of the posterior margin	mm
SL	The maximum straight-line length of the scape	mm
MandL	The straight-line length from the mandibular apex to the anterior clypeal margin	mm
WL	The diagonal length of the mesosoma in profile from the point at which the pronotum meets the cervical shield to the posterior basal angle of the metapleuron	mm
HFL	Maximum length of hind femur, measured in anterior view	mm

Data analysis

Standard deviation (± SD) and the coefficient of variation (CV%) were calculated for all measured traits. HFL was used as an indicator of body size, and allometric relationships with the other traits were calculated after log-log transformation. The slope of the regression was calculated by the regression of the maximum axis, as the least squares method tends to produce devaluations (McArdle, 1988). When two measured traits change accordingly, the slope of the regression is equal to 1 (Zar,

1999). When the regression slope = 1, we have an isometric relation. A slope < 1 indicates negative allometry, ie the organs grow at a slower rate compared to the overall size, while when the slope > 1 we have positive allometry. Then we made an estimate of the shape of the head independent of its size with the log-log ratio of the two dimensions (Log10 HW / HL) to log of the index of the total size, HFL (Mosiman & James, 1979). When the slope does not differ statistically significantly from zero (t-test) it shows us that the shape of the head does not change with increasing body size, ie it is isometric with the body, while a slope other than zero shows an allometric relationship of the shape with the overall size. The distribution of all measurements was found to be normal with homogeneous variations. IBM SPSS Statistics v.23 was used for the analyses and Past v2.17c was used for the graphs.

RESULTS

All nests in both study areas were found to belong to *Formica lugubris* Zetterstedt, 1838. The results of the measurements of 150 individuals in Elatia and 150 individuals in Lailias, as well as the standard deviation and the coefficient of variation are presented in Table 2. In general, no large morphological variations were found in the measured traits or significant statistical differences, but the samples from Lailias were found to be slightly larger. Little variation was found in the head, scape and mandible indexes (Table 3).

Table 2. Measured traits in *F. lugubris* workers in Elatia and Lailias.

Elatia						
	HFL (mm)	**HW (mm)**	**HL (mm)**	**SL (mm)**	**MandL(mm)**	**WL (mm)**
N	150	150	150	150	150	150
Mean	1,96102	1,591993	1,222197	1,490527	0,817434	2,44576
SD	0,2249747	0,1728537	0,1267583	0,1587119	0,083282	0,25175
CV %	11,47233	10,85769	10,37135	10,64804	10,18822	10,2934
Lailias						
	HFL (mm)	**HW (mm)**	**HL (mm)**	**SL (mm)**	**MandL(mm)**	**WL (mm)**
N	150	150	150	150	150	150
Mean	2,157107	1,66512	1,262226	1,631273	0,8471	2,61252
SD	0,182989	0,138613	0,108357	0,111026	0,066746	0,20873
CV %	8,483065	8,324524	8,584612	6,80607	7,879305	7,98942

HW found linearly related to HFL (and consequently to the total body size both in Elatia (R^2 = 0.693, p <0.0001, log-log slope = 0.783) (Fig. 1a), and in Lailias (R^2 = 0.747, p <0.0001, log-log slope = 0.853) (Fig. 2a). Respectively, HL is related to HFL in Elatia (R^2 = 0.587, p <0.0001, log-log slope = 0.678) (Fig. 1b) and in Lailias (R^2 = 0.707, p <0.0001, log-log slope = 0.85) (Fig. 2b). SL grows at slower rate than to the overall size, as does the length of the Mandible. For Elatia, for SL (R^2 = 0.714,

p <0.0001, log-log slope = 0.78) (Fig. 1c), while for MandL (R^2 = 0.563, p <0.0001, log-log slope = 0.684) (Fig. 1d). For Lailias, for SL (R^2 = 0.684, p <0.0001, log-log slope = 0.662) (Fig. 2c), while for MandL (R^2 = 0.699, p <0.0001, log-log slope =0.771) (Fig. 2d). Finally, WL found to have negative allometry with body size in both Elatia (R^2 = 0.69, p <0.0001, log-log slope = 0.736) (Fig. 1e), and in Lailias (R^2 = 0.788, p <0, 0001, log-log slope = 0.831) (Fig 2e).

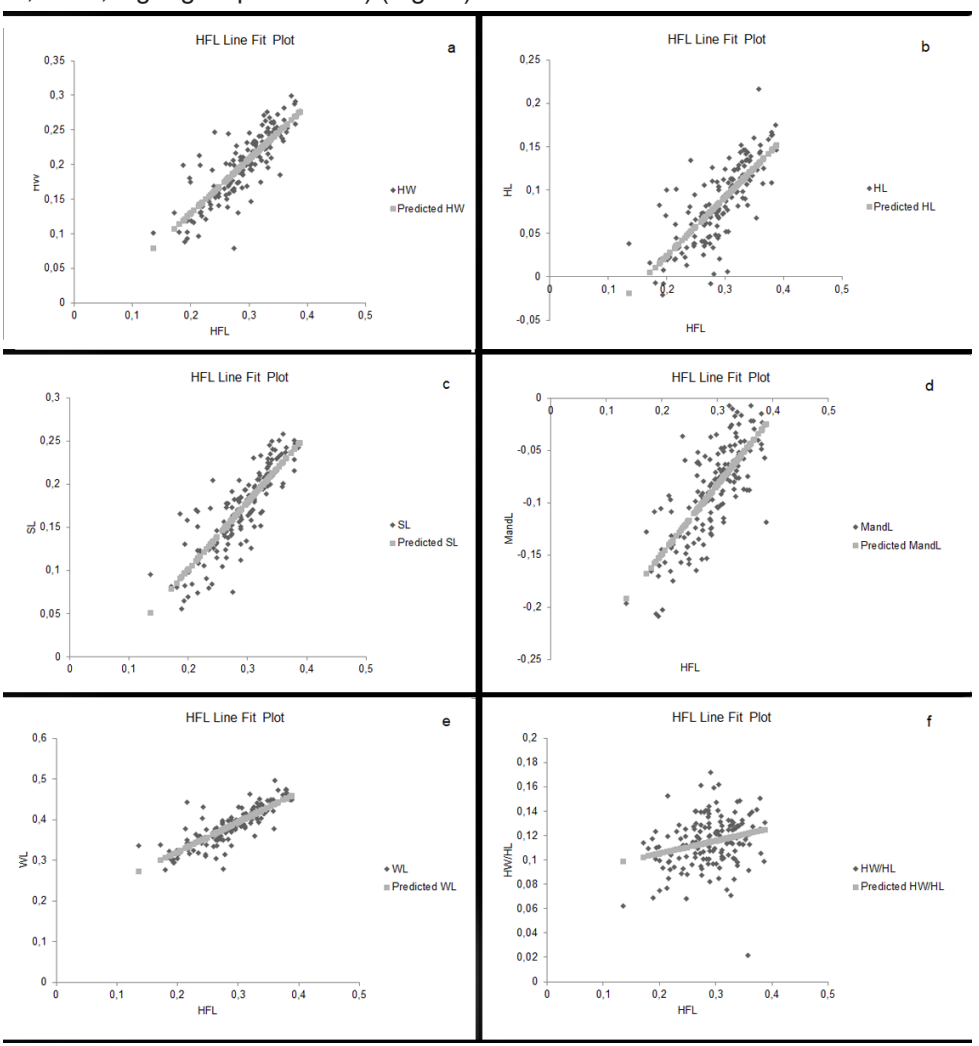


Fig 1. The relationship between HFL and a) HW (y = 0,783x – 0,027), b) HL (y= 0,678x – 0,112), c) SL (y = 0,78x – 0,055), d) MandL (y = 0,684x - 0,282), e) WL (y = 0,736x + 0,173), and f) Head Ratio (y = 0,105x + 0,084) in Elatia.

Despite the allometric rules in both populations in all measured traits, there is a difference in the head ratio, and consequently the shape of the head. Thus, for Elatia we found the shape of the head capsule to have an allometric relationship with the body (R^2 = 0.061, p = 0.002264, log-log slope = 0.105) (Fig. 1f), while for Lailias the shape of the head does not change with increase in body size and has an isometric relationship with the body (R2 = 0.0001, p = 0.9, log-log slope = 0.003) (Fig. 2f).

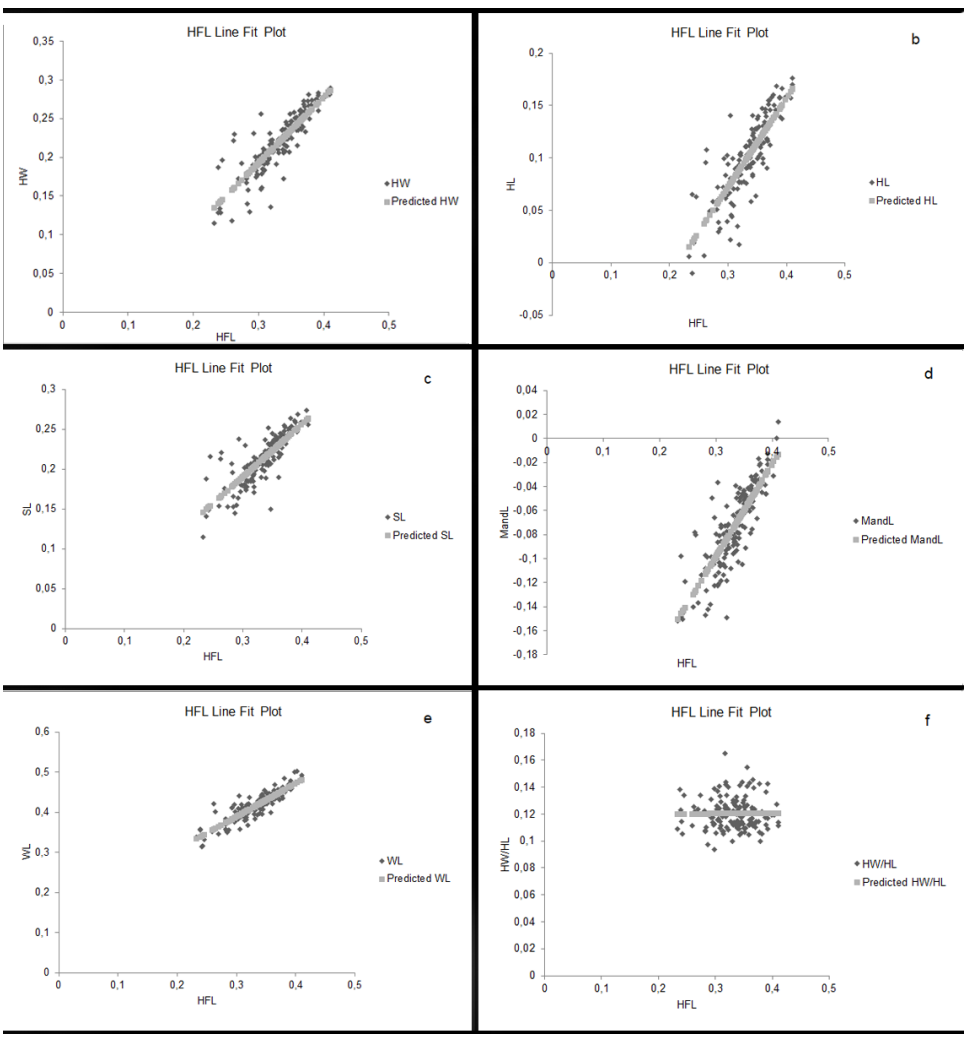


Fig 2. The relationship between HFL and a) HW (y = 0,853x – 0,064), b) HL (y = 0,85x – 0,183), c) SL (y = 0,662x – 0,008), d) MandL (y = 0,771x - 0,330), e) WL (y = 0,831x + 0,140), and f) Head Ratio (y = 0,003x + 0,119) in Lailias.

Table 3. Cephalic Index (CI), Scape Index (SI) and Mandible Index (MandIndex) in *F. lugubris* workers in Elatia and Lailias.

Elatia			
	CI	**SI**	**MandIndex**
N	150	150	150
Mean	130,332	93,8729	67,1004
SD	6,38762	6,55454	5,364791
CV %	4,90103	6,98236	7,995169
Lailias			
	CI	**SI**	**MandIndex**
N	150	150	150
Mean	124,013	98,1469	60,10378
SD	3,58943	3,50959	2,633692
CV %	2,71943	3,57586	3,91895

DISCUSSION

Red wood ant workers, although monomorphic and not divided into castes, show a wide range of size in their body. Using the length of the femur of the hind leg as a measure of size, we created models with allometric rules governing the size of the workers' organs. These rules, although linear for both populations, differ in the two study areas.

As the size of the worker's body increases, so does the size of the head, the scapi, the mandible and the mesosoma, but with negative allometry. Consequently, smaller workers have relatively larger organs compared to larger ones. These relationships occur in all nests we studied in both study areas. Seifert (2016) found similar results for *F. lugubris*, while Perl, Rossoni, & Niven (2017) found grade shifts in the allometric scaling of the compound eye among four *Formica* species in terms of eye scaling, but no grade or slope shifts in the scaling of mean facet diameter. Also, similar relationships have been observed in other ants such as e.g. *Melophorus bagoti* (Schwarz et al, 2011), as well as in other Hymenoptera, such as *Bombus terrestris* (Spaethe & Chittka, 2003). However, the comparison between the population of Elatia and Lailias, shows us significant differences in the allometric rules between the populations. Consequently, we cannot create allometric models for red wood ants from two different areas, even when they belong in the same species. Also, regarding the shape of the head, it is observed that in Elatia it also has allometric relations with the size, while on the contrary in Lailias it does not seem to change. Therefore, the whole shape and size of red wood ants seems to vary greatly even within the same species. Future research should focus on the factors that lead us to these results. A genetic analysis of the two populations may have shown us possible differences in their genome. The genetic diversity is expected to be low in these isolated

populations, due to the genetic bottleneck. So, the two populations are expected to differ significantly due to zero gene flow, or even it is possible to have two different species. Molecular analyses have been used for the identification and monitoring of cryptic species (e.g. Bickford et al, 2007; Vogler &Monaghan, 2007; Moreau, 2009). Morphologically similar ant species have been found to differ significantly in mtDNA analyses (Smith, Fisher, & Hebert, 2005; Steiner et al, 2005; Pusch, Seifert, Foitzik, & Heinze, 2006; Schlick-Steiner et al, 2006a; 2006b; Steiner et al, 2006; Bernasconi, Pamilo, & Cherix, 2010) or microsatellites (Macaranas, Colgan, Major, Cassis, & Gray, 2001; Gyllenstrand, Seppä, Pamilo, 2004; Knaden, Tinaut, Cerda, Wehner, & Wehner, 2005; Bernasconi et al, 2010).

Also, the two populations have different environmental conditions, such as vegetation and temperature, and consequently diet, which could explain the differences in allometric relations. Larvae diet (Thomas, 1993) and temperature (Atkinson, 1994; Mirth & Riddiford, 2007) directly affect adult size. Both diet and temperature were found to affect allometric relationships in the fly *Drosophila melanogaster* (Shingleton et al, 2009), which is also holometabolous, like red wood ants. The various factors that affect growth, like the differences in scots pine and spruce-sucking aphids' honeydew nutrients, should be studied in the future in different ecosystems for possible correlations.

REFERENCES

Agosti, D. & Collingwood, C.A. (1987). A provisional list of the Balkan ants (Hym. Formicidae) with a key to the worker caste. II. Key to the worker caste, including the European species without the Iberian. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 60, 261-293.

Atkinson, D. (1994). Temperature and organism size - A biological law for ectotherms? *Advances in Ecological Research*, 25, 1-58.

Bargum, K., Boomsma, J.J., & Sundström, L. (2004). A genetic component to size in queens of the ant *Formica truncorum*. *Behavioral Ecology and Sociobiology*, 57, 9-16.

Bernasconi, C., Pamilo, P., & Cherix, D. (2010) Molecular markers allow sibling species identification in red wood ants (*Formica rufa* group). *Systematic Entomology*, 35, 243-249.

Bickford, D., Lohman, D.J., Sohdi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K. & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, 22, 148-155.

Bonduriansky, R., Day, T., & Pitnick, S. (2003). The evolution of static allometry in sexually selected traits. *Evolution,* 57, 2450-2458.

Currie, D.A., Milner, M.J., & Evans, C.W. (1988). The growth and differentiation in vitro of leg and wing imaginal disc cells from *Drosophila melanogaster*. *Development*, 102, 805-814.

Eberhard, W.G., Huber, B.A., Rodriguez R.L.S., Briceño, R.D., Salas, I., & Rodriguez, V. (1998). One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution*, 52(2), 415-431.

Emlen, D.J. (1994). Environmental control of horn length dimorphism in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 256, 131-136.

Emlen, D.J. (1997). Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London. Series B*, 264, 567-574.

Emlen, D.J. & Nijhout, H.F. (2000). The development and evolution of exaggerated morphologies in insects. *Annual Review of Entomology*, 45, 661-708.

Emlen, D.J., Warren, I.A., Johns, A., Dworkin, I., & Lavine, L.C. (2012). A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science*, 337(6096), 860-4.

Fedoseeva, E.F. (2011). Morphometric characteristics of *Formica aquilonia* ants in monitoring of their settlements. *Entomological Review*, 91(2), 152-168.

Feener, D.H, Lighton, J.J.R.B., & Bartholomew, G.A. (1988). Curvilinear allometry, energetics and foraging ecology: A comparison of leaf-cutting ants and army ants. *Functional Ecology*, 2(4), 509-520.

Flocas, A.A., Giles, B.D., & Angouridakis, V.E. (1983). On the estimation of annual and monthly mean-values of air-temperature over Greece using stepwise multiple-regression analysis. *Archives for Meteorology Geophysics and Bioclimatology Series B-Theoretical and Applied Climatology*, 32(2-3), 287-295.

Geraghty, M.J., Dunn, R.R., & Sanders, N.J. (2007). Body size, colony size, and range size in ants (Hymenoptera: Formicidae): are patterns along elevational and latitudinal gradients consistent with Bergmann's Rule? *Myrmecological News*, 10, 51-58.

Gould, S.J. (1973). Positive allometry of antlers in the 'Irish Elk' *Megaloceros giganteus*. *Nature*, 244, 375-376.

Gyllenstrand, N., Seppä, P., & Pamilo, P. (2004). Genetic differentiation in sympatric wood ants, *Formica rufa* and *F. polyctena*. *Insectes Sociaux*, 51, 139-145.

Hurlbert, A.H., Ballantyne I.V.F., & Powell, S. (2008). Shaking a leg and hot to trot: the effects of body size and temperature and running speed in ants. *Ecological Entomology*, 33, 144-154.

Huxley, J.S. & Tessier, G. (1936). Terminology of relative growth. *Nature*, 137, 780-787.

Kaspari, M. & Weiser, M.D. (1999). The size-grain hypothesis and interspecific scaling in ants. *Functional Ecology*, 13, 530-538.

Kawano, K. (1995). Horn and wing allometry and male dimorphism in giant Rhinoceros beetles (Coleoptera: Scarabaeidae) of tropical Asia and America. *Annals of the Entomological Society of America*, 88(1), 92-99.

Knaden, M., Tinaut, A., Cerda, X., Wehner, S., & Wehner, R. (2005). Phylogeny of three parapatric species of desert ants, *Cataglyphis bicolor*, *C. viatica* and *C. savigny*: a comparison of mitochondrial DNA, nuclear DNA and morphological data. *Zoology*, 108, 169-177.

Kodric-Brown, A., Sibly, R.M., & Brown, J.H. (2006). The allometry of ornaments and weapons. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 8733-8738.

Macaranas, J.M., Colgan, D.J., Major, R.E., Cassis, G., & Gray, M.R. (2001). Species discrimination and population differentiation in ants using microsatellites. *Biochemical Systematics and Ecology*, 29, 125-136.

McArdle, B.H. (1988). The structural relationship: regression in biology. *Canadian Journal of Zoology*, 66, 2329-2339.

Mirth, C.K. & Riddiford, L.M. (2007). Size assessment and growth control: How adult size is determined in insects. *Bioessays*, 29, 344-355.

Morata, G. & Lawrence, P.A. (1979). Development of the eye-antenna imaginal disc of *Drosophila*. *Developmental Biology*, 70, 355-371.

Moreau, C.S. (2009). Inferring ant evolution in the age of molecular data (Hymenoptera: Formicidae). *Myrmecological News*, 12, 201–210.

Mosiman, J. & James, F. (1979). New statistical methods for allometry with application to Florida red-winged blackbirds. *Evolution*, 33, 444-459.

Nicholls, E., Rossi, M., & Niven, J.E. (2021). Larval nutrition impacts survival to adulthood, body size and the allometric scaling of metabolic rate in adult honeybees. *Journal of Experimental Biology*, 224(14), jeb242393.

Nijhout, H.F. & Wheeler, D.E. (1996). Growth models of complex allometries in holometabolous insects. *The American Naturalist*, 148, 40-56.

Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y., & Callier, V. (2014). The developmental control of size in insects. *WIREs Journal of Developmental Biology*, 3, 113-134.

Perl, C.D. & Niven, J.E. (2016). Colony-level differences in the scaling rules governing wood ant compound eye structure. *Scientific Reports*, 6, 24204.

Perl, C.D., Rossoni, S., & Niven, J.E. (2017). Conservative whole-organ scaling contrasts with highly labile suborgan scaling differences among compound eyes of closely related *Formica* ants. *Ecology and Evolution*, 7, 1663-1673.

Pusch, K., Seifert, B., Foitzik, S., & Heinze, J. (2006). Distribution and genetic divergence of two parapatric sibling ant species in Central Europe. *Biological Journal of the Linnean Society*, 88, 223-234.

Schlick-Steiner, B.C., Steiner, F.M., Konrad, H., Marko, B., Csosz, S., Heller, G., Ferencz, B., Sipos, B., Christian, E., & Stauffer, C. (2006a). More than one species of *Messor* harvester ants (Hymenoptera: Formicidae) in Central Europe. *European Journal of Entomology*, 103, 469-476.

Schlick-Steiner, B.C., Steiner, F.M., Moder, K., Seifert, B., Sanetra, M., Dyreson, E., Stauffer, C., & Christian, E. (2006b). A multidisciplinary approach reveals cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, 40, 259-273.

Schwarz, S., Narendra, A., & Zeil, J. (2011). The properties of the visual system in the Australian desert ant *Melophorus bagoti*. *Arthropod Structure and Development*, 40, 128-134.

Seifert, B. (2016). The supercolonial European wood ant *Formica paralugubris* SEIFERT, 1996 (Hymenoptera: Formicidae) introduced to Canada and its predicted role in Nearctic forests. *Myrmecological News*, 22, 11-20.

Shingleton, A.W., Das, J., Vinicius, L., & Stern, D.L. (2005). The temporal requirements for insulin signaling during development in *Drosophila*. *PLoS Biology*, 3, e289

Shingleton, A.W., Estep, C.M., Driscoll, M.V., & Dworkin, I. (2009). Many ways to be small: different environmental regulators of size generate distinct scaling relationships in *Drosophila melanogaster*. *Proceedings of the Royal Society of London. Series B*, 276, 2625-2633.

Smith, A., Fisher, B.L., & Hebert, P.D.N. (2005). DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society B London*, 360, 1825-1834.

Sorvari, J. & Hakkarainen, H. (2009). Forest clear-cutting causes small workers in the polydomous wood ant *Formica aquilonia*. *Annales Zoologici Fennici*, 46, 409-416.

Spaethe, J. & Chittka, L. (2003). Interindividual variation of eye optics and single object resolution in bumblebees. *Journal of Experimental Biology*, 206, 3447-3453.

Steiner, F.M., Schlick-Steiner, B.C., Sanetra, M., Ljubomirov, T., Antonova, V., Christian, E., & Stauffer, C. (2005). Towards DNA-aided biogeography: an example from *Tetramorium* ants (Hymenoptera, Formicidae). *Annales Zoologici Fennici*, 42, 23-35.

Steiner, F.M., Schlick-Steiner, B.C., Konard, H., Moder, K., Christian, E., Seifert, B., Crozier, R.H., Stauffer, C., & Buschinger, A. (2006). No sympatric speciation here: multiple data sources show that the ant *Myrmica microrubra* is not a separate species but an alternate reproductive morph of *Myrmica rubra*. *Journal of Evolutionary Biology*, 19, 777-787.

Stevenson, R.D., Hill, M.F., & Bryant, P.J. (1995). Organ and cell allometry in Hawaiian *Drosophila*: How to make a big fly. *Proceedings of the Royal Society of London. Series B*, 259, 105-110.

Stockan, J.A., Robinson, E.J., Trager, J.C., Yao, I., & Seifert, B. (2016). Introducing wood ants: evolution, phylogeny, identification and distribution. In J.A., Stockan, and E.J.H., Robinson, (Eds), *Wood ant ecology and conservation* (pp 1-36). Cambridge University Press.

Thomas, R.H. (1993). Ecology of body size in *Drosophila buzzatii*: Untangling the effects of temperature and nutrition. *Ecological Entomology*, 18, 84-90.

Tschinkel, W.R. (2013). The morphometry of *Solenopsis* fire ants. *Plos One*, 8(11), e79559.

Vogler, A.P. & Monaghan, M.T. (2007). Recent advances in DNA taxonomy. J*ournal of Zoological Systematics and Evolutionary Research*, 45(1), 1-10.

Véle, A. & Modlinger, R. (2019). Body size of wood ant workers affects their work division. *Sociobiology*, 66(4), 614-618.

Wheeler, D.A. (1991). The developmental basis of worker caste polymorphism in ants. *The American Naturalist*, 138(5), 1218-1238.

Zagas, T. (1990). *Conditions for natural establishment of Pinus sylvestris in an area of Rhodopi Mountain, northern Greece* (In Greek). Ph.D. thesis, Aristotle University, Thessaloniki.

Zar, J.H. (1999). *Biostatistical analysis*. Prentiss-Hall, Inc. Upper Saddle River, N.J.

Received: February 03, 2021 *Accepted: September 06, 2021*

J. Entomol. Res. Soc., 23(3): 225-238, 2021 *Review Article*
Doi: 10.51963/jers.v23i3.2016 *Online ISSN:2651-3579*

Insecticide Resistance in the Brown Planthopper, *Nilaparvata lugens* (Stål): Mechanisms and Status in Asian Countries

Juel DATTA[1]* Sujan Chandra BANIK[2]

[1]Entomology Division, Bangladesh Rice Research Institute, Gazipur-1701, BANGLADESH
[2]Department of Horticulture, Sylhet Agricultural University, Sylhet-3100, BANGLADESH
e-mails: [1,]*datta.juel@outlook.com, [2]rahul0707sau@gmail.com
ORCID IDs: [1]0000-0001-7008-0833, [2]0000-0001-5828-0680

ABSTRACT

The brown planthopper, *Nilaparvata lugens* (Stål) is a destructive rice pest found in almost all the rice-growing areas across the globe. In pest management strategies, insecticides are the vital element to control this insect pest. But recently their heavy use poses a risk of control failure because of the development of insecticide resistance. Quick insecticide resistance development nature in *N. lugens* intrigued scientists to understand the complex resistance mechanism(s), side by side pledge the importance of regular monitoring to know the trend of resistance development. Resistance mechanisms like, target-site insensitivity and enhanced activity of detoxifying enzymes, have been extensively studied and identified in governing the resistance development of *N. lugens*. Both the field collected and laboratory selected pest populations were tested against commonly used insecticides to detect insecticide resistance ratio. In this review, recent findings of resistance mechanisms, candidate genes those contribute in resistance development have been summarized. We also provide an insight into the metabolic resistance mechanisms that confer significant levels of resistances and the current status of insecticide resistance in *N. lugens*. This review will help to get a clearer view on present research directions of insecticide resistance in *N. lugens*.

Key words: Nilaparvata lugens, insecticide resistance, monitoring, metabolic mechanisms.

Datta, J. & Banik, S.C. (2021). Insecticide resistance in the brown planthopper, *Nilaparvata lugens* (Stål): mechanisms and status in Asian countries. *Journal of the Entomological Research Society*, 23(3), 225-238.

INTRODUCTION

Brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) is a major rice pest that causes significant losses in rice-growing areas (Masaya et al, 2009). Since 2003, many Asian countries have seen frequent outbreaks of *N. lugens* (Bottrell & Schoenly, 2012). Chemical control is the key element of integrated pest management strategies to control rice insect pests (Min, Lee, Choi, Lee, & Kwon, 2014). Because of higher efficiency, neonicotinoid is widely used to control many insect pests including *N. lugens* (Matsuda, Ihara, & Sattelle, 2020; Datta et al, 2021a). However, it has developed low to moderate levels of resistance to neonicotinoids. Overdose and constant use of insecticides considers as the key reason for the fast resistance development in *N. lugens* (Matsumura et al, 2018).

Research on insecticide resistance in *N. lugens* has been doubled in recent years, and studies were attempting to explain complex mechanisms conferring resistance. The study of insecticide resistance mechanisms is vital to manage resistance problems, to reduce the threat of pest outbreak and to introduce more improved control measures. Numerous resistance mechanisms have been found governing insect resistance to insecticides (Garrood et al, 2016). Among the two mostly reported resistance mechanisms, the enhanced metabolic detoxification of xenobiotics has been commonly studied in *N. lugens* (Latif, Omar, Tan, Siraj, & Ismail, 2010). Increased activities of detoxifying enzymes have been constantly found in resistant *N. lugens*. Through gene amplification it has been proved that multiple resistance genes are directly correlated with enhanced detoxifying enzyme activities (Hamada, Stam, Nakao, Kawashima, & Banba, 2020). Cytochrome P450 monooxygenase (P450) displayed significant roles in conferring insecticide resistance in *N. lugens* in response to neonicotinoids (Hamada et al, 2020). Functional analysis through RNA interference (RNAi) confirmed the function of multiple P450 genes (Jin et al, 2019). The levels of enhanced detoxifying enzyme activities and the expression levels of the genes encoded for the enzymes vary with the insecticide resistance levels (Mao et al, 2020).

Currently, *N. lugens* developed resistance to frequently used insecticides and there are threats of future resistance development to less used insecticides (Fujii et al, 2020; Matharu & Tanwar, 2020). Previous findings suggest extremely higher levels of resistance to imidacloprid, a principal neonicotinoid insecticide, and potentiality to develop resistance to other insecticides of the same group (Datta et al, 2021a). This review mainly focuses metabolic resistance mechanism as one of the main mechanism to confer insecticide resistance in *N. lugens*. Side by side, the status of resistance to commonly used insecticides developed in this pest has been shortly described.

Nilaparvata lugens a destructive pest of rice

One of the major destructive dominant herbivore of rice is *N. lugens* that is found in all rice-growing areas of Indonesia, Thailand, India, Japan, Vietnam, China, Bangladesh, Solomon Island and north-eastern Australia, the Philippines, and Malaysia (Masaya et al, 2008; Latif et al, 2010; Ali et al, 2014; Hereward et al, 2020). The long distance migratory behavior and population development patterns sometimes make the control measure more complex and most of the rice field of different places became

vulnerable to the pest (Khoa, Thang, Liem, Holst, & Kristensen, 2018). It damaged rice plant by sucking sap during different growth stages of rice plant, which caused wilting and drying, known as "hopper-burn" and it also, transmits several viral diseases (Liao et al, 2019). The outbreaks of *N. lugens* in several rice producing countries have frequently occurred in the last few decades, which is threatening food security for the growing populations (Bottrell & Schoenly, 2012).

Evolution of insecticide resistances in *N. lugens*

Resurgence of *N. lugens* was thought to be associated with different factors likely use of synthetic insecticides, fertilizers and susceptible rice varieties (Uddin, Islam, Jahan, Ara, & Afrin, 2020). Precautions have been taken to control this pest and a significant amount of money have been invested to improve pest control strategies, for instance, in Indonesia alone 100 million US dollar have been invested to control this pest per year (Cheng, 2015). However, the investments to improve control programs sometimes failed to uphold the destruction of rice yield as pest outbreaks in recent years occurs in some Asian countries. Chemical control became the only means to control this destructive insect pest in rice farming practices and since then the use of insecticides considers as the vital element to control this pest (Bottrell & Schoenly, 2012). However, heavy use of common insecticides poses enormous risks not only to environmental elements but the reduced toxicity of insecticides against *N. lugens* (Mu et al, 2016).

Insecticide Resistance Action Committee (IRAC) defined insecticide resistance as the repeated failure of a chemical product to damage insect pest population in an expected level even after applying recommended doses (Sparks & Nauen, 2015). Evolution of insecticide resistance in insect pest has become a threat in choosing efficient insecticide for its management (Bolzan et al, 2019). Even after the continuous use of insecticides, farmers witnessed the resurgence of *N. lugens*; studies later find out the development of low to high level of resistance in *N. lugens* (Cheng, 2015; Uddin et al, 2020). The demand and use of synthetic insecticides to control *N. lugens* never been clogged but increased proportionately in some places, which extends the resistance problems. It has been reported that *N. lugens* field populations already developed moderate to extremely high level of resistance to imidacloprid, thiamethoxam, buprofezin, dinotefuran (Liao et al, 2021). In addition, the cross-resistance between different insecticides among the field populations is also posing a threat to select competent insecticide classes to control BPH (Mu et al, 2016).

Mechanism of insecticide resistance

Development of insecticide resistance relies on particular resistance mechanisms of insects. Four resistance mechanisms have been reported in insect pests, however, according to previous studies two major mechanisms are mainly contributing in developing resistance in *N. lugens*, target-site modifications and metabolic resistance. Majority of the research described metabolic resistance mechanisms as the principal mechanism to induce resistance in this pest (Mao et al, 2020). Here, both of the reported mechanisms are discussed, although metabolic mechanisms cover most of the portion of this review.

Target-site insensitivity

Several insecticides affect specific target sites of insect nervous system. It has been found that resistant insect shows modifications in their target sites to confer resistance against particular insecticide (Steinbach et al, 2015). In two nicotinic acetylcholine receptor (nAChR) subunits, point mutations (Y151S) have been identified in association with imidacloprid resistance in *N. lugens* (Zewen et al, 2005). However, the mutations in target site have only been found in laboratory selected imidacloprid resistant-strain and never been reported in resistant field populations of *N. lugens* (Zewen et al, 2005; Liang et al, 2018; Sanada-Morimura et al, 2019). For this reason most of the recent research only emphasizes complex metabolic resistance mechanisms that found both in lab selected strain and field collected *N. lugens*. These findings shed light to study insecticide resistance mechanisms in this pest in response to distinct insecticide classes.

Metabolic resistance mechanism

Metabolic resistance is the principal mechanism and widely studied topic in *N. lugens.* Its resistance evolution to common insecticides is mostly attributed to the enhanced detoxification of enzymes, and expression of resistance genes encoded for detoxifying enzymes (Wen, Liu, Bao, & Han, 2009). The metabolic resistance mechanisms reported in previous findings have been summarized.

Metabolic enzyme activities

The involvement of a metabolic enzyme in detoxifying insecticide has proved by measuring the enhanced levels of detoxifying enzyme activities in insects. The elevated activities of metabolic enzymes P450, esterases (EST) and glutathione S-transferases (GST) have been reported in several insect species resistant to insecticides (Bass & Field, 2011; Liang et al, 2018). Through the synergistic and enzymatic assay, increased activity of detoxifying P450 enzyme has been found in many insects in response to neonicotinoids insecticides (Chen, Shan, Liu, Shi, & Gao, 2019). Similarly, insecticide resistance of *N. lugens* against different insecticides is mostly controlled by increased enzyme activity, especially P450 enzyme. The level of P450 was significantly high in *N. lugens* resistant to imidacloprid, thiamethoxam, and dinotefuran compare to susceptible pest (Sun, Gong, Ali, & Hou, 2018). Detection of enhanced P450 activities in resistant *N. lugens* strain, suggesting P450-mediated detoxification occurs in imidacloprid-, thiamethoxam-, and dinotefuran-resistant strains. In contrast, significantly increased activities of two detoxifying enzymes, P450 and EST, were found in nitenpyram and sulfoxaflor selected strain, but P450 might be the major detoxifying enzyme (Liao et al, 2019). Although the finding of enzymatic assay was consistent with the synergistic assay, characterization of gene expression and functional validation of specific gene are needed to confirm the xenobiotic mechanism in response to nitenpyram and sulfoxaflor. The elevated levels of EST activities have been reported higher in chlorpyrifos-resistant strain compare to susceptible strain of *N. lugens* (Lu et al, 2017). This report suggests that enhanced EST activity could account for resistance to organophosphate insecticides.

Characterization of resistance genes

Studies have successfully characterized gene expression that revealed significant information of metabolic enzymes associated with detoxification of insecticides (Table 1). A number of metabolic resistant genes individually and/or in group have been reported overexpressed in resistant *N. lugens* through gene amplification, transcriptional up-regulation and genome sequencing (Zhang et al, 2016a; Xu et al, 2017). Three major metabolic detoxification genes have been reported in *N. lugens* that involves in the detoxification of insecticides (Liang et al, 2018). Among them P450s are considered as the principal contributor to confer resistance that found in all the living organisms (Wang et al, 2018). Reported P450 genes are belonged to microsomal CYP4, CYP6, CYP9, and mitochondrial CYP12 families and are mostly correlated with the resistance development to neonicotinoids (Feyereisen, 1999; Scott, 1999). Many researchers have also been found several P450s involvement in *N. lugens* resistance to distinct classes of insecticides. Overexpression of one or multiple P450 genes have been reported in this pest resistance to imidacloprid, thiamethoxam, dinotefuran, buprofezin, nitenpyram, sulfoxaflor, clothianidin and etofenprox (Pang et al, 2014; Garrood et al, 2016; Liao et al, 2021; Datta et al, 2021b). Similarly, the up-regulation of esterase gene *NlCarE* was found related to chlorpyrifos resistance in *N. lugens* (Lu et al, 2017). It is obvious that P450s as the superfamily has repeatedly been verified that confer insecticide resistance in *N. lugens*. Recent advances in research have been very useful to generate knowledge about the resistance genes those are significantly contributing in insecticide metabolism in *N. lugens*.

Functional validation of resistance genes

Bao et al, (2016) characterized imidacloprid metabolism by determining *CYP6ER1* and *CYP6AY1* expression in vitro through recombinant P450 proteins. Similarly, using the recombinant P450 proteins, enzymatic activities of five P450s were determined to analyze their roles in developing resistance to imidacloprid (Zhang, Yang, Sun, & Liu, 2016b). Among the five P450 proteins, the fastest metabolite formation was observed in incubation with *CYP6CW1*, *CYP6AY1*, *CYP6ER1*, and *CYP4CE1* (Zhang et al, 2016b). These approaches confirmed the contribution of these genes in resistance development in *N. lugens*.

The overexpression of metabolic detoxification genes displayed its significance in resistance development in *N. lugens*, which implies the importance to characterize individual gene function involving in resistance. Studying the metabolic mechanisms has been improved in this genomic era with the advancement of the molecular tools and techniques including gene silencing technique, RNAi and CRISPR/Cas9 (Unniyampurath, Pilankatta, & Krishnan, 2016; Zhu, Chereddy, Howell, & Palli, 2020). Silencing of overexpressed P450 gene, *CYP6ER1*, in lab strain of *N. lugens* increased susceptibility to imidacloprid, thiamethoxam, dinotefuran, nitenpyram and sulfoxaflor, which demonstrated the involvement of *CYP6ER1* as the functional gene in resistance development (Jin et al, 2019; Liao et al, 2019). Although most of the findings outlined *CYP6ER1* as the key P450 gene contributes in conferring

insecticide resistance in *N. lugens*, overexpressed *CYP6AY1* is another important P450 gene reported by several authors. The *in vivo* study through RNAi reduced the mRNA levels of *CYP6AY1* in imidacloprid resistant strain and increased the mortality rate of the pest after the imidacloprid application (Bao et al, 2016; Ding et al, 2013). This indicated that *CYP6AY1* dsRNA feeding successfully suppressed insecticide resistance to imidacloprid and confirmed the role of *CYP6AY1* gene in insecticide resistance. The overexpression of P450 genes has also reported in *N. lugens* resistant to pyrethroid insecticides. Multiple P450 genes were silenced through RNAi that cause major changes in resistance levels in BPH against etofenprox, a non-ester pyrethroid insecticide (Sun, Yang, Zhang, & Liu, 2017). The functional analyses of resistance genes by knocking down of multiple genes have considerably extended our understanding on the complex mechanisms of conferring resistance to different group of insecticides. Additionally, the expression level of *NlCarE* was significantly reduced after dsRNA injection in chlorpyrifos resistant *N. lugens* (Lu et al, 2017).

Table 1. Genetic characterization of insecticide resistance mechanism in *Nilaparvata lugens*.

Insecticide	Population/Strain	Methods	Resistance gene(s)	Reference
Imidacloprid[1]	Field	qRT-PCR	*CYP6ER1*	Garrood et al, (2016)
Imidacloprid	Field	qRT-PCR, RNAi	*CYP6ER1, CYP6AY1*	Bao et al, (2016)
Imidacloprid, Thiamethoxam[1], Dinotefuran[1]	Lab	qRT-PCR, RNAi	*CYP6ER1*	Sun et al, (2018)
Imidacloprid	Lab	qRT-PCR, RNAi	*CYP6ER1, CYP6AY1, CYP6CE1, CYP6CW1*	Zhang et al, (2016b)
Imidacloprid	Field	qRT-PCR	*CYP6ER1, CYP6AY1, CYP6CS1*	Zhang et al, (2016a)
Imidacloprid	Field	qRT-PCR, RNAi, transgenic[5]	*CYP6ER1*	Pang et al, (2016)
Imidacloprid	Lab	qRT-PCR, RNAi	*CYP6AY1*	Ding et al, (2013)
Imidacloprid, Buprofezin[2]	Field	qRT-PCR	*CYP6AY1*	Pang et al, (2014)
Imidacloprid	Lab	qRT-PCR, RNAi	*CYP6ER1*	Yang et al, (2016)
Nitenpyram[1]	Lab	qRT-PCR, RNAi	*CYP6ER1*	Mao et al, (2019)
Sulfoxaflor[1]	Lab	qRT-PCR, RNAi	*CYP6ER1*	Liao et al, (2019)
Chlorpyrifos[3]	Lab	qRT-PCR, RNAi	*NlCarE*	Lu et al, (2017a)
Clothianidin[1]	Lab	qRT-PCR, RNAi	*CYP6ER1*	Jin et al, (2019)
Etofenprox[4]	Lab	qRT-PCR, RNAi	*CYP6FU1, CYP425A1,CYP6AY1*	Sun et al, (2017)
Imidacloprid, Thiamethoxam, Dinotefuran, Clothianidin[1], Buprofezin	Field	qRT-PCR	*CYP6AY1, CYP6ER1*	Liao et al, (2021)
Imidacloprid, Thiamethoxam, Dinotefuran, Buprofezin	Field	qRT-PCR	*CYP6ER1*	Datta et al, (2021b)

[1]Neonicotinoids, [2]Insect growth regulator, [3]Organophosphate, [4]Pyrethroids, [5]Transgenic approach utilizing the GAL4/UAS system of *D. melanogaster*

The *Drosophila* transgenic technique is another approach to determine functions of genes in insect resistance to insecticides (Daborn et al, 2012; Pang et al, 2016). The expression of *CYP6ER1* of *N. lugens* in *Drosophila* evolved significant levels of resistance to imidacloprid compare to dinotefuran for the variant of *CYP6ER1* gene (Hamada et al, 2020). This provides important information on the *in vivo* metabolism of imidacloprid resistance by the variants of *CYP6ER1*.

The metabolic resistance mechanism has been depicted with the possible transcription factors regulating gene expression in Fig. 1. Several signaling pathways were found to mediate the up-regulation of detoxification enzymes in insects and play a key role in metabolic resistance to insecticides (Amezian, Nauen, & Le Goff, 2021). The signaling pathways includes transcription factors (TF) namely, cap "n" collar (CncC), musculoaponeurotic fibrosarcoma (MaF), aryl hydrocarbon receptor (AhR), G-protein coupled receptor (GPCR) (Amezian et al, 2021). The expression of two P450 genes contributes to insecticide resistance in *Drosophila* regulated by the transcription factors CncC and Maf (Gaddelapati, Kalsi, Roy, & Palli, 2018; Bo et al, 2020). However, little is known about these gene regulatory factors in gene expression in insects. Further studies are needed to describe the principal regulatory routes of detoxification gene expression in *N. lugens*. It is necessary to clarify what are the specific functions of those genes, what is the exact number of genes, how the genes are correlated with insecticide resistance ratio, and what are the functions that regulate the overexpression of resistance genes.

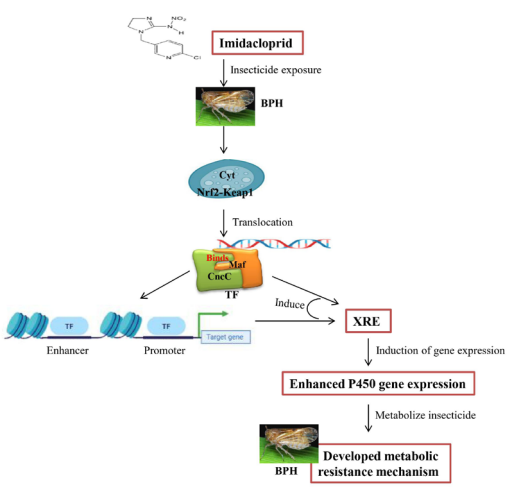


Fig. 1. Flowchart of the development of metabolic resistance mechanism in BPH started in cytoplasm of insect cell. Here, imidacloprid represents the neonicotinoids insecticide group. Through translocation Nrf2 with Keap1 transferred to nucleus. Cyt-cytoplasm; Nrf2-nuclear factor erythroid 2; Keap1-kelch-like ECH-associated protein 1 pathway; XRE-xenobiotic response element. The description of transcription factors and their mechanism in insect pests may found in Palli, 2020.

Current status of insecticide resistance

Since the first outbreak of *N. lugens*, the pest distribution and the population development of the pest has been frequently reported in many studies (Bottrell

& Schoenly, 2012). Additionally, the status of the insecticide resistance has been monitored by different authors, providing details information, which would help forecast pest outbreak hence to improve pest management strategies (Liao et al, 2021). The insecticide resistance status in *N. lugens* has been detected either by topical application of insecticides or by rice stem dipping method (Priyadharshini, Muthukrishnan, Sathiah, & Prabakar, 2020). Studies used field collected pest populations to detect insecticide resistance, which helped to know the exact scenario of the levels of resistance. In contrast, laboratory selected strain has been used to monitor pests ability to develop resistance in controlled condition, to provide information on cross-resistance among insecticides and to compare with field-evolved resistance. The resistance ratio of *N. lugens* to commonly used insecticides reported during 2011 to 2021 has been presented in Fig. 2. The data was collected from multiple studies and the resistance ratio to tested insecticides has been summarized (research findings from where the data was extracted mentioned in Supplementary file).

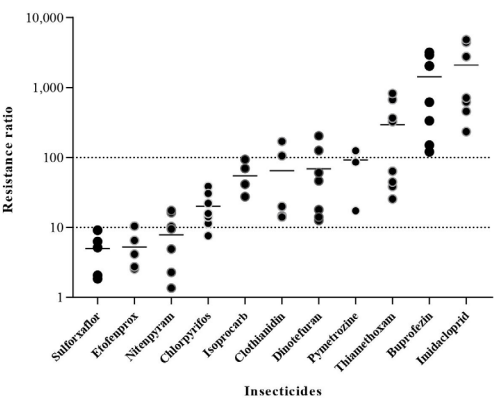


Fig. 2. Previously reported resistance ratio of *Nilaparvata lugens* field populations to several tested insecticides. The resistance ratio (ranges and calculation) was described by Wang et al, (2018). Data of resistance ratio of *N. lugens* field populations is summarized from eighteen different research articles published during 2011-2020.

Resistance to organophosphates and carbamates

Organophosphates and carbamates insecticides target AChE inhibitor of small brown planthopper and have been using to control the pest after the World War II (Kwon, Kim, Jeong, & Lee, 2019). Common insecticides of these two groups includes chlorpyrifos, diazinon, carbufuran, carbosulfan and fenobucarb, has been using to control *N. lugens* field populations. However, the threat of *N. lugens* resurgence is still presence as low to high levels of resistance to chlorpyrifos has been documented (Fig. 2) (Lu et al, 2017; Yang & Lai, 2019).

Resistance to neonicotinoids

Imidacloprid a widely used neonicotinoid has become an effective solution to control many chewing and sucking pest, including *N. lugens* since 1991 (Masaya et al, 2008).

However, the continuous and overuse of the insecticide faced a challenge when reduced toxicity of imidacloprid has been reported in *N. lugens* field populations across Asian countries (Bao et al, 2016). High to extremely high levels of resistance to imidacloprid has been reported for several consecutive years in China, Japan, Vietnam, Bangladesh and Thailand (Wang et al, 2014; Bao et al, 2016; Garrood et al, 2016; Sanada-Morimura et al, 2019; Datta et al, 2021b). Other neonicotinoids such as thiamethoxam, dinotefuran, sulfoxaflor, clothianidin, cycloxaprid, and nitenpyram were effective, but in recent year's development of resistance in *N. lugens* to these insecticides also reported (Pang et al, 2014; Mu et al, 2016; Fang et al, 2018; Sun et al, 2018; Mao et al, 2019; Zhang et al, 2020). These findings suggest that reduced toxicities of several neonicotinoids have been threatened pest control measure of *N. lugens*.

Resistance to other insecticides

Resistance to insecticides belong to pyrethroids (pymetrozine, etofenprox), and insect growth regulator (buprofezin) have been found in *N. lugens* in recent years (Yang et al, 2016; Sun et al, 2017; Liao et al, 2019; Datta et al, 2021b). In contrast, a new mesoionic insecticide triflumezopyrim still shows its efficiency to control *N. lugens* (Liao et al, 2021). Although the levels of resistance to these insecticides are comparatively low than neonicotinoids, there is a threat of complete control failure in future by these insecticides. Therefore, it is important to monitor insecticide resistance development in rice insect pests regularly.

Resistance selections - method to understand resistance potentiality

Various experiments designed to find out how quick insecticide resistance could increase or reduce in an insect pest in response to a single or multiple insecticides and what are the responses of the pest against different insecticides after certain generations of rearing in laboratory condition (Jin et al, 2019; Liao et al, 2019). For these objectives, researchers, rear the pest with insecticides termed as resistance selection. This method helps to understand the potentiality of resistance development in *N. lugens*, to advance molecular study to gather more knowledge on resistance mechanisms, and suggests rational use of insecticides. Susceptible *N. lugens* developed low to high levels of resistances to imidacloprid, etofenprox and clothianidin when resistance selection was done with the same insecticide for several generations (Zhang et al, 2015; Sun et al, 2017; Jin et al, 2019). Resistance selection method helps to get a highly resistant strain to a single insecticide that make it possible to conclude the contribution of a sole resistance mechanism in developing resistance.

CONCLUSIONS

In last decades side by side the resurgence of *N. lugens,* use of insecticides has also been significantly increased. This major rice pest already developed low to high levels of resistances to commonly used insecticides in most of the rice producing Asian countries. Increased detoxifying enzyme activities have been found as the principal resistance mechanism in *N. lugens.* Enhanced activities of P450, EST, and

GST have been significantly contributes in evolution of resistance to neonicotinoids, pyrethroids, insect growth regulator and to organophosphate insecticides. Thus it shows that this pest have the potentiality to develop resistance to multiple insecticides in field condition. Hence, many studies have been carried out to understand particular molecular mechanisms presence in *N. lugens.* To delay or abandoned the resistance development in this pest, rotational use of insecticides, introducing new class of insecticides, understanding multi-resistance mechanism and adapting the insecticide resistance management strategies are recommended.

REFERENCES

Ali, M.P., Huang, D., Nachman, G., Ahmed, N., Begum, M.A., & Rabbi, M.F. (2014). Will climate change affect outbreak patterns of planthoppers in Bangladesh? *Plos One,* 9(3), e91678. 10.1371/journal.pone.0091678

Amezian, D., Nauen, R., & Le Goff, G. (2021). Transcriptional regulation of xenobiotic detoxification genes in insects-An overview. *Pesticide Biochemistry and Physiology,* 104822.

Bao, H., Gao, H., Zhang, Y., Fan, D., Fang, J., & Liu, Z. (2016). The roles of *CYP6AY1* and *CYP6ER1* in imidacloprid resistance in the brown planthopper: Expression levels and detoxification efficiency. *Pesticide Biochemistry and Physiology, 129,* 70-74. 10.1016/j.pestbp.2015.10.020

Bass, C. & Field, L.M. (2011). Gene amplification and insecticide resistance. *Pest Management Science,* 67(8), 886-890. 10.1002/ps.2189

Bo, H., Miaomiao, R., Jianfeng, F., Sufang, H., Xia, W., Elzaki, M.E.A., Chris, B., Palli, S.R., & Jianya, S. (2020). Xenobiotic transcription factors CncC and maf regulate expression of *CYP321A16* and *CYP332A1* that mediate chlorpyrifos resistance in *Spodoptera exigua*. *Journal of Hazardous Materials*, 398, 122971.

Bolzan, A., Padovez, F.E., Nascimento, A.R., Kaiser, I.S., Lira, E.C., Amaral, F.S., & Omoto, C. (2019). Selection and characterization of the inheritance of resistance of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to chlorantraniliprole and cross-resistance to other diamide insecticides. *Pest Management Science,* 75, 2682-2689. 10.1002/ps.5376

Bottrell, D.G. & Schoenly, K.G. (2012). Resurrecting the ghost of green revolutions past: The brown planthopper as a recurring threat to high-yielding rice production in tropical Asia. *Journal of Asia-Pacific Entomology,* 15, 122-140. 10.1016/j.aspen.2011.09.004

Chen, C., Shan, T., Liu, Y., Shi, X., & Gao, X. (2019). Identification of a novel cytochrome P450 *CYP3356A1* linked with insecticide detoxification in *Bradysia odoriphaga*. *Pest Management Science, 75*(4), 1006-1013. doi:10.1002/ps.5208

Cheng, J. (2015). Rice Planthoppers in the Past Half Century in China. In *Rice Planthoppers* (pp. 1-32).

Daborn, P.J., Lumb, C., Harrop, T.W., Blasetti, A., Pasricha, S., Morin, S., & Batterham, P. (2012). Using *Drosophila melanogaster* to validate metabolism-based insecticide resistance from insect pests. *Insect Biochemistry and Molecular Biology,* 42(12), 918-924.

Datta, J., Monsur, M.B., Chakraborty, P., Chakrabarty, S., Fahad, S., Hossain, A., Mondal, M.F., Ahmed, S., Ali, M.P., & Sabagh, A. (2021a). Obstacle in controlling major rice pests in Asia: insecticide resistance and the mechanisms to confer insecticide resistance. In S. Fahad, O. Sönmez, S. Saud, et al. (Eds.). *Engineering tolerance in crop plants against abiotic stress* (p. 336). CRC Press.

Datta, J., Wei, Q., Yang, Q., Wan, P.J., He, J.C., Wang, W.X., Lai, F.X., Ali, M.P., & Fu, Q. (2021b). Current resistance status of the brown planthopper *Nilaparvata lugens* (Stål) to commonly used insecticides in China and Bangladesh. *Crop Protection*, 150, 105789. 10.1016/j.cropro.2021.105789

Ding, Z., Wen, Y., Yang, B., Zhang, Y., Liu, S., Liu, Z., & Han, Z. (2013). Biochemical mechanisms of imidacloprid resistance in *Nilaparvata lugens*: over-expression of cytochrome P450 *CYP6AY1*. *Insect Biochemistry and Molecular Biology,* 43(11), 1021-1027. 10.1016/j.ibmb.2013.08.005

Fang, Y., Xie, P., Dong, C., Han, Y., Tang, T., Liu, Y., & Zhou, X. (2018). Cross-resistance and baseline susceptibility of brown planthopper *Nilaparvata lugens* (Hemiptera: Delphacidae) from China to cycloxaprid. *Journal of Economic Entomology,* 111(5), 2359-2363. 10.1093/jee/toy222

Feyereisen, R. (1999). Insect P450 enzymes. *Annual Review of Entomology,* 44(1), 507-533.

Fujii, T., Sanada-Morimura, S., Oe, T., Ide, M., Van Thanh, D., Van Chien, H., & Matsumura, M. (2020). Long-term field insecticide susceptibility data and laboratory experiments reveal evidence for cross resistance to other neonicotinoids in the imidacloprid-resistant brown planthopper *Nilaparvata lugens*. *Pest Management Science,* 76(2), 480-486. 10.1002/ps.5533

Gaddelapati, S.C., Kalsi, M., Roy, A., & Palli, S. R. (2018). Cap 'n' collar C regulates genes responsible for imidacloprid resistance in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Insect Biochemistry and Molecular Biology,* 99, 54-62. 10.1016/j.ibmb.2018.05.006

Garrood, W.T., Zimmer, C.T., Gorman, K.J., Nauen, R., Bass, C., & Davies, T.G. (2016). Field-evolved resistance to imidacloprid and ethiprole in populations of brown planthopper *Nilaparvata lugens* collected from across South and East Asia. *Pest Management Science,* 72(1), 140-149. 10.1002/ps.3980

Hamada, A., Stam, L., Nakao, T., Kawashima, M., & Banba, S. (2020). Differential metabolism of neonicotinoids by brown planthopper, *Nilaparvata lugens, CYP6ER1* variants. *Pesticide Biochemistry and Physiology,* 165, 104538. 10.1016/j.pestbp.2020.02.004

Hereward, J.P., Cai, X., Matias, A.M.A., Walter, G.H., Xu, C., & Wang, Y. (2020). Migration dynamics of an important rice pest: The brown planthopper (*Nilaparvata lugens*) across Asia-Insights from population genomics. *Evolutionary Applications,* 13, 2449-2459. 10.1111/eva.13047

Jin, R., Mao, K., Liao, X., Xu, P., Li, Z., Ali, E., & Li, J. (2019). Overexpression of *CYP6ER1* associated with clothianidin resistance in *Nilaparvata lugens* (Stål). *Pesticide Biochemistry and Physiology,* 154, 39-45. 10.1016/j.pestbp.2018.12.008

Khoa, D.B., Thang, B.X., Liem, N.V., Holst, N., & Kristensen, M. (2018). Variation in susceptibility of eight insecticides in the brown planthopper *Nilaparvata lugens* in three regions of Vietnam 2015-2017. *PloS one*, 13(10), p.e0204962.

Kwon, D.H., Kim, H., Jeong, I.-H., & Lee, S.H. (2019). F331H mutation and reduced mRNA in type-1 acetylcholinesterase is associated with carbofuran resistance development in the small brown planthopper (*Laodelphax striatellus* Fallén). *Journal of Asia-Pacific Entomology,* 22(1), 6-14. doi:10.1016/j.aspen.2018.11.014

Latif, M.A., Omar, M.Y., Tan, S.G., Siraj, S.S., & Ismail, A.R. (2010). Biochemical studies on malathion resistance, inheritance and association of carboxylesterase activity in brown planthopper, *Nilaparvata lugens* complex in Peninsular Malaysia. *Insect Science,* 17(6), 517-526. doi:10.1111/j.1744-7917.2010.01331.x

Liang, Z., Pang, R., Dong, Y., Sun, Z. X., Ling, Y., & Zhang, W.Q. (2018). Identification of SNPs involved in regulating a novel alternative transcript of P450 *CYP6ER1* in the brown planthopper. *Insect Science,* 25(5), 726-738.

Liao, X., Jin, R., Zhang, X., Ali, E., Mao, K., Xu, P., Li, J., & Wan, H. (2019). Characterization of sulfoxaflor resistance in the brown planthopper, *Nilaparvata lugens* (Stål). *Pest Management Science,* 75(6), 1646-1654. doi:10.1002/ps.5282

Liao, X., Xu, P.F., Gong, P.P., Wan, H., & Li, J.H. (2021). Current susceptibilities of brown planthopper *Nilaparvata lugens* to triflumezopyrim and other frequently used insecticides in China. *Insect Science,* 28(1), 115-126. doi:10.1111/1744-7917.12764

Lu, K., Wang, Y., Chen, X., Zhang, Z., Li, Y., Li, W., & Zhou, Q. (2017). Characterization and functional analysis of a carboxylesterase gene associated with chlorpyrifos resistance in *Nilaparvata lugens* (Stål). *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology,* 203, 12-20. doi:10.1016/j.cbpc.2017.10.005

Mao, K., Ren, Z., Li, W., Cai, T., Qin, X., Wan, H., & Li, J. (2020). Carboxylesterase genes in nitenpyram-resistant brown planthoppers, *Nilaparvata lugens*. *Insect Science,* doi:10.1111/1744-7917.12829

Mao, K., Zhang, X., Ali, E., Liao, X., Jin, R., Ren, Z., & Li, J. (2019). Characterization of nitenpyram resistance in *Nilaparvata lugens* (Stål). *Pesticide Biochemistry and Physiology,* 157, 26-32. doi:10.1016/j.pestbp.2019.03.001

Masaya, M., Hiroaki, T., Masaru, S., Sachiyo, S.-M., Akira, O., Tomonari, W., & Van, T. D. (2008). Species-specific insecticide resistance to imidacloprid and fipronil in the rice planthoppers *Nilaparvata lugens* and *Sogatella furcifera* in East and South-east Asia. *Pest Management Science,* 64, 1115-1121. doi:10.1002/ps.164110.1002/ps

Masaya, M., Takeuchi, H., Satoh, M., Sachiyo, S.-M., Akira, O., Tomonari, W., & Van, T. D. (2009). Current status of insecticide resistance in rice planthoppers in Asia. In K.L. Heong & B. Hardy (Eds.). *Planthoppers: new threats to the sustainability of intensive rice production systems in Asia* (pp. 233-244). Los Baños (Philippines): International Rice Research Institure.

Matharu, K.S. & Tanwar, P.S. (2020). Efficacy of different insecticides against brown planthopper, *Nilaparvata lugens* (Stål) in rice. *International Journal of Chemical Studies,* 8(3), 870-873. 10.22271/chemi.2020.v8.i3k.9310

Matsuda, K., Ihara, M., & Sattelle, D.B. (2020). Neonicotinoid insecticides: molecular targets, resistance, and toxicity. *Annual Review of Pharmacology and Toxicology*, 60, 241-255.

Matsumura, M., Sanada-Morimura, S., Otuka, A., Sonoda, S., Van Thanh, D., Van Chien, H., & Huang, S.H. (2018). Insecticide susceptibilities of the two rice planthoppers *Nilaparvata lugens* and *Sogatella furcifera* in East Asia, the Red River Delta, and the Mekong Delta. *Pest Management Science,* 74(2), 456-464. 10.1002/ps.4729

Min, S., Lee, S.W., Choi, B.-R., Lee, S.H., & Kwon, D.H. (2014). Insecticide resistance monitoring and correlation analysis to select appropriate insecticides against *Nilaparvata lugens* (Stål), a migratory pest in Korea. *Journal of Asia-Pacific Entomology,* 17(4), 711-716. 10.1016/j.aspen.2014.07.005

Mu, X.C., Zhang, W., Wang, L.X., Zhang, S., Zhang, K., Gao, C.F., & Wu, S.F. (2016). Resistance monitoring and cross-resistance patterns of three rice planthoppers, *Nilaparvata lugens*, *Sogatella furcifera* and *Laodelphax striatellus* to dinotefuran in China. *Pesticide Biochemistry and Physiology,* 134, 8-13. doi:10.1016/j.pestbp.2016.05.004

Palli, S.R. (2020). CncC/Maf-mediated xenobiotic response pathway in insects. *Archives of Insect Biochemistry and Physiology,* 104(2), p.e21674.

Pang, R., Chen, M., Liang, Z., Yue, X., Ge, H., & Zhang, W. (2016). Functional analysis of *CYP6ER1*, a P450 gene associated with imidacloprid resistance in *Nilaparvata lugens*. *Scientific Reports,* 6, 34992. doi:10.1038/srep34992

Pang, R., Li, Y., Dong, Y., Liang, Z., Zhang, Y., & Zhang, W. (2014). Identification of promoter polymorphisms in the cytochrome P450 *CYP6AY1* linked with insecticide resistance in the brown planthopper, *Nilaparvata lugens*. *Insect Molecular Biology,* 23(6), 768-778. doi:10.1111/imb.12121

Priyadharshini, E., Muthukrishnan, N., Sathiah, N., & Prabakar, K. (2020). Baseline toxicity study of sulfoxaflor against rice brown planthopper, *Nilaparvata lugens* (Stål) populations of Tamil Nadu. *Journal of Entomology and Zoology Studies,* 8(5), 2176-2180.

Sanada-Morimura, S., Fujii, T., Chien, H.V., Cuong, L.Q., Estoy, G.F., Jr., & Matsumura, M. (2019). Selection for imidacloprid resistance and mode of inheritance in the brown planthopper, *Nilaparvata lugens*. *Pest Management Science,* 75(8), 2271-2277. 10.1002/ps.5364

Scott, J.G. (1999). Cytochrome P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology,* 29(9), 757-777.

Sparks, T.C. & Nauen, R. (2015). IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology,* 121, 122-128. 10.1016/j.pestbp.2014.11.014

Steinbach, D., Gutbrod, O., Lummen, P., Matthiesen, S., Schorn, C., & Nauen, R. (2015). Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella*. *Insect Biochem Mol Biol, 63*, 14-22. doi:10.1016/j.ibmb.2015.05.001

Sun, H., Yang, B., Zhang, Y., & Liu, Z. (2017). Metabolic resistance in *Nilaparvata lugens* to etofenprox, a non-ester pyrethroid insecticide. *Pesticide Biochemistry and Physiology,* 136, 23-28. doi:10.1016/j.pestbp.2016.08.009

Sun, X., Gong, Y., Ali, S., & Hou, M. (2018). Mechanisms of resistance to thiamethoxam and dinotefuran compared to imidacloprid in the brown planthopper: Roles of cytochrome P450 monooxygenase and a P450 gene *CYP6ER1*. *Pesticide Biochemistry and Physiology,* 150, 17-26. 10.1016/j.pestbp.2018.06.014

Uddin, A.B.M.A., Islam, K.S., Jahan, M., Ara, A., & Afrin, S. (2020). Farmer`s Perception about Resurgence of Brown Planthopper, *Nilaparvata Lugens* (Stål) in Bangladesh. *Bangladesh Rice Journal,* 23(1), 21-33. 10.3329/brj.v23i1.46078

Unniyampurath, U., Pilankatta, R., & Krishnan, M.N. (2016). RNA Interference in the Age of CRISPR: Will CRISPR Interfere with RNAi? *International Journal of Molecular Sciences,* 17(3), 291. 10.3390/ijms17030291

Wang, H.Y., Gao, F.C., Zhu, C., Chen, J., Li, H., Zhuang, L.Y., & Shen, L.J. (2014). Imidacloprid susceptibility survey and selection risk assessment in field populations of *Nilaparvata lugens* (Homoptera: Delphacidae). *Journal of Economic Entomology,* 101(2), 515-522.

Wang, K., Zhang, M., Huang, Y., Yang, Z., Su, S., & Chen, M. (2018). Characterisation of imidacloprid resistance in the bird cherry-oat aphid, *Rhopalosiphum padi*, a serious pest on wheat crops. *Pest Management Science,* 74(6), 1457-1465. 10.1002/ps.4834

Wen, Y., Liu, Z., Bao, H., & Han, Z. (2009). Imidacloprid resistance and its mechanisms in field populations of brown planthopper, *Nilaparvata lugens* Stål in China. *Pesticide Biochemistry and Physiology,* 94(1), 36-42. 10.1016/j.pestbp.2009.02.009

Xu, L., Zhao, C.Q., Xu, D.J., Xu, G.C., Xu, X.L., Han, Z.J., Zhang, Y.N., & Gu, Z.Y. (2017). RNAi suppression of nuclear receptor genes results in increased susceptibility to sulfoxaflor in brown planthopper, *Nilaparvata lugens*. *Journal of Asia-Pacific Entomology,* 20(2), 645-653. 10.1016/j.aspen.2017.03.022

Yang, Y.Y. & Lai, C.T. (2019). Synergistic effect and field control efficacy of the binary mixture of permethrin and chlorpyrifos to brown planthopper (*Nilaparvata lugens*). *Journal of Asia-Pacific Entomology,* 22(1), 67-76. 10.1016/j.aspen.2018.11.004

Yang, Y., Huang, L., Wang, Y., Zhang, Y., Fang, S., & Liu, Z. (2016). No cross-resistance between imidacloprid and pymetrozine in the brown planthopper: status and mechanisms. *Pesticide Biochemistry and Physiology,* 130, 79-83. 10.1016/j.pestbp.2015.11.007

Zewen, L., Williamson, M.S., Lansdell, S.J., Denholm, I., Han, Z., & Millar, N.S. (2005). A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *PNAS,* 102(24).

Zhang, J., Zhang, Y., Wang, Y., Yang, Y., Cang, X., & Liu, Z. (2016a). Expression induction of P450 genes by imidacloprid in *Nilaparvata lugens*: A genome-scale analysis. *Pesticide Biochemistry and Physiology,* 132, 59-64. 10.1016/j.pestbp.2015.10.016

Zhang, Y.C., Feng, Z.R., Zhang, S., Pei, X.G., Zeng, B., Zheng, C., Gao, C.F., & Yu, X.Y. (2020). Baseline determination, susceptibility monitoring and risk assessment to triflumezopyrim in *Nilaparvata lugens* (Stål). *Pesticide Biochemistry and Physiology,* 167, 104608. 10.1016/j.pestbp.2020.104608

Zhang, Y., Wang, X., Yang, B., Hu, Y., Huang, L., Bass, C., & Liu, Z. (2015). Reduction in mRNA and protein expression of a nicotinic acetylcholine receptor alpha8 subunit is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*. *Journal of Neurochemistry,* 135(4), 686-694. 10.1111/jnc.13281

Zhang, Y., Yang, Y., Sun, H., & Liu, Z. (2016b). Metabolic imidacloprid resistance in the brown planthopper, *Nilaparvata lugens*, relies on multiple P450 enzymes. *Insect Biochemistry and Molecular Biology,* 79, 50-56. 10.1016/j.ibmb.2016.10.009

Zhu, G.H., Chereddy, S., Howell, J.L., & Palli, S.R. (2020). Genome editing in the fall armyworm, *Spodoptera frugiperda*: Multiple sgRNA/Cas9 method for identification of knockouts in one generation. *Insect Biochemistry and Molecular Biology,* 122, 103373. 10.1016/j.ibmb.2020.103373

Received: February 16, 2021 *Accepted: August 27, 2021*

J. Entomol. Res. Soc., 23(3): 239-255, 2021 *Research Article*
Doi: 10.51963/jers.v23i3.2026 *Online ISSN:2651-3579*

Wing Morphometric and DNA Barcoding Analysis of Two Different Public Health Important *Anopheles* Mosquito Species

Deepika PANDA[1] Ipsita BISWAL[2] Jaya Kishor SETH[3] Tapan Kumar BARIK[1,4*]

[1,2,3,4]Post Graduate Department of Zoology, Berhampur University, Bhanjabihar-760007, Odisha, INDIA
[4] Post Graduate Department of Biotechnology, Berhampur University, Bhanjabihar-760007, Odisha, INDIA
e-mails: [1]deepika.02104@gmail.com, [2]ipsita.biswal@gmail.com, [3]jkseth52@gmail.com, [4*]tkbarik@rediffmail.com
ORCID IDs: [1]0000-0001-8637-6823, [2]0000-0002-4720-4495, [3]0000-0002-1331-5971, [4*]0000-0003-3814-4061

ABSTRACT

Accurate identification and bio-ecology of mosquito vectors are of paramount importance in several respects including the development of species-specific vector control strategies. In the current study, we explored two different advanced tools such as Geometric morphometric (GM) and DNA barcoding analysis, to supplement morpho-taxonomy-based identification of malaria vectors. In landmark-based GM analysis, the pattern of wing shape was examined and we noticed a clear variation in their shape among different *Anopheles* species. Similarly, in the DNA barcoding studies, the phylogenetic analysis is based upon the marker genes such as mitochondrial Cytochrome oxidase subunit- I (mt COI), Cytochrome b (mt Cytb), and nuclear D3 domain of 28s rDNA gene sequences were carried out. The Neighbor-joining tree was formed by distinctive conspecific clusters illustrating genetic variation among different groups of *Anopheles* species. Further, genetic exchange among different Anopheline populations was analyzed by considering the COI gene sequences. In the gene flow study, the frequent genetic divergence, as well as gene exchange among *Anopheles stephensi* mosquito populations, was observed. Our findings suggested that GM analysis of the wing shape along with the DNA barcoding approach can effectively be used together for accurate identification of mosquito vectors including *Anopheles stephensi* and *Anopheles barbirostris*.

Key words: Integrated Taxonomic approach, Geometric morphometric, Molecular analysis, Mosquito vector, *Anopheles stephensi, Anopheles barbirostris*

Panda, D., Biswal, I., Seth J.K., & Barik, T.K. (2021). Wing morphometric and DNA barcoding analysis of two different public health important *Anopheles* mosquito species. *Journal of the Entomological Research Society,* 23(3), 239-255.

INTRODUCTION

In India, Malaria, Dengue, Lymphatic filariasis, Japanese encephalitis, Chikungunya, Zika are major vector-borne diseases. Mosquitoes are the well-known vector-borne disease-transmitting agent. There are about 3,583 different Culicidae species reported across the globe (Harbach, 2021). The family Culicidae is a huge and most abundant group which is classified into two different medically important subfamilies such as Anophelinae and Culicinae, among those subfamilies, Anophelinae comprises about 488 valid species. In India, around 58 *Anopheles* mosquito species have been reported to date (Dev and Sharma 2013). Most *Anopheles* mosquito *species* act as a vector for malarial parasites and some other also transmits microfilaria and arboviruses to humans and other animals (Trent, 2005).

For surveillance as well as control of vectors and mosquito-borne diseases, precise identification of mosquito vector species, as well as an understanding of their biology, ecology, and geographical distribution, are crucial (Amini, Hanafi-Bojd, Aghapour, & Chavshin, 2020). The accurate identification of vector species is essential for developing efficient control strategies. The knowledge of taxonomy has evolved in many different ways, but still, morpho- taxonomy is rated high in mosquito species identification. Traditional mosquito identification has relied on dichotomous keys that explain the physical attributes of a certain life stage. Although this technique has proven to be extremely useful and is still widely used to distinguish many mosquito species, it does have some limitations. It requires taxonomic experts to perform accurate identifications, and it is prone to inappropriate identifications due to the loss of key morphological characters during specimen collection and preservation (Madeira, Duarte, Boinas, & Costa Osorio, 2021). Further, an integrated taxonomic approach is the need of the hour in order to resolve taxonomic ambiguity, systematics study and phylogenetic analysis in biological sciences (Joshi & Agarwal, 2021). Keeping in view the importance of the integrated taxonomic approach, during this study we applied two tools i.e. Geometric morphometric and DNA barcoding along with a traditional morpho-taxonomic approach for accurate identification and molecular analysis of mosquito vectors, *Anopheles stephensi* and *Anopheles barbirostris.*

The Geometric morphometric (GM) analysis has been introduced as a supplement for error-free identification of species. It has been demonstrated to be an incredible tool for evaluating the correlation between shapes of various organs and structures, including 2D, 3D points representing landmarks (LMs), curves and outlines. It also permits several statistical analyses and allows depicting the shape and size graphically (Lorenz et al, 2017). It became popular because of the current accessibility of modest computational power-specific software (Rohlf, 2003; 2004; 2006; Dujardin, 2010) and digitizing applications suitable even for less experienced users. The advantage of the use of this technique by multivariate regression investigation is that the allometric impact can effortlessly be evacuated from shape analysis, making it conceivable to compare shapes with the least interference from varying sizes (Adams, Rohlf, & Slice, 2004). Mosquito geometric morphometrics has previously been used to identify

between genera (Wilke et al, 2016), species within the same genus (Sumruayphol et al, 2016; Chaiphongpachara et al, 2019) and populations within a species (Vidal & Suesdek, 2012; Morales Vargas et al, 2013). In mosquitoes, wings are the excellent structures generally utilized for morphometric comparisons since it contains veins that encompass natural anatomical landmarks which are ideal for landmarking (Mondal, Devi & Jauhari 2015).

In recent time, the molecular taxonomic approaches have gained momentum in numerous taxonomic studies on dipterans (Stahls, Vujic, & Perez-Banon, 2009; Pramual, Wongpakam, & Adler, 2011) and also employed in identifying several mosquito species (Gonzalez et al, 2010; Laboudi et al, 2011; Ruiz-Lopez et al, 2012). The standard mitochondrial DNA, COI barcode region, has enough information and is very proficient for species identification. It is also helpful in resolving the identification related to ambiguous and cryptic species. In some cases, multiple gene markers have been used to distinguish closely related cryptic as well as sibling species (Lin & Danforth, 2004). Hence, nuclear gene along with mitochondrial DNA may be used as a supplementary marker to support any conclusions. Moreover, molecular data are broadly utilized for producing molecular phylogenies such as phylogenetic, population genetics, genetic variation and species identification studies. Geographical barriers and climatic differences are the main factors of genetic difference which prevents the geneflow within the same species which gradually leads to speciation. A study on the rate of geneflow gives an idea about the genetic exchange among the different populations of a particular species.

Malaria affects an estimated 219 million people worldwide each year, resulting in over 400,000 deaths (WHO, 2021). India contributes substantially to the global malaria burden with approximately 1,86,532 malaria cases reported in the year 2020 (NVBDCP, 2021). Odisha state of India accounts for about 22% of malaria cases and 10% mortality due to malaria alone (NVBDCP, 2021). Out of thirty districts of this state, most of the cases were reported from the Western, Northern and Southern regions of the state (Pradhan et al, 2016). Several mosquito vectors of *Anopheles, Culex* and *Aedes* are found in Odisha state and study site in particular. *An. culicifacies*, *An. stephensi, An. minimus* mainly act as malaria vectors and *Cx. vishnui, Cx. tritaeniorhynchus, Cx. gelidus, Cx. quinquefaciatus, An. barbirostris, An. peditaeniatus, An. subpictus* etc acts as vectors of JE in the studied area. Further, there is no sufficient data available on mosquito faunal diversity of southern districts of Odisha state and therefore we have considered this region as our study area.

The primary goal of this study is to identify the public health important *Anopheles* mosquito species through the geometric-morphometric examination of wing shape variation and by DNA barcoding approach using marker genes such as mitochondrial COI (mt COI), Cytochrome b and nuclear D3 domain of 28s rDNA. In addition, we also analyzed the genetic exchange between different *Anopheles* mosquitoes based on COI sequences to understand the genetic divergence among Anopheline mosquito species.

MATERIALS AND METHODS

Mosquito sampling

A survey of different mosquito breeding habitats was carried out for the updation of mosquito faunal diversity in some southern districts of Odisha state, India (Fig.1). Mosquitoes in their different developmental stages were collected throughout the year by using a variety of standard procedures from multiple locations and transported to the laboratory in preserved condition for identification. Morphological identification of all the field-collected samples was performed using the available identification keys (Christophers, 1933; Barraud, 1934; Tyagi, Munirathinam & Venkatesh, 2015). After successful identification; the mosquito samples were vouchered and stored for future study.

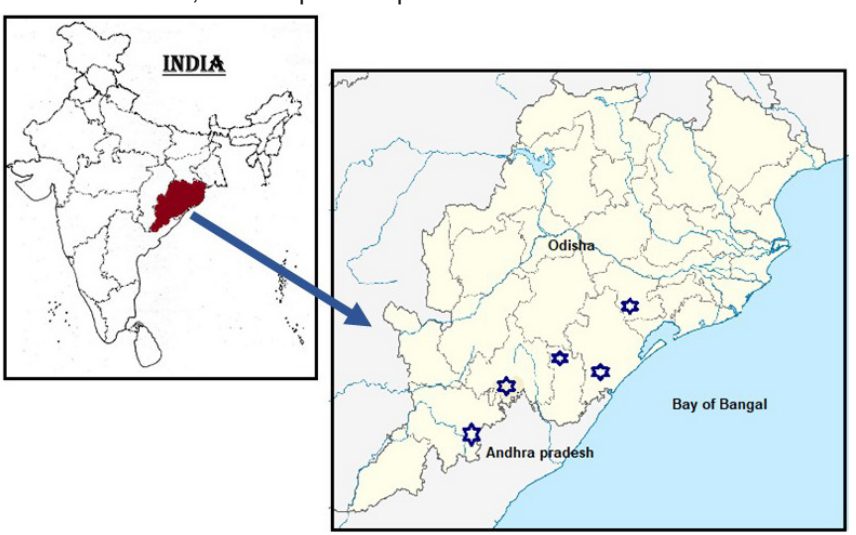


Fig.1. Map showing locations of the mosquito collection sites.

Material preparation for geometric morphometric analysis

The right-wing of individual adult female mosquito was segregated from the thorax and mounted over a microscope slide with a coverslip. Each wing was then photographed under 40x magnification using Leica DFC320 digital camera coupled with a Leica S6 microscope.

Landmarking for GM analysis

About 62 photographs of two different mosquito species (*Anopheles stephensi* (n=31), *Anopheles barbirostris* (n=31)) were chosen and saved as TPS files using the program TPSutil32. The coordinates of 19 landmarks represented through vein intersections were carried out using TPS Dig version 2.31. All the coordinates of landmarks and their descriptions are provided in (Fig.2). In this study, the software Morpho J (Klingenberg, 2011) was used for Principal Component Analysis (PCA) and Discriminate Function Analysis (DFA).

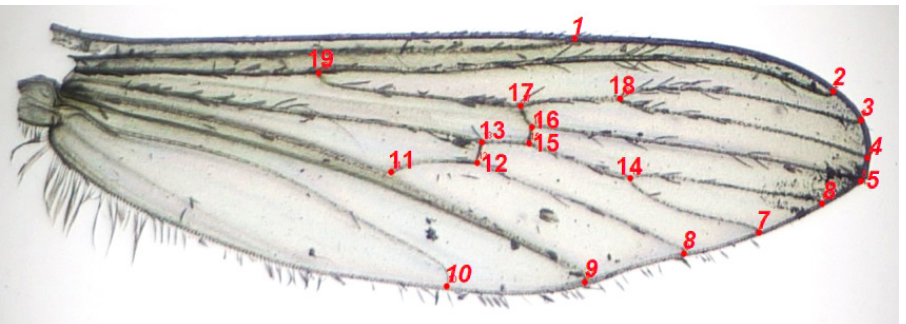


Fig. 2. Landmarks used for PCA and DFA analysis: (1) Intersection of costa(c), (2) Distal end of the radius(R), (3) Radical branch 2, (4) Radical branch 3, (5) Distal end of radius branches 4 and 5, (6) Distal end of M1 and 2, (7) Distal end of M3 and 4, (8) Distal end of cubital vein 1, (9) Distal end of cubital vein 2, (10) Anal vein, (11) Origin of cubital 1, (12) Midpoint branch of cubital 3, (13) Medio-cubital cross vein, (14) Radio-sectoral vein, (15) Midpoint branch Radio medial vein, (16) Radio medial cross vein, (17) Mid-point branch of radial vein, (18) Origin of radius branches 2 and 3, (19) Radial cross vein.

Genomic DNA isolation, PCR amplification and sequencing

DNA extraction from the whole adult mosquito was carried out as per the Bender Buffer method (Collins et al, 1987) with minor modifications. Further, the isolated DNA was used as a templet for the amplification of mitochondrial COI, Cytb and nuclear 28s rDNA gene. DNA amplification was carried out using previously described primer pairs by Folmer, Black, Hoeh, Lutz, & Vrijenhoek 1994; Lyman et al, 1999, Singh et al, 2004, respectively. The reaction mixture was the same for all the studied genes. All the gene amplification consisting of 1X PCR buffer, 0.5 U Taq DNA, 2.5mM $MgCl_2$, 200 µM dNTPs, 10pmol of each primer, 100 pmol template DNA, total dilution was made up to 25 µl. The thermal profile for the COI gene consisted of one cycle of 95℃ for 5min followed by 35 cycles of 95℃ for 30sec, 45℃-55℃ for 30sec and 72℃ for 1min with a final extension step of 7 min at 72℃. The thermal profile for Cytb gene amplification includes an initial denaturation at 94℃ for 5 min followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 45–47℃ for 30 seconds, and extension at 72℃ for 2 min; followed by a final extension of 72℃ for 7 min. The PCR condition for the 28s rDNA gene was similar to COI amplification except for the annealing temperature, which is 48℃ for 30 sec. The amplicons were resolved in 1.5% agarose gel. Finally, the purified PCR products were outsourced for sequencing. Large no. of individual specimens were sequenced for each species but the only good quality sequence of COI, Cytb and 28S rDNA was selected and used in this study for analysis of both the species.

DNA sequence analysis

The trace files of COI, Cytb and 28s rDNA sequences were edited and assembled using Geneious version 9.0.5 (Biomatters Ltd, Auckland, NZ) (http://www.geneious.com) software and low-quality sequences were excluded at the time of data analysis. The generated nucleotide sequence from each specimen was compared with barcode sequences available on NCBI using nucleotide Basic Local Alignment Search Tool

(BLASTn), and the final obtained sequences were submitted to NCBI to get the accession number. The accession numbers of the submitted sequences are listed in Table 1. Further, to resolve the genetic relationship among different species, some mt COI gene sequences representing the same and related species of *Anopheles* taxa under study were retrieved from GenBank as replicate data for evaluating the taxonomic position of our target species.

Table 1. Sequence characteristics of COI, Cytb and 28s rDNA (D3).

Gene	*Anopheles stephensi*		*Anopheles barbirostris*	
COI	Sequence length	Accession No.	Sequence length	Accession No.
	668bp	MN329060	615bp	MN166188
Cytb	469bp	MN954649	434bp	MT036262
28s rDNA	398bp	MN319577	381bp	MN318470

Phylogenetic analysis of COI, Cytb and D3 28s rDNA sequences

Multiple sequence alignment was carried out by the CLUSTAL W algorithm embedded in software package MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) to find out the distance matrix which can be used to make a phylogenetic tree. Further, to study the evolutionary relationships among the mosquito species, the generated mitochondrial COI and Cytb gene sequences as well as the 28s rDNA gene sequences were subjected to phylogenetic analysis. The phylogenetic analysis of each gene was carried out with a published set of sequences of different mosquitoes for the mapping of the gene phylogenies by Neighbor-joining (NJ) algorithm (Saitou& Nei, 1987). The evolutionary distances were estimated using the (Kimura 2 Parameter model) K_2P-distance method in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) with a bootstrap test of 1000 replicates (Felsenstein, 1985). $1^{st}+2^{nd}+3^{rd}$+noncoding codon positions were included in the study and for each sequence pair, all the ambiguous positions were discarded.

Gene flow estimation

Estimates of long-term inbreeding effective population sizes and rates of gene flow among regions were made under a Bayesian inference framework using the program DnaSP version 6 (Rozas et al, 2017) separately for different species. Population genetic indices were calculated separately for each mosquito species using nucleotide data of the mt COI gene. Apart from this, the haplotype diversity (Hd), nucleotide diversity (Pi), genetic distances among haplotypes (Fst), number of migration (Nm) were analyzed. Obtained datasets were compared with datasets reported from various regions of India, Pakistan, Sri Lanka, Iran, Thailand, and China.

RESULTS

Both the immature and mature stages of different types of mosquitoes were collected from different sites of the study area. The immature stages of mosquitoes were reared to the adult stage for morphological identification. The larval habitat from which specimens were obtained includes both natural and artificial container habitats

whereas adults were collected resting on the various surface from cattle sheds and other human habitats. All the adult mosquitoes were identified morphologically as *Anopheles stephensi* and *Anopheles barbirostris* using the available identification keys (Christophers, 1933; Barraud, 1934; Tyagi, Munirathinam & Venkatesh, 2015).

In the geometric morphometric analysis, the Procrustes sums of squares and the tangent sum of the square were found to be 0.1314 and 0.1310, respectively. In Principal component (PC) analysis, out of 34 principal components, PC1 and PC2 have the highest eigenvalues and % variance and thus, these two PCs were chosen for further analysis (Fig.3). The eigenvalues of PC1 and PC2 were found to be 0.0011 and 0.0002 with % variance 52.091% and 9.941% respectively, representing more than 62% of cumulative variance. The scatter plot of the first two PCs for the 62 specimens showed two distinct clusters thereby the *Anopheles barbirostris* clustered themselves in the negative axis of PC1 and *Anopheles stephensi* formed a cluster in the positive axis of PC1 (Fig.3 C). The transformation grid of PC1 showed that the lollipop structure of LM 7, 9, 10, 11, 12, 13, 14 and 18 had maximum variation (Fig.3 A). In the PC2 transformation grid, the lollipop of LM 1, 7, 8, 9, 10, 14, 18 and 19 showed maximum variation (Fig.3 B). *Anopheles stephensi* showed a significant shape difference from *Anopheles barbirostris* which is evident from the Procrustes distance, Mahalanobis distance and T- square value obtained from Discriminant Function Analysis (DFA) having values 0.0642, 15.6633, 3802.7658 respectively with p-value < 0.0001.

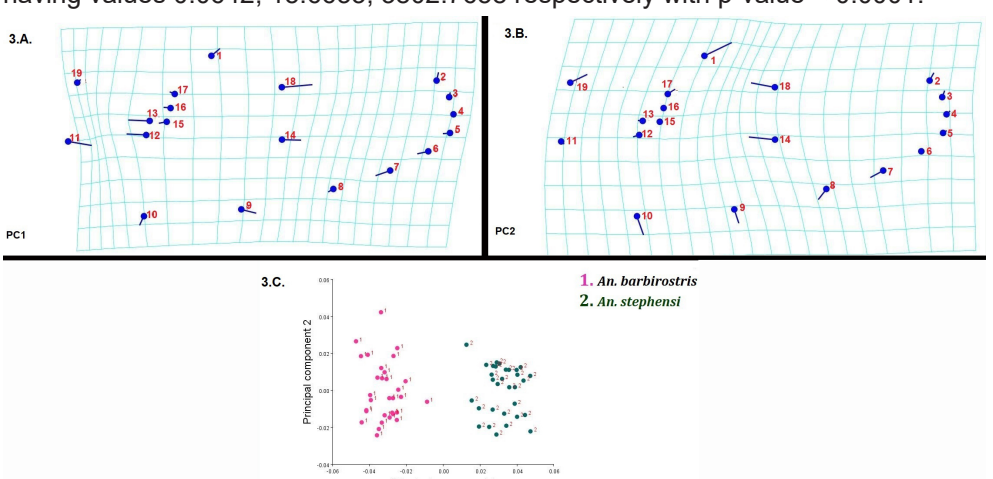


Fig.3. Scatter plot of principal component (PC1 and PC2) and transformation grid representation of variance of 62 samples. Arrows indicate the changes in the relative position of the landmarks.

The COI gene sequences for *Anopheles stephensi* (668bp) and *Anopheles barbirostris* (615bp) were obtained after the final annotation. AT- richness ranges from 67-68% in these sequences. The Neighbor-joining is conceptually related to the clustering pattern. In this study, the COI gene fragments provide phylogenetic signals and also revealed species boundaries accurately. The optimal NJ tree with the sum of branch length =0.43167063 (Fig.4). In the resulted phylogenetic tree, all

the above-studied specimens form clusters with their respective species. Likewise, the generated Cytb sequences of *Anopheles stephensi* (469 bp) and *Anopheles barbirostris* (434bp) were rich in AT content, which is about 74%. A similar NJ tree topology was observed in the case of Cytb geneset, supporting the process of molecular identification (Fig.5). Final annotation obtained from D_3 gene sequences for *Anopheles stephensi* (398 bp) and *Anopheles barbirostris* (381bp) were rich in GC content, which ranges from 55.6%-57.3%. Similar to both the mitochondrial genes, these 28s rDNA sequences of the same species cluster together in the phylogeny (Fig.6).

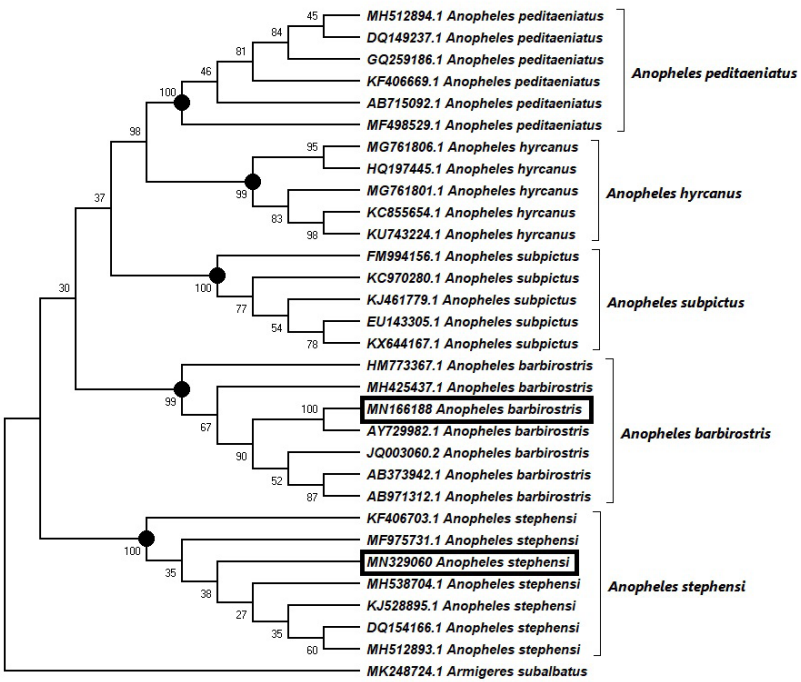


Fig.4. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among COI sequences and are in the units of the number of base substitutions per site. All the ambiguous positions were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

Haplotype diversity (Hd) and nucleotide diversity (π) are two important indicators to measure the diversity of species populations among different geographical strains. Haplotype diversity (Hd) for the 34 COI sequences of *Anopheles stephensi* was calculated to be 0.631, an average number of nucleotide differences (k), and nucleotide diversity (Pi) was found to be 42.146 and 0.125, respectively. Genetic differences among the populations of *Anopheles stephensi* species are provided in Table 2. For *Anopheles barbirostris,* by considering 41 COI sequences, the haplotype diversity (Hd) was calculated to be 0.897. An average number of nucleotide differences (k) and nucleotide

diversity (π) was found to be 89.092 and 0.342, respectively. Genetic differences among the population of *An. barbirostris* are provided in Table 3. The Nm value between Indian and Pakistan *An. stephensi* population was greater than 1, which was an indication of frequent genetic exchange between these two places. However, the Nm value was less than 1 between Indian and other studied populations of *An. barbirostris* species which indicates a less or no genetic exchange observed among these mosquito populations.

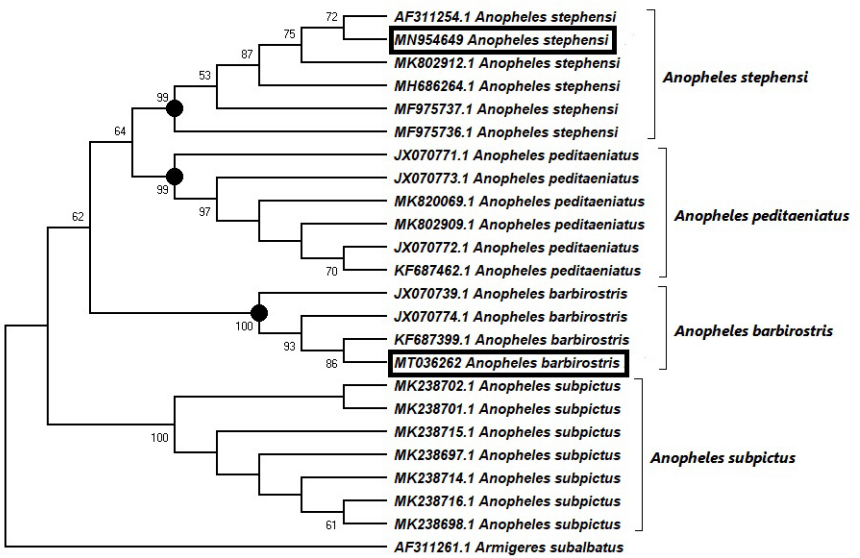


Fig. 5. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among Cytb sequences and are in the units of the number of base substitutions per site. All the ambiguous position were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

Table 2. Gene flow properties between different populations of *Anopheles stephensi* calculated from nucleotide sequence of mtCOI gene. AN1- India, AN2- Pakistan, AN3- Sri Lanka, AN4- Iran. (Hd=Haplotype diversity, Pi= nucleotide diversity, Kt=average number of nucleotide difference, Kxy= inter-population nucleotide differences between the populations, Gst= Genetic differentiation index based on the frequency of haplotypes, Fst= genetic distances among haplotypes, Dxy= average number of nucleotide substitutions per site between all the populations, Da= average no. of net nucleotide substitutions per site between all the populations, Nm= number of migration)

Population 1	Population 2	Hd	Pi	Kt	Kxy	Gst	Fst	Dxy	Da	Nm (Based on Fst)
AN1	AN2	0.395	0.089	30.193	30.333	0.096	0.092	0.090	0.008	2.46
AN1	AN3	0.699	0.110	37.921	30.416	0.096	0.093	0.088	0.008	2.42
AN2	AN3	0.404	0.001	0.500	0.833	0.348	0.480	0.002	0.001	0.27
AN2	AN4	0.058	0.136	66.858	123.40	0.617	0.499	0.252	0.125	0.25
AN3	AN4	0.854	0.205	78.690	107.833	0.197	0.495	0.281	0.139	0.25
AN1	AN4	0.794	0.213	72.882	89.816	0.171	0.184	0.262	0.048	1.10

Table 3. Gene flow properties between different populations of *Anopheles barbirostris* calculated from nucleotide sequence of mtCOI gene. P1- Thailand, P2- India, P3-Srilanka, P4- China. (Hd=Haplotype diversity, Pi=nucleotide diversity, Kt=average number of nucleotide difference, Kxy= inter-population nucleotide differences between the populations, Gst= Genetic differentiation index based on the frequency of haplotypes, Fst= genetic distances among haplotypes, Dxy= average number of nucleotide substitutions per site between all the populations, Da= average no. of net nucleotide substitutions per site between all the populations, Nm= number of migration)

Population 1	Population 2	Hd	Pi	Kt	Kxy	Gst	Fst	Dxy	Da	Nm (Based on Fst)
P1	P2	0.839	0.198	65.930	93.433	0.169	0.539	0.281	0.151	0.21
P2	P3	0.757	0.234	65.990	115.175	0.281	0.849	0.409	0.348	0.04
P2	P4	0.774	0.224	77.792	125.00	0.292	0.749	0.360	0.269	0.08
P1	P4	0.942	0.368	160.100	209.66	0.065	0.499	0.481	0.240	0.25
P1	P3	0.923	0.191	70.456	74.466	0.096	0.153	0.202	0.031	1.38
P3	P4	0.830	0.276	60.566	97.944	0.273	0.746	0.447	0.334	0.08

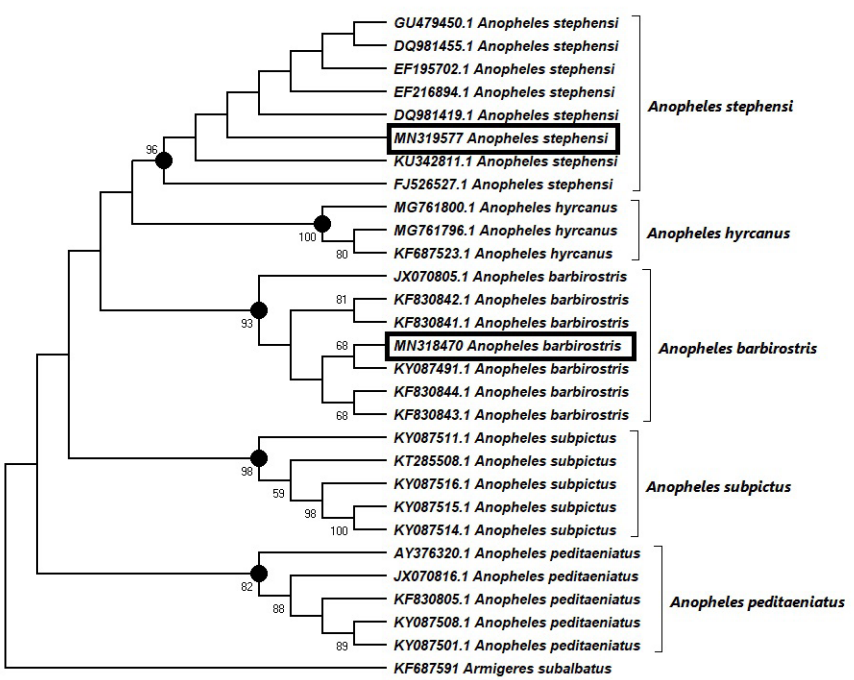


Fig. 6. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among 28s rDNA sequences and are in the units of the number of base substitutions per site. All the ambiguous position were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

DISCUSSION

Vector-borne diseases account for more than 17% of all infectious diseases, causing more than 7,00,000 deaths annually (WHO, 2020). The species *Anopheles stephensi* is a dominant urban vector for both *Plasmodium falciparum* and *Plasmodium vivax,* distributed throughout the geographical range of middle-east Indian subcontinent (Nalin et al, 1985; Sharma, 1995; Oshaghi, Yaaghoobi, & Abaie, 2006). *Anopheles barbirostris* is also reported as a vector of Japanese encephalitis in India (Tyagi, Munirathinam & Venkatesh, 2015). Therefore, the unequivocal identification of mosquito specimens is one of the backbones of mosquito and vector surveillance programs and therefore major attention should be given to their genetic diversity study. The combined use of genetic markers provides an idea about the population structure, genetic differentiation and gene flow of species, which is an essential component to design strategies for the management of vector-borne disease (Weeraratne, Surendran, Walton, & Karunaratne, 2018b).

Even though morpho-taxonomy is regarded as the gold standard method for discrimination of mosquito species, it seems to be quite difficult in the identification of field-collected mosquitoes as they may lose some of their important identifying features during handling. For this reason, there is a need for an alternative technique for identification especially in the case of cryptic and ambiguous species. Various other techniques were also available for mosquito species identification such as cytotaxonomy involving karyotyping of polytene chromosome (Tyagi et al, 2015), isozyme analysis (Knight & Nayar, 2004), next-generation sequencing approach (NGS) (Muturi, Dunlap, & Tchouassi, 2021), SNP barcoding with a decision tree algorithm based on machine-learning approach (Swain, Makunin, Dora, & Barik, 2019), deep learning technique (Mulchandani, Siddiqui, & Kanani, 2019), convolutional neural networks (CNNs) using image database (Goodwin et al, 2021) etc. Herein, we examined the accuracy of the integrated taxonomic tool using the geometric-morphometrics and DNA barcoding technique.

Geometric morphometric analysis based on quantitative characterization of mosquito wing venation has proved to be a reliable technique for identifying cryptic mosquito species (Morais, Moratore, Suesdek, & Marrelli, 2010; Lorenz, Marques, Sallum, & Suesdek, 2012) and also efficiently classify up to species level (Wilke et al, 2016). This was proved to be an effective tool for the rapid, inexpensive and reliable classification of six species of the *Aedes* genus in France (Martinet et al, 2021). The use of multiple gene markers coupled with landmark-based wing morphometric analysis of insect species including Lepidoptera and Diptera was evidenced from the various studies of Laparie, et al, (2016); Sontigun, et al, (2017); Jin, Hu, Han, & Chen, (2018). Earlier studies on morphological features of mosquito wings for the identification purpose were also reported by Sumruayphol et al, (2016); Lorenz, et al, (2017); Haarlem, & Vos, (2018). In the present study, PCA analysis compares two different Anopheline mosquito groups that segregate, forming two separate clusters. LM7, LM9, LM10, LM14 and LM18 showed the highest displacement along the transformation grid of both PC1 and PC2 which specifies that both species are separated from each

other at these four landmarks. Further, in DFA analysis between two species, the P-value is < 0.0001 which indicates that each group is significantly different from each other with high variation. Thus, our finding indicates that two epidemiologically critical mosquitoes *Anopheles stephensi* and *Anopheles barbirostris* were accurately recognized by utilizing wing shape through GM analysis.

DNA-based methodologies for mosquito identification (Manonmani et al, 2001; Kang, & Sim, 2013), molecular phylogeny (Shepard, Andreadis, & Vossbrinck, 2006) and genetic diversity (Pfeiler, Lopez Flores-Lopez, Mada-Velez, Escalante-Verdugo, & Markow, 2013) have gained increasing adoption in recent years as it is faster to perform and more reliable. Phylogenetic trees are utilized for analysis of gene duplication, estimating rates of diversification, polymorphism, recombination, population dynamics and inferring organismal phylogenies by combining it with other data sources.

Earlier studies have proved the use of the mitochondrial COI marker in finding more biodiversity and increasing species richness than traditional taxonomic approaches by uncovering undescribed and cryptic species (Hebert, Ratnasingham, &de Waard, 2003; Schmidt, Schmid-Egger, Morinière, Haszprunar, & Hebert, 2015; Wilson, Sing, Floyd, & Hebert, 2017). This COI gene as a molecular marker was used to infer the phylogeny of various dipteran taxa within the genera *Aedes, Anopheles* and *Culex* mosquitoes (Ashfaq et al, 2014; Weeraratne, Surendran, & Parakrama Karunaratne, 2018a; Chan-Chable, Martínez-Arce, Mis-Avila, & Ortega-Morales, 2019). Recently, a DNA barcode reference library was developed for identifying the mosquito species from Portuguese mosquito fauna, including the most significant vector species (Madeira, Duarte, Boinas, & Costa Osorio, 2021). In previous reports, rDNA such as ITS_2, D_3, and Mt DNA COII sequences were also used for differentiation of siblings from five species of Maculatus group (Ma, Li, & Xu, 2006) collected from China. Similarly, phylogenetic relationships among the Anopheline species was inferred using COI, COII, D3, ITS genes in various other regions of Odisha state of India which proved useful in properly defining species distribution and resolving the ambiguity that normally arises due to morphological taxonomy (Mohanty, Swain, Kar & Hazra, 2009). Therefore, multiple genes may be required as a supplementary marker to draw any conclusion. Thus, in addition to geometric morphometric analysis of wing shape variation, we employed DNA barcoding of COI, Cytb and D_3 domain of 28s rDNA sequences for mosquito species identification in the present study. We observed that the composition of generated COI sequences was AT-rich which is similar to the findings of Cywinska, Hunter, & Hebert, (2006); Rivera, & Currie, (2009) on dipterans. In this study, the mt COI-based analysis strongly supports the positioning of *Anopheles stephensi* and *Anopheles barbirostris* by forming distinct clusters with their respective group. Similarly, the phylogenetic repositioning with other two markers such as Cytb and 28s rDNA also supports the cladogenesis of both species. The clustering patterns agreed with the morphological identification, enable distinguishing the individual species based on both the studied gene sequences. Further, in a study, the genetic diversity and evolutionary relationships among *An. tessellatus* in nine Asian countries at the COI gene were described by Bourke, Wilkerson and Linton in 2021 which revealed

the exceptional levels of genetic diversity in populations across its known range and identify up to six putative species in the newly determined Tessellatus Complex. The existence of such cryptic diversity has potentially important consequences for vector management and disease control.

Population genetic study illustrated the occurrence of genetic differentiation within the population and the frequent exchange of genes among the population. Fang, et al, (2018) noticed a positive correlation between differences in genetic material among *Aedes albopictus* population with respect to their geographical distances and concluded that the genetic diversity might occur due to genetic mutation or due to ecological factors which accumulate genetic differences that may lead to reproductive isolation and gradually formation of a new species. We also analyzed the rates of gene flow of two different *Anopheles* mosquito species in the present study. When the gene flow Nm value is greater than 1, it means that the gene exchange is frequent, which can prevent the inter-population differentiation caused by genetic drift (Morton, 1977). It may be inferred from this study that due to geographical barriers and differences in climatic conditions between India and other studied regions except for Pakistan, there was a slight difference in COI gene sequences of *An. stephensi*. A frequent genetic exchange between Indian and Pakistan populations might be happening due to human activity or fewer physical barriers. However, the Nm value between different studied populations of *An. barbirostris* indicates, there might be less or no genetic exchange occurs between Indian with other populations might be due to different environmental and climatic conditions.

In conclusion, although *Anopheles stephensi* and *Anopheles barbirostris* are two different species that can be distinguished easily by using identification keys based on morpho-taxonomy. Our study proved here the efficiency of the combined use of wing geometric morphometric analysis and DNA barcoding approach for mosquito identification which can be utilized in the future for the identification of species that are difficult to distinguish. No doubt, only the COI gene as a molecular marker alone can confirm the species identification along with morpho-taxonomy and geomorphometric analysis but in the present study, we used multiple marker genes to validate the data to strengthen our findings. Furthermore, the result also validated the use of COI gene sequences for genetic flow analysis that helps to understand genetic diversity among geographically distant populations of different mosquito species.

ACKNOWLEDGMENTS

The first author would like to thank the Department of Science and Technology, Government of Odisha for financial support.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

REFERENCES

Adams, D.C., Rohlf, F.J., & Slice, D.E. (2004). Geometric morphometrics: ten years of progress following the revolution. *Italian Journal of Zoology,* 71(1), 5-16.

Amini, M., Hanafi-Bojd, A.A., Aghapour, A.A., & Chavshin, A.R. (2020). Larval habitats and species diversity of mosquitoes (Diptera: Culicidae) in West Azerbaijan Province, North western Iran. *BMC Ecology*, 20 (1), 60.

Ashfaq, M., Hebert, P.D., Mirza, J.H., Khan, A.M., Zafar, Y. & Mirza, M.S. (2014). Analyzing mosquito (Diptera: Culicidae) diversity in Pakistan by DNA barcoding. *Plos One,* 9**,** 263 e97268.

Barraud, P.J. (1934). *The fauna of British India including Ceylon and Burma. Diptera vol. V. family Culicidae Tribes Megarhinini and Culicini. Taylor & Francis*, London, United Kingdom.

Bourke, B., Wilkerson, R., & Linton, Y. (2021). Molecular species delimitation reveals high diversity in the mosquito *Anopheles tessellatus* Theobald, 1901 (Diptera, Culicidae) across its range. *Acta Tropica,* 215, 105799.

Chaiphongpachara, T., Sriwichai, P., Samung, Y., Ruangsittichai, J., Morales Vargas, R.E., Cui, L., Jetsumon, S., Dujardin, J.P., & Suchada, S. (2019). Geometric morphometrics approach towards discrimination of three member species of Maculatus group in Thailand. *Acta Tropica,*192, 66-74.

Chan-Chable, R.J., Martínez-Arce, A., Mis-Avila, P.C., & Ortega-Morales, A.I. (2019). DNA barcodes and evidence of cryptic diversity of anthropophagous mosquitoes in Quintana Roo, Mexico. *Ecology and Evolution,* 9, 4692-4705.

Christophers, S.R. (1933). *Family Culicidae Tribes Anophelini: The fauna of British India, including Ceylon and Burma- Diptera*. *Taylor & Francis, London*, 4, 1-271.

Collins, F.H., Mendez, M.A., Rasmussen, M.O., Mehaffey, P.C., Besansky, N.J., & Finnerty, V. (1987). A Ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene,*37, 37-41.

Cywinska, A., Hunter, F.F. & Hebert, P.D.N. (2006). Identifying Canadian mosquito species through DNA barcodes. *Medical & Veterinary Entomology*, 20, 413-424.

Dujardin, J.P., Kaba, D. & Henry, A.B. (2010). The exchangeability of shape. *BMC Research Notes,*3 (1), 266.

Fang, Y., Zhang, J., Wu, R., Xue, B., Qian, Q. & Gao B. (2018). Genetic polymorphism study on *Aedes albopictus* of different geographical regions based on DNA barcoding. *Biomed Research International,* 1-10.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,*39,783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology,* 3, 294-299.

Gonzalez, R., Carrejo, N., Wilkerson, R.C., Alarcon, J., Alarcon-Ormasa, J., Ruiz, F., Bhatia, R., Loaiza, J., & Linton, Y.M. (2010). Confirmation of *Anopheles* (Anopheles) *calderoni* Wilkerson, 1991 (Diptera: Culicidae) in Colombia and Ecuador through molecular and morphological correlation with topotypic material. *Memorias do Instituto Oswaldo Cruz,*105,1001-1009.

Goodwin, A., Padmanabhan, S., Hira, S., Glancey, M., Slinowsky, M, Immidisetti, R., Scavo, L., Brey, J., Sudhakar, B.M.M.S., Ford, T., Heier, C., Linton, Y.M., Pecor, D.B., Quiroga, L.C., & Acharya, S. (2021). Mosquito species identification using convolutional neural networks with a multitiered ensemble model for novel species detection. *Scientific Reports,* 11, 13656.

Haarlem, C. & Vos, R. (2018, Sept 7). *Inspecting morphological features of mosquito wings for identification with image recognition tools*. doi: http://dx.doi.org/10.1101/410449. Retrieved from https://www.biorxiv.org/content/biorxiv/early/2018/09/07/410449.full.pdf

Harbach, R.E. (2021, March 1). *Mosquito taxonomic inventory*. Retrieved from http:// mosquito-taxonomic-inventory.info.

Hebert, P.D., Ratnasingham, S., & de Waard, J.R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 270, 96-99.

Jin, Q., Hu, X., Han, H., & Chen, F. (2018). A two-step DNA barcoding approach for delimiting moth species: moths of Dongling Mountain (Beijing, China) as a case study. *Scientific Reports,*8, 14256.

Joshi, J. & Agarwal, I. (2021). Integrative taxonomy in the Indian subcontinent: current progress and prospects. *Journal of the Indian Institute of Science,* 101, 125-149.

Kang, D. & Sim, C. (2013). Identification of Culex complex species using SNP markers based on high-resolution melting analysis. *Molecular Ecology Resources*, 13(3), 369-76.

Klingenberg, C.P. (2011). Morphoj: An integrated software package for geometric morphometrics. *Molecular Ecology Resources*,11, 353-357.

Knight, J., W., & Nayar, J., K, (2004). Identification of four common Culex (Culex) (Diptera: Culicidae) species from florida with isozyme analysis. *Florida Entomologist,* 87(1), 1-5.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution, 35,* 1547-1549.

Laboudi, M., Faraj, C., Sadak, A., Harrat, Z., Boubidi, S.C., Harbach, R.E., Aouad, R.E., & Linton, Y.M. (2011). DNA barcodes confirm the presence of a single member of the *Anopheles maculipennis* group in Morocco and Algeria: *An. sicaulti* is conspecific with *An. labranchiae*. *Acta Tropica,*118, 6-13.

Laparie, M., Vernon, P., Cozic, Y., Frenot, Y., Renault, D., & Debat, V. (2016). Wing morphology of the active flyer *Calliphoravicina* (Diptera: Calliphoridae) during its invasion of a sub-Antarctic archipelago where insect flightlessness is the rule. *Biological Journal of the Linnean Society*, 119, 179-183.

Lin, C.P. & Danforth, B.N. (2004). How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. *Molecular Phylogeny and Evolution*, 30(3), 686-702.

Lorenz, C., Almeida, F., Almeida-Lopes, F., Louise, C., Pereira, S. N., Petersen, V., Vidal, P.O., Virginio, F., & Suesdek, L. (2017). Geometric morphometrics in mosquitoes: What has been measured? *Infection, Genetics and Evolution*, 54, 205-215.

Lorenz, C., Marques, T., Sallum, M.A., & Suesdek, L. (2012). Morphometrical diagnosis of the malaria vectors *Anopheles cruzii*, *An. homunculus* and *An. bellator*. *Parasites & Vectors*, 5, 257.

Lyman, D.F., Monteiro, F.A., Escalante, A.S., Cordon-Rosales, C., Wesson, D.M., Dujardin, J., & Beard, C.B. (1999). Mitochondrial DNA sequence variation among Triatomine vectors of Chargas' disease. *American Journal of Tropical Medicine and Hygiene,* 60(3), 377-386.

Ma, Y., Li, S. & Xu, J. (2006). Molecular identification and phylogeny of the Maculatus group of Anopheles mosquitoes (Diptera: Culicidae) based on nuclear and mitochondrial DNA sequences. *Acta Tropica*, 99, 272-280.

Madeira, S., Duarte, A., Boinas, F., & Costa Osório, H. (2021). A DNA barcode reference library of Portuguese mosquitoes. *Zoonoses and Public Health*, 00, 1-11.

Manonmani, A., Townson, H., Adeniran, T., Jambulingam, P., Sahu, S., & Vijayakumar, T. (2001). rDNA-ITS2 polymerase chain reaction assay for the sibling species of *Anopheles fluviatilis*. *Acta Tropica,*78,3-9.

Martinet, J.P., Ferte, H., Sientzoff, P., Krupa, E., Mathieu, B., & Depaquit, J. (2021). Wing Morphometrics of Aedes Mosquitoes from North-Eastern France. *Insects*, 12, 341.

Mohanty, A., Swain, S., Kar, S.K, & Hazra, R.K. (2009). Analysis of the phylogenetic relationship of Anopheles species, subgenus Cellia (Diptera: Culicidae) and using it to define the relationship of morphologically similar species. *Infection, Genetics and Evolution,* 9, 1204-1224.

Mondal, R., Devi, N.P., & Jauhari, R.K. (2015). Landmark-based geometric morphometric analysis of wing shape among certain species of Aedes mosquitoes in District Dehradun (Uttarakhand). *Journal of Vector Borne Diseases.* 52, 122-128.

Morais, S.A. de, Moratore, C., Suesdek, L., & Marrelli, M.T. (2010). Genetic-morphometric variation in *Culex quinquefasciatus* from Brazil and La Plata, Argentina. *Memórias do Instituto Oswaldo Cruz,*105, 672-676.

Morales Vargas, R.E., Phumala-Morales, N., Tsunoda, T., Apiwathnasorn, C., & Dujardin, J.P. (2013). The phenetic structure of *Aedes albopictus*. *Infection, Genetics and Evolution*, 13, 242-251.

Morton, N.E. (1977). Isolation by distance in human populations. *Annals of Human Genetics*, 40 (3), 361-365.

Mulchandani, P., Siddiqui, M.U. ,& Kanani, P. (2019). Real-Time mosquito species identification using Deep learning techniques. *International Journal of Engineering and Advanced Technology,* 2249-8958.

Muturi, E.J., Dunlap, C., Tchouassi, D.P., & Swanson, J. (2021). Next generation sequencing approach for simultaneous identification of mosquitoes and their blood- meal hosts. *Journal of Vector Ecology,* 46(1), 116-121.

Nalin, D.R., Mahood, F., Rathor, H., Muttab, A., Sakai., R., & Chowdhary, M.A. (1985). A point survey of periurban and urban malaria in Karachi. *The Journal of Tropical Medicine and Hygiene,* 88, 7-15.

National Vector Borne Disease Control Program (NVBDCP), S*tate wise number of Malaria Cases and Deaths from 2017-2021* (2021, September 23). Retrieved from https://nvbdcp.gov.in/WriteReadData/l892s/41082350651617959011.pdf.

Oshaghi, M.A., Yaaghoobi, F., & Abaie, M.R. (2006). Pattern of mitochondrial DNA variation between and within *Anopheles stephensi* (Diptera: Culicidae) biological forms suggests extensive gene flow. *Acta Tropica,* 99, 226-33.

Pfeiler, E., Lopez Flores-Lopez, C.A., Mada-Velez, J.G., Escalante-Verdugo, J. & Markow, T.A. (2013). Genetic Diversity and Population Genetics of Mosquitoes (Diptera: Culicidae: *Culex* spp.) from the Sonoran Desert of North America. *The Scientific World Journal*, 1-11.

Pradhan, A., Anasuya, A., Pradhan, M.M., Kavitha A.K., Kar P., Sahoo K.C., Panigrahi P., & Dutta A (2016). Trends in Malaria in Odisha, India-an analysis of the 2003-2013 time-series data from the national vector borne disease control program. *Plos One*, 11(2), e0149126-e0149126.

Pramual, P., Wongpakam, K., & Adler, P.H. (2011). Cryptic biodiversity and phylogenetic relationships revealed by DNA barcoding of Oriental black flies in the subgenus Gomphostilbia (Diptera: Simuliidae). *Genome*, 54,1-9.

Rivera, J. & Currie, D. (2009). Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). *Molecular Ecology Resources*, *9,* 224-236.

Rohlf, F.J. (2003). *Tps regr, shape regression, version 1.28*. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F.J. (2004). *Tps dig. department of ecology and evolution state university of New York*, Stony Brook, NY.

Rohlf, F.J. (2006). *Tps series*. Department of Ecology and Evolution, State University of New York, Stony Brook, NY.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA Sequence Polymorphism Analysis of Large Datasets. *Molecular Biology Evolution,* 34, 3299-3302.

Ruiz-Lopez, F., Wilkerson, R.C., Conn, J.E., McKeon, S.N., Levin, D.M., Quiñones, M.L., Póvoa, M.M., & Linton, Y.M. (2012). DNA barcoding reveals both known and novel taxa in the Albitarsis Group (Anopheles:Nyssorhynchus) of Neotropical malaria vectors. *Parasites & Vectors*, 5, 44.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution, 4,* 406-425.

Schmidt, S., Schmid-Egger, C., Morinière, J., Haszprunar, G., & Hebert, P.D. (2015). DNA barcoding largely supports 250 years of classical taxonomy: identifications for Central European bees (Hymenoptera, Apoidea partim). *Molecular Ecology Resources,*15, 985-1000.

Sharma, R.S. (1995). Urban malaria and its vectors *Anopheles stephensi* and *Anopheles culicifacies* Diptera: Culicidae) in Gurgaon, India. *Southeast Asian Journal of Tropical Medicine and Public Health*, 26,172-176.

Shepard, J.J., Andreadis, T.G., & Vossbrinck, C.R. (2006). Molecular Phylogeny and Evolutionary Relationships among Mosquitoes (Diptera: Culicidae) from the Northeastern United States Based on Small Subunit Ribosomal DNA (18S rDNA) Sequences. *Journal of Medical Entomology,* 43(3), 443-454.

Singh, O.P., Chandra, D., Nanda, N., Raghavendra, K., Sunil, S., Sharma, S.K., Dua, V.K., & Subbarao, S.K. (2004). Differentiation of members of the *Anopheles fluviatilis* species complex by an Allele-specific polymerase chain reaction based on 28s ribosomal DNA sequences. *American Journal of Tropical Medicine and Hygiene,* 70(1), 27-32.

Sontigun, N., Sukontason, K.L., Zajac, B.K., Zehner, R., Sukontason, K., Wannasan, A. & Amendt, J. (2017). Wing morphometrics as a tool in species identification of forensically important blow flies of Thailand. *Parasites & Vectors*,10, 229.

Stahls, G., Vujic, A., & Perez-Banon, C. (2009). COI barcodes for identification of Merodon hoverflies (Diptera, Syrphidae) of Lesvos Island, Greece.*Molecular Ecology Resources*,9, 1431-1438.

Sumruayphol, S., Apiwathnasorn, C., Ruangsittichai, J., Sriwichai, P., Attrapadung, S., Samung, Y., & Dujardin, J.P. (2016). DNA barcoding and wing morphometrics to distinguish three Aedes vectors in Thailand. *Acta Tropica*, 159, 1-10.

Swain, S.N., Makunin, A., Dora, A.S., & Barik, T.K. (2019). SNP barcoding based on decision tree algorithm: A new tool for identification of mosquito species with special reference to *Anopheles*. *Acta Tropica*, 199, 105152.

Trent, R.J. (2005). Infectious diseases: *Molecular medicine.* 3rd ed., 193-220. https://doi.org/10.1016/B978-012699057-7/50008-4

Tyagi, B., Munirathinam, A., & Venkatesh, A. (2015). A catalogue of Indian mosquitoes. *International Journal of Mosquito Research,* 50, 50-97.

Tyagi, V., Sharma, A.K., Yadav, R., Sukumaran, D., Agrawal, O.P., & Veer, V. (2015). Cytotaxonomic examination for identification of *Anopheles culicifacies* sibling species in central India. *Journal of Mosquito Research,* 20(5), 1-7.

Vidal, P.O. & Suesdek, L. (2012). Comparison of wing geometry data and genetic data for assessing the population structure of *Aedes aegypti. Infection, Genetics and Evolution*, 12, 591-596.

Weeraratne, T.C., Surendran, S.N., & Karunaratne, S.H.P.P. (2018a). DNA barcoding of morphologically characterized mosquitoes belonging to the subfamily Culicinae from Sri Lanka. *Parasites Vectors*, 11, 266.

Weeraratne, T.C., Surendran, S.N., Walton, C., & Karunaratne, S.H.P.P. (2018b). Genetic diversity and population structure of malaria vector mosquitoes *Anopheles subpictus, Anopheles peditaeniatus* and *Anopheles vagus* in five districts of Sri Lanka. *Malaria Journal,*17, 271.

Wilke, A.B.B., de Oliveira Christe, R., Multini, L.C., Vidal, P.O., Wilk-da-Silva, R., d Carvalho, G.C., & Marrelli, M.T. (2016). Morphometric wing characters as a tool for mosquito identification. *Plos One,* 11(8), e0161643.

Wilson, J.J., Sing, K.W., Floyd, R.M., & Hebert P.D. (2017). DNA barcodes and insect biodiversity. In R. G. Foottit, & P. H. Adler (Eds.). *Insect biodiversity: science and society* (pp 575-592), Chapter 17, John Wiley & Sons, United States.

World Health Organization (WHO) (2021, Sept 20). Vector-borne diseases. Retrieved from https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases.

World Health Organization (WHO) (2021, Sept 20). Retrieved from https://www.who.int/news-room/fact-sheets/detail/malaria.

Received: February 25, 2021 *Accepted: October 05, 2021*

J. Entomol. Res. Soc., 23(3): 257-277, 2021
Doi: 10.51963/jers.v23i3.2054

Research Article
Online ISSN:2651-3579

Ethology of *Holcocephala calva* (Loew, 1872) (Diptera: Asilidae) in Northeastern Florida, U.S.A.

D. Steve DENNIS †

1105 Myrtle Wood Drive, St. Augustine, Florida 32086-4838, U.S.A.
e-mail: dstevedennis@msn.com
ORCID ID: 0000-0002-4832-4026

ABSTRACT

Holcocephala calva (Loew, 1872) forages primarily from dead twig tips, capturing and immobilizing prey in flight or in a hover near the foraging/feeding site. Identified prey is in eight orders (Araneae, Blattodea (Isoptera), Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Psocoptera), with Hymenoptera (42.3%) and Diptera (33.1%) making up 75.4%. Mating occurs in the tail-to-tail position. Females oviposited by dropping one egg at a time onto the ground. *Holcocephala calva* exhibit a daily rhythm of activity with peak periods between 6:00-10:00 AM of foraging, male searching flights for females with which to mate, mating, and ovipositing. Grooming occurs throughout the day. The most common grooming type consisting of the hind tarsi and tibiae being rubbed together and one or both hind tarsi and tibiae being used to stroke the sides of the abdomen. Habitat, flight patterns, daytime and nighttime resting behavior, light levels and vision, and predators and parasites also are discussed.

Key words: behavior, robber flies, Diptera, Asilidae.

Dennis, D. S. (2021). Ethology of *Holcocephala calva* (Loew, 1872) (Diptera: Asilidae) in Northeastern Florida, U.S.A. *Journal of the Entomological Research Society*, 23(3), 257-277.

INTRODUCTION

Holcocephala is predominantly a genus of Neotropical robber flies with 40 described species (Carrera, 1958; Geller-Grimm, 2021 January 4). There are three Nearctic species: *H. abdominalis* (Say, 1823), *H. calva* (Loew, 1872), and *H. fusca* Bromley, 1951. These three species are usually found in mesic habitats such as along margins of streams, fields, and woods (Scarbrough, 1982) and wet meadows. They occur from the eastern United States west to eastern Texas, Kansas and Nebraska (Fisher & Wilcox, 1997; Martin & Wilcox, 1965).

Some behavior and habitat information has been published on all three Nearctic species: *H. abdominalis* (Baker & Fischer, 1975; Blanton, 1939; Brimley, 1922; Bromley, 1931, 1946, 1950; Johnson, 1976; McAtee & Banks, 1920; Scarbrough, 1974, 1982); *H. calva* (Baker & Fischer, 1975; Bromley, 1931; McGravy & Baxa, 2011; Scarbrough, 1974, 1982); and *H. fusca* (Dennis, 1979; Douglass & Wehling, 2016; Fabian, Sumner, Wardill, Rossoni, & Gonzalez-Bellido, 2018; Wardill, Fabian, Pettigew, Stavenga, Nordström & Gonzalez-Bellido, 2017).

This paper provides information on the ethology of *H. calva* in the Moses Creek Conservation Area (MCCA) in St. Augustine (St. Johns County) in northeastern Florida. The main behaviors described are flight patterns, resting, foraging and feeding, mating, ovipositing, grooming, and daily rhythm of activity. Habitat, prey, light levels and vision, and predators and parasites are also discussed.

MATERIALS AND METHODS

In the MCCA, *H. calva* was studied from 22.04.2020-24.12.2020; and 09.03.2021-14.04.2021. Observations involved an average of 7 individuals per day, each for up to 7 hours. Total number of hours of observation equaled approximately 146.

Holcocephala calva was studied by the author standing and observing single flies for as long as possible in order to collect information on their diurnal activities. Additionally, the author would slowly walk around a study area and observe the specific activities of individual flies, primarily in order to collect prey and to locate mating pairs and ovipositing females. Pentax Papilio 8.5x21 binoculars were used to observe flies so as not to disturb them.

Most prey were collected when prey were dropped into a net placed beneath the feeding robber flies that had ceased to feed. Collected prey were placed in glass vials with labels indicating the date, time, and location. It was difficult to accurately determine the sex of the robber flies because of their height on vegetation, and so this was not recorded. After prey were pinned, they were examined in the laboratory with a Wild Heerbrugg M8 stereomicroscope with an incident light base, a 1.6x objective, and a 20x focusing eyepiece for magnifications up to 160x. The eyepiece was equipped with a 5 mm/100 division reticle for measuring the prey. The eyepiece reticle was calibrated using a dual axis 1 mm/100 division/0.01 mm and linear 50 mm/500 division/0.1 mm multi-function scale/stage micrometer.

While in the field, a hand-held Cooper-Atkins DPP400W Digital Thermometer was used to take air temperatures. A Dwyer Hand-Held Wind Meter measured wind speed. Also a hand held UYIGAO Digital Light Meter (UA-962) measured light levels in lux, the metric International System of Units (SI). One lux equals one lumen/square meter and 0.0929 foot candle in the Imperial System.

RESULTS AND DISCUSSION

Morphology

Holcocephala calva is a small, 7.5-9.4 mm (n=20; 10 males and 10 females) long, blackish-gray species. Its head is dark brown to black with a sparse, white mystax that is confined to the oral margin. Head broader than thorax and compressed anterio-posteriorly. The thorax is white and arched, in particular the anterior 1/2, and the scutum is dark gray to black. The scutellum has a grayish pollinose margin without bristles. The wings are covered with black microtrichiae and the veins are black. The legs are dark reddish yellow to reddish brown, and the femora and tibiae are darker distally. The abdomen is brownish with distinct grayish bands on the posterior margins of the first three segments, which are also constricted. The female terminalia do not have apical spines or acanthophorites.

Habitat

Holcocephala calva was found at three locations in the MCCA: (1) along an electrical transmission line (corridor/road (Red Trail)) in the Moses Creek floodplain and along the tree line between the floodplain and the mesic flatwoods vegetation community; (2) along the edge of a floodplain of a perennial unnamed tributary northwest of Moses Creek between roller chopped sandhill and scrubby flatwoods communities; and (3) west of Hidden Creek along a road (Yellow Trail) in an upland mixed forest community. The vegetation communities are shown in Table 1. In all three habitats a number of individuals (10-14) were found close together, within 20-45 cm of each other. Bromley (1946), Johnson (1976), LaPierre (2000), and Scarbrough (1982) also commented on large numbers of *Holcocephala* congregating in small areas.

The habitat in the Moses Creek floodplain (Fig. 1) extends approximately 54.7 m to the north of the Creek along the tree line with the approximate 33.8 m wide transmission line corridor to the east and forest immediately to the west. The dominant vegetation is groundsel tree (*Baccharis halimifolia (L.)*), sweetgum (*Liquidambar styraciflua* L.), southern bayberry/wax myrtle (*Morella* (*Myrica*) *cerifera* (L.) Small), dahoon holly (*Ilex cassine* L. var. *cassine*), saw palmetto (Arecaceae, *Serenoa repens* (W. Bartram)), and red maple (*Acer rubrum* L.). After heavy rains, some of the vegetation hangs over or is resting in 10-15 cm deep water.

The habitat 7.6 m to the east of the unnamed tributary to Moses Creek is in an open area approximately 20.9 m^2 in size surrounded by taller trees and other vegetation. The dominant vegetation in the habitat is muscadine vine (*Vitis rotundifolia* Michx.), common buttonbush (*Cephalanthus occidentalis* (L.)), dahoon holly, and southern bayberry/wax myrtle.

Fig. 1. *Holcocephala calva* habitat along road in Moses Creek floodplain (Photograph: D.S. Dennis, 29 May 2020, 8:52 AM).

Table 1. Vegetation communities in which *Holcocephala calva* was studied in the Moses Creek Conservation Area.

Vegetation Type	Vegetation Community		
Family/Genus/Species/Common Name	Moses Creek Floodplain Marsh and Along Edge of Mesic Flatwoods	Perennial Unnamed Tributary to Moses Creek	West of Hidden Creek Along Road in Upland Mixed Forest
Altingiaceae			
Liquidambar styraciflua L./ Sweetgum	X[1]	—[2]	X
Aquifoliaceae			
Ilex cassine (L.) var. *cassine*/ Dahoon	X	X	X
Ilex glabra (L.) A. Gray/Gallberry	—	—	X
Ilex opaca Alton var. *opaca* / American holly	—	X	—
Arecaceae			
Sabal palmetto (Walter) Lodd. ex Schult. & Schult. f./Cabbage palm	—	X	—
Serenoa repens (W. Bartram) Small/ Saw palmetto	X	X	X
Asteraceae			
Baccharis halimifolia (L.)/Groundsel tree; sea myrtle	X	—	X
Coreopsis leavenworthii Torr. & A. Gray/ Leavenworth's tickseed	X	—	—
Erechtites hieraciifolius (L.) Raf. Ex DC./American burnweed	X	—	X
Eupatorium capillifolium (Lam.) Small ex Porter & Britton/Dogfennel	X	X	X
Symphyotrichum sp./Aster	—	—	X

Table 1. Continued.

Vegetation Type	Vegetation Community		
Family/Genus/Species/Common Name	Moses Creek Floodplain Marsh and Along Edge of Mesic Flatwoods	Perennial Unnamed Tributary to Moses Creek	West of Hidden Creek Along Road in Upland Mixed Forest
Cupressaceae			
Taxodium ascendens Brongn./ Pond-cypress	X	—	X
Cyperaceae			
Cyperus spp./Flatsedges	X	X	—
Dennstaedtiaceae			
Pteridium aquilinum L. (Kuhn) var. *pseudocaudatum* (Clute) Clute ex A. Heller/Tailed bracken	—	—	X
Ericaceae			
Lyonia ferruginea (Walter) Nutt./ Rusty lyonia	—	—	X
Vaccinium myrsinitas Lam./Shiny blueberry	—	—	X
Euphorbiaceae			
Triadica sebifera (L.) Small/ Popcorntree; Chinese tallowtree	X	—	X
Fabaceae			
Sesbania herbacea (Mill.) McVaugh/ Danglepod	X	—	—
Sesbania sericea (Wild.) Link/ Silky sesban	X	—	—
Fagaceae			
Quercus geminate Small/Sand live oak	—	—	X
Quercus laurifolia Michx./Laurel oak	X	—	X
Quercus myrtifolia Willd./Myrtle oak	X	X	X
Quercus nigra L./Water oak	—	—	X
Gelsemiaceae			
Gelsemium sempervirens (L.) W.T. Alton/Yellow (Carolina) jessamine	X	—	X
Lauraceae			
Persea borbonia (L.) Spreng. var *borbonia*/Red bay	X	—	X
Magnoliaceae			
Magnolia virginiana L./Sweetbay	—	X	—
Myricaceae			
Morella (*Myrica*) *cerifera* (L.) Small/ Southern bayberry; wax myrtle	X	X	X
Osmundaceae			
Osmundastrum cinnamomeum (L.) C. Presl/ Cinnamon fern	—	X	X

Table 1. Continued.

Vegetation Type	Vegetation Community		
Family/Genus/Species/Common Name	Moses Creek Floodplain Marsh and Along Edge of Mesic Flatwoods	Perennial Unnamed Tributary to Moses Creek	West of Hidden Creek Along Road in Upland Mixed Forest
Osmundaceae			
Osmundastrum cinnamomeum (L.) C. Presl/ Cinnamon fern	—	X	X
Pinaceae			
Pinus elliotti Engelm./Slash pine	—	—	X
Pinus serotina Michx./Pond pine	X	X	—
Poaceae			
Andropogon glomeratus (Walter) Britton et al. var *glomeratus*/Bushy bluestem	—	—	X
*Andropogon sp./B*luestem	X	—	—
Eustachys glauca Chapm./ Saltmarsh fingergrass	X	—	—
Setaria sp./Foxtail	X	—	X
Pontederiaceae			
Pontederia cordata L./Pickerelweed	X	—	—
Rosaceae			
Rubus cuneifolius Pursh/Sand blackberry	X	—	—
Pontederia cordata L./Pickerelweed	X	—	—
Rubiaceae			
Cephalanthus occidentalis (L.)/ Common buttonbush	—	X	—
Sapindaceae			
Acer rubrum L./Red maple	X	X	—
Smilacaceae			
Smilax auriculata Walter/Earleaf greenbrier vine	—	—	X
Smilax bona-nox L./Saw greenbrier vine	X	X	—
Smilax laurifolia L./Laurel greenbrier vine	X	—	X
Viburnaceae			
Viburnum obovatum Walter/Small leaf arrow wood; Walter's viburnum			
Vitaceae			
Vitis rotundifolia Michx./ Muscadine grape vine	X	X	X

1- present; 2 - not present

The habitat west of Hidden Creek consists of an open area north of the road, approximately 50.9 m^2 in size, 15.8 m from the Creek, and an area along the southern edge of the road that extends from approximately 3.7-34.7 m from the Creek. The dominant vegetation in the open area is muscadine vine, Chinese tallow tree (*Tridica sebifera* (L.) Small), bushy bluestem (*Andropogon glomeratus* (Walter) Britton et al.), and dog fennel (*Eupatorium capillifolium* (Lam.) Small ex Porter & Britton). Along the southern edge of the road the dominant vegetation is sweetgum, southern bayberry/ wax myrtle, muscadine vine, earleaf greenbrier vine (*Smilax auriculata* Walter), and laurel greenbrier vine (*S. laurifolia* L.).

Holcocephala calva are most frequently found on dead twig tips of muscadine vine, dahoon holly, groundsel tree, sweetgum, southern bayberry/wax myrtle and Chinese tallow tree. However, a few individuals would briefly land on muscadine vine leaves and tips of live vine tendrils.

Hull (1962), commented that *Holcocephala* are "…frequently abundant in swamp areas where they are found on the tips of twigs and leaves among rank undergrowth." Brimley (1922) noted that *H. abdominalis* in North Carolina can be found "… in rank herbage in damp shady places…" The term rank indicates grassland or marsh vegetation that has grown abundantly, without being cut or grazed for some time.

McGravy & Baxa (2011) found *H. calva* in an oak-hickory forest that had not been burned for at least 5 years. Bromley (1931) observed that *H. calva* is generally associated with *H. abdominalis* "…in rather damp situations, alighting on the tips of twigs and grasses." Bromley (1946, 1950) also noted that *H. abdominalis* can be found in low country or moist edges of meadows or low woodland landing on the tips of dead twigs or grass with a number of individuals occurring on the same plant or low shrub.

Back (1909) found *H. abdominalis* in damp grass areas clinging to grass stems. McAtee and Banks (1920) said that *H. abdominalis* is "…usually seen perched on tips of grass blades in damp situations," and Blanton (1939) found this species at the edge of a field and near woods on the dead stems of yankeeweed (as dog fennel; Asteraceae, *Eupatorium compositifolium* Walter). Johnson (1976) studied *H. abdominalis* in a field surrounded by woods. Dennis (1979) found *H. fusca* on the edge of a forested area next to the lawn associated with apartment buildings.

Where *H. calva* and *H. abdominalis* have been found occupying the same habitat, the former is usually less abundant than the latter (McAtee & Banks, 1920; Bromley, 1931; Baker & Fischer, 1975; Scarbrough, 1982).

Fisher (2009) indicated that in Central America *Holcocephala* perch on twig and plant tips and "…show distinct preferences in forest type and light exposure. Some *Holcocephala* species prefer open, sunny habitats, such as edges of forest or sparse second-growth vegetation. Other species are only found in more closed habitat, such as mature forest, including the shaded forest understory."

Flight patterns

Robber fly flight patterns have been classified as orientation, investigatory, foraging, and searching flights (Dennis & Lavigne, 1975). Orientation flights are often short

flights around a robber fly's location to change its position or field of vision and are not directed towards potential prey. Investigatory flights are directed toward potential prey without the robber fly making contact. Because *H. calva* prey are difficult to see by a human observer, it is believed that many orientation flights are investigatory flights, and it is not possible to distinguish between the two and so they are discussed together.

Foraging flights occur when an individual makes contact with potential prey. Searching flights are when males search for receptive females with which to mate, with the males often weaving in and out of the vegetation.

Holcocephala calva orientation/investigatory and foraging flights are also discussed below under Foraging and Feeding Behavior. Searching flights are discussed under Courtship and Mating Behavior.

Daytime and nighttime resting behavior

During the daytime *H. calva* rests and forages from 1-3 mm diameter twig tips 32.5 cm to 4.65 m (average 1.8 m; n=80) above the ground. Individuals are active in both sun and shade, although their habitats are in the shade of surrounding vegetation most of the day, in particular along the unnamed tributary to Moses Creek and near Hidden Creek. Bromley (1950) said that H. *calva* "rests on tips of twigs of bushes and small trees." When their habitats are in the sun, most *H. calva* rest and forage with their backs to the sun.

Other species of *Holcocephala* are generally found on vegetation at heights from 20 cm to 5 m above the ground (Dennis, 1979; Johnson, 1976; Scarbrough, 1982). Johnson (1976) observed *H. abdominalis* moving to lower levels (10-30 cm above the ground) in vegetation as temperatures decreased later in the flight season. Scarbrough (1982) also found that *H. abdominalis* foraged from perches on lower vegetation than did *H. calva*.

When *H. calva* is ready to "rest," they back down or crawl around to the top, side or underside (Fig. 2) of the branch 1-30 cm (average 5 cm; n=53) from the tip and hold their bodies close to and parallel to the branch. They rest in one location for an average of 7.7 minutes (range 1/2-40 minutes; n=22) before moving to another location to rest or resume foraging. One individual moved nine times over a period of 1 hour and 31 minutes. While resting, individuals are motionless or move very little and do not react to other insects that fly around them.

Many species of robber flies maintain their body temperature by changing their position in relation to the sun, resting on the shady side of vegetation, or flattening themselves against the substrate they are on (Dennis, 2018; Dennis & Lavigne, 1975; Lavigne & Holland, 1969). A few *H. calva* move to the shade of the twig they are on when the sun shines on them and then move back to the top of the branch when they are no longer in the sun. Also, some individuals turn their right or left sides to the sun.

When resting and/or feeding, a number of *H. calva* expel a drop of creamy-white to shiny white liquid from their anus. Lehr (1958) commented that the expulsion of liquid from the anal opening is quite common in robber flies.

Fig. 2 *Holcocephala calva* in daytime and nighttime resting position (Photograph: D.S. Dennis, 3 July 2020, 1:39 PM).

Some *H. calva* spend the night on the same twigs where they rest and forage during the daylight hours. However, in the unnamed tributary to Moses Creek habitat most move down to slightly lower twigs 1.5-1.9 m above the ground. At night individuals are usually positioned on the top of or underneath the twig 1-17.5 cm (average 5.4 cm; n=11) from the tip, with their bodies held flat against the twig. They remain in this position until approximately 45-60 minutes after sunrise when they stand up and often groom their hind tarsi and tibiae before they start daily activities.

Dennis (1979) observed *H. fusca* spending the night on twig tips or flattened against the top or side of a twig 1-3.5 cm from the tip.

Because the *H. calva* habitats are in limited areas surrounded by vegetation, there is usually very little wind to disturb the resting and foraging individuals and the wind was typically calm to less than 3.2 km/hr.

Foraging and feeding behavior

When foraging, *H. calva*, (1) stand up on or near a twig tip with their bodies parallel to the twig or at a 30-45º angle with their fore and mid legs extended and the tips of their abdomen often touching the twig, or (2) hang vertically from the twig tip with their head and at least one-half of their thorax above the twig tip (Fig. 3). They usually face open areas and forage from one position/location for an average of 69.6 minutes (range from 2 minutes to 7 hours; n=29). Thus, they capture prey that are usually outlined or backlit against a blue or bright sky. Scarbrough (1982) observed similar foraging behavior for both *H. calva* and *H. abdominalis*, although the latter foraged more frequently in sunlight. The majority of *H. fusca* prey were captured in sunlit areas (Dennis, 1979).

Female *H. calva* appear to remain on the same twig tips for longer periods of time than do males and they also may forage from the same twig tip for several days. It is assumed that males move around more because they are performing searching flights for receptive females with which to mate.

Holcocephala calva make orientation/investigatory flights within 3.75-37.5 cm (average 21 cm; n=26) of their perch and usually return to the same perch. These

flights are made all around and generally at the same level as their perch. Only three orientation/investigatory flights were made 7.5-12.5 cm below their perch.

Fig. 3. *Holcocephala calva* in foraging position (Photograph: D.S. Dennis, 12 June 2020, 12:10 PM).

Ten foraging flights were observed when prey was captured in flight and released, usually in a hover adjacent to or slightly below the foraging perch while the individual manipulated the prey with all six tarsi. There was only one prey capture and release without the individual hovering and manipulating its potential prey. Seven of these prey capture and release flights were made around and at the same level as the foraging perch, within an average 21.8 cm (range 15-37.5 cm) of the perch. Two flights were in front of and 7.5 and 10 cm below the perch, and one flight was straight up 12.5 cm above the perch. After releasing the discarded prey, two individuals held their wings open to a 45º angle as if "frustrated" that they had not captured an acceptable prey. One individual also immediately re-captured the prey that it had just released and again discarded it. Sometimes *Holopogon phaeonotus* Loew, 1874 exhibits similar wing spreading when captured prey are not acceptable (Dennis, 2014).

Based on 54 prey captures, *H. calva* captured 36 of its prey at the same level and 5-45 cm (average 21.3 cm) around its foraging position. Ten prey were captured 20-38 cm (average 27.8 cm) in front of or to the right or left, and 6.3-45 cm (average 19.8 cm) above or below the foraging position. Eight prey were captured 8.8-45 cm (average 19.5 cm) straight above the individuals foraging position. Scarbrough (1982) said that *H. calva* and *H. abdominalis* capture over approximately 88% of their prey between 15-100 cm in front of and to one side or the other of their foraging sites. *Holcocephala fusca* successfully captured its prey within 12.5-30 cm in front of and to the side of its foraging position (Dennis, 1979).

Wardill et al. (2017) found that *H. fusca* uses a constant bearing angle (CBA) strategy to initially fly an interception course with its prey. When an individual is within 29 cm of the prey, the robber fly "locks-on" to the prey and adjusts its velocity and direction so that it comes alongside the prey to make its capture.

After prey capture, the proboscis is usually immediately inserted in the prey and the individual lands on the same foraging perch. Only a few *H. calva* hover next to or 2.5-10 cm below their feeding site and manipulate the prey with all six tarsi before inserting their proboscis. Hovering and manipulating prey usually lasts only a few seconds, but two individuals hovered and manipulated prey for 13-15 seconds.

During feeding, prey hang free from the individual's proboscis without being held by the tarsi (Fig. 4). Some prey, such as Hymenoptera (Eucharitidae, *Orasema* sp. and winged Formicidae) may be manipulated one or two times with all six tarsi and the proboscis reinserted in the prey as the individual hovers near the feeding site. Also, an individual will continue to observe other insects as they fly by, as indicated by rapid movements of its head and body. Similar behavior has been observed for other species of *Holcocephala* (Dennis, 1979; Scarbrough, 1982).

The average length of 10 male and female *H. calva is* 8.5 mm. The average length of 88 prey is 2.4 mm with a range from 0.8-5.6 mm. This results in a mean predator to prey ratio of 3.5, which indicates that *H. calva* is approximately 3 1/2 times larger than its prey. Scarbrough (1982) said that the mean predator to prey ratios for *H. calva* and *H. abdominalis* is 3.7 and 3.4, respectively. The overall mean predator to prey ratio for both *H. fusca*, (Dennis, 1979) and *H. oculata* is 3.5 (LaPierre, 2000). Mean predator to prey ratios for other species of robber flies range from 0.9:1.0 to 8.4:1.0 (Dennis, 2016), with an overall mean of 2.8.

Fig. 4. *Holcocephala calva* with winged *Pheidole navigans* as prey (Photograph: D.S. Dennis, 23 June 2020, 7:01 AM).

Holcocephala calva feedings range from 1-36 minutes with an average of 10.7 minutes (n=54). The time spent feeding generally correlates with prey length. *Holcocephala calva* fed on wasps (Eucharitidae, *Orasema* sp.) with lengths ranging from 2.15-3.00 mm (average, 2.39 mm; n=6), for 12-30 minutes (average 21.9 minutes). Long-legged flies (Dolichopodidae, *Chrysotus* sp.) with lengths ranging from 1.12-2.47 mm (average 1.69 mm; n=3), were fed on for 4-25.5 minutes (average 14.2 minutes). As for a number of other species of robber flies, including *H. fusca*, the time spent feeding usually depends on prey length (Dennis, 1979, 2020).

At the completion of feeding, *H. calva* discards prey as follows, from most to least common: (1) it pushes prey off its proboscis with the fore tarsi while still at the feeding site (35.5%; n=43); (2) it allows prey to drop off its proboscis at the feeding site (29.8%; n=36); (3) it drops prey in flight and captures another prey (24.8%; n=30); (4) it drops prey in flight, but does not capture another prey (9.1%; n=11); and (5) while hovering and manipulating prey, it drops the prey (0.8%; n=1). Other species of *Holcocephala* use similar methods to discard prey (Dennis, 1979; Johnson, 1976; Scarbrough, 1982) with pushing prey off the proboscis at the feeding site being the most common.

Thirty-nine interfeedings were observed with an average of 8.8 minutes and a range from 0-68.5 minutes. There were 20, 0-minute interfeedings when the robber flies dropped the prey in flight that they were feeding on and immediately captured another prey.

The theoretical number of prey an individual *H. calva* can feed on in one day can be calculated if one assumes that: (1) it continually forages and feeds between 6:00 AM and 3:00 PM (the time when 87.7% of the feedings were observed), and (2) it captures and feeds on prey every 19.5 minutes (which includes the average feeding and inter-feeding times). Therefore, over a 9-hour period an individual could feed on approximately 27 prey. However, it is of interest to note that one individual fed on five prey over 41.5 minutes and another fed on six prey over an 88 minute period.

This compares favorably with Scarbrough (1982) who noted that *H. calva* fed on prey for an average of 9.2 minutes and could dispatch approximately 25 prey per day. *Holcocephala abdominalis* (Scarbrough, 1982) and *Holcocephala fusca* (Dennis, 1979) fed on prey for an average of 8.6 and 10.9 minutes and approximately 22 and 26 prey per day, respectively. Dennis (2016) reported that other investigators estimate robber flies feed on 1 to 35 prey per day.

Some prey (in particular Diptera) ballooned or swelled while *H. calva* was feeding. This also was observed for *H. fusca* (Dennis, 1979). Lavigne & Holland (1969) attributed prey ballooning to asilids pumping proteolytic enzymes and digested material in and out of prey. Also, during feeding some robber flies contract the first one to three segments of their abdomen.

Abdominal pumping (or contractions) has been associated with the pumping of enzymes and digested material while feeding on prey (Musso, 1968; Lavigne & Holland, 1969), as well as thermoregulation (Morgan & Shelly, 1988; Morgan, Shelly, & Kimsey, 1985). No abdominal pumping was recorded for *H.* calva, *H. abdominalis* (Johnson, 1976), and *H. fusca* (Dennis, 1979)..

Of interest, Drukewitz et al (2018) have described and illustrated the hypothesized feeding process of robber flies starting with envenomation of the prey through feeding on the liquefied prey contents.

Prey

The following is a list of prey taken by *H. calva* with the number of prey following the date, if more than one.

ARANEAE, Unidentified, 02.05.20, 08.05.20, 22.08.20. BLATTODEA (Isoptera), Kalotermitidae (alate; winged): *Calcaritermes nearcticus* Snyder, 1933, 21.05.20. COLEOPTERA, Bostrichidae: *Lyctus* sp., 25.04.20; Curculionidae: *Xyleborinus* sp., 05.06.20; *Xyleborinus*, poss. *saxsenii* (Ratzeburg. 1837), 30.05.20; *Xyleborus* sp., prob. *pubescens* Zimmermann, 1868, 16.06.20, 27.06.20; Mordellidae: *Tolidomordella discoidea* (Meisheimer, 1845), 28.04.20 (2); Nitidulidae: unidentified, 25.5.20, 15.06.20. Staphylinidae: *Proteinus* sp., 25.05.20; 15.06.20. DIPTERA, Cecidomyiidae: unidentified, 28.04.20, 13.10.20; Ceratopogonidae: unidentified, 20.06.20;

Chironomidae: unidentified, 05.08.20, 21.08.20 (2), 07.10.20, 13.10.20; Chloropidae: unidentified, 27.04.20, 19.08.20; Dolichopodidae: *Chrysotus* sp., 28.04.20 (2), 01.05.20, 11.05.20, 20.05.20 (3), 21.05.20, 02.06.20, 09.06.20, 13.10.20; unidentified, 01.05.20; Empididae: unidentified, 18.05.20; Ephydridae: unidentified, 23.09.20; Hybotidae: *Euhybos* sp., 26.08.20; *Syneches* sp., 19.05.20, 17.06.20, 29.08.20; unidentified, 18.06.20; Muscidae: unidentified, 16.06.20; Phoridae: *Borophaga* sp., 13.10.20; Sciaridae: unidentified, 09.06.20 (2), 16.06.20, 18.06.20, 20.06.20, 19.08.20; Simuliidae: *Simulium* sp., 11.12.20; Unidentified Family, 02.06.20, 05.06.20 (2), 14.06.20, 16.06.20, 20.06.20 (2), 22.06.20, 26.06.20 (2), 27.06.20, 29.08.20, 16.09.20, 07.10.20 (3). HEMIPTERA, Cicadellidae: unidentified, 21.11.20 (2), Delphacidae: unidentified, 19.06.20 (2); Miridae: unidentified, 18.05.20, 25.05.20 (3), 09.06.20, 05.08.20. HYMENOPTERA, Chalcidoidea: unidentified, 02.05.20; Eucharitidae: *Orasema* sp., 27.04.20 (4), 28.04.20, 04.05.20 (5), 05.05.20 (4), 08.05.20 (3), 15.05.20 (3), 18.05.20, 23.05.20; unidentified, 17.06.20; Eupelmidae: unidentified, 09.06.20; Formicidae (alates; winged): *Camponotus* sp. poss. *inaequalis* Roger, 1863, 16.09.20; *Crematogaster* sp., 27.04.20, 27.05.20 (3), 29.05.20, 09.06.20; 11.06.20; *Nylanderia* sp., 05.06.20 (3), 27.06.20; *Pheidole navigans* Ford, 1901 (Fig. 4), 15.05.20, 27.05.20, 18.06.20; 20.06.20, 26.06.20; *Solenopsis invicta* Buren, 1972, 25.05.20, 11.06.20 (2), 16.06.20; unidentified: 01.05.20, 16.06.20 (3), 19.06.20, 22.06.20 (2), 23.06.20 (5), 26.06.20 (2), 27.06.20 (4), 08.07.20, 10.07.20, 05.08.20, 18.09.20; Unidentified Family, 19.05.20. LEPIDOPTERA, Tortricidae: *Eucosma* sp., 28.04.20, 02.05.20, 25.05.20, 09.06.20, 18.06.20. PSOCOPTERA: Unidentified, 27.04.20 (2), 04.05.20, 05.05.20, 08.05.20, 18.05.20, 17.06.20, 23.09.20 (2), 30.09.20. UNIDENTIFIED Order, 22.04.20, 25.04.20 (12), 27.04.20 (2), 28.04.20, 02.05.20, 04.05.20 (12), 05.05.20 (16), 08.05.20 (3), 15.05.20, 21.05.20 (5), 23.05.20 (4), 25.05.20 (3), 29.05.20 (4), 30.05.20 (22), 02.06.20 (3), 05.06.20 (17), 09.06.20 (5), 11.06.20 (8), 12.06.20 (3), 13.06.20 (3), 16.06.20, 17.06.20 (3), 18.06.20 (4), 22.06.20, 23.06.20 (4), 26.06.20 (3), 27.06.20 (2), 03.07.20 (5), 07.07.20, 08.07.20, 29.07.20, 05.08.20, 15.08.20 (2), 21.08.20 (2), 16.09.20, 18.09.20, 30.09.20, 02.10.20, 13.10.20 (2).

Of the prey identified to at least Order, the majority are Hymenoptera (42.3%), followed by Diptera (33.1%), Coleoptera (6.7%), Hemiptera and Psocoptera (each 6.2%), Lepidoptera (3.1%), Araneae (1.8%), and Blattodea (Isoptera, 0.6%). Other investigators have documented *Holcocephala* feeding on insects representing the same orders (Johnson, 1976; Dennis, 1979; Scarbrough, 1982; LaPierre, 2000). Similarly, Scarbrough (1982) reported *H. calva* and *H. abdominalis* feeding primarily on Diptera followed by Hymenoptera. Johnson (1976) said that *H. abdominalis* fed mainly on Diptera and then Hymenoptera. Dennis (1979) also observed male *H. fusca* feeding mainly on Diptera and then Hymenoptera; whereas, females fed in the reverse order.

It is believed that *H. calva* captured the Araneae while they were ballooning or floating through the air on silk threads. Johnson (1976) observed *H. calva* "…feeding on a spiderling with a silk thread trailing from it." Dennis, Lavigne & Dennis (2012) documented other species of robber flies capturing spiders while they were ballooning.

For other species, McAtee and Banks (1920) indicated that *H. abdominalis* fed on *Solenopsis* sp. and *Lasius* sp. (Hymenoptera, Formicidae) while Bromley (1950) noted that they fed on *Culicoides* sp. (Diptera, Ceratopogonidae).

Mating behavior

Male *H. calva* perform searching flights for receptive females with which to mate. Searching flights consist of males flying around the twigs of branches in their habitat for 2-23 seconds with an average of 8.6 seconds (n=23). During searching flights, the males slowly fly with the femora of the fore- and mid-legs held up against the thorax, and the tibiae and tarsi hanging down at about a 30-45º angle or the tibiae and tarsi are held closer to the thorax and extend forward. The hind legs also hang below the individual at about a 45º angle. Male searching flights have been described for *H. fusca* (Dennis, 1979) and *H. oculata* (LaPierre, 2000).

Like *H*. fusca (Dennis, 1979), male *H. calva* did not hover in front of or near females or exhibit courtship as described for *H. abdominalis* (Johnson, 1976) and *H. oculata* (LaPierre, 2000).

Holcocephala calva males attempt to initiate matings by landing on the dorsum of both males and females that are on twig tips. Non-receptive individuals spread their wings at a 30-90º angle and sometimes curve their abdomen down and/or crawl on the twig away from the male. If the male does not fly off after landing on a non-receptive individual, the pair will fall off the twig and separate in flight or when they hit leaves of vegetation or the ground below. Before separating some males will attempt to clasp the genitalia of non-receptive individuals for several seconds. Also, males will repeatedly attempt to mate with the same non-receptive individuals. Dennis (1979) reported similar behavior for *H. fusca*.

When the male is able to successfully clasp a female's genitalia, he will then fall backwards to below the female in the tail-to-tail position (Fig. 5). In this position the wings of both the male and female are folded over their backs and the asilids remain still while mating. The male faces the opposite direction of the female or slightly to her side and his legs hang "below" his body. On one occasion one mating pair was in the tail-to-tail position when another male landed on the female and attempted to clasp her genitalia for 2 minutes before flying away.

At the completion of mating, the male usually swings up and grabs a nearby twig to the side of or below the female, and then unclasps the female. The male either then flies off or remains on the twig for up to 1.5 minutes before flying off. One male swung up and grabbed the female before unclasping her and flying off at the completion of mating.

Seven mating pairs were observed with four complete matings that lasted 18.0-19.5 minutes with an average of 18.9 minutes. *Holcocephala fusca* (Dennis, 1979) and *H oculata* (LaPierre, 2000) also mate in the tail-to-tail position, with the latter mating for an average of 11 minutes.

Holcocephala calva mating pairs were observed at heights ranging from 1.1-3.3 m (average height of 2.1 m) above the ground where air temperature ranged from 14.0-27.4ºC (average of 20.1ºC).

Fig. 5. Mating pair of *Holcocephala calva* in the tail-to-tail position with the female above and male hanging below (Photograph: D.S. Dennis, 30 September 2020, 8:37 AM).

Oviposition behavior

Four ovipositions were observed; females were on branch tips or up to 2.5 cm back from the tips on top of the branch with their abdomens slightly extended over the side of the branch. The females were approximately 1.4-1.5 m (average 1.5 m) above the ground. Three females each dropped one egg with their abdomens parallel to the ground. The fourth female quickly dipped her abdomen and dropped an egg. The eggs were a glistening light to dark amber and slightly elongate or oval with one end narrower than the other. It was not possible to recover the eggs because the ovipositions were being watched through binocularis. *Holcocephala abdominals* (Johnson, 1976) and *H. fusca* (Dennis, 1979) have similar eggs. Johnson (1976) suggested that the former species probably deposits eggs one at a time.

The ultrastructure of the chorion of the eggs of *H. calva* and *H. abdominalis* was described by Candan, Suludere, Hasbenli, Çağıran, Lavigne & Scarbrough (2004). The chorion of the eggs of both species is thick, but *H calva* had tall, thin, uniform ridges in a hexagonal pattern; whereas, *H. abdominalis* had low, thick, uniform ridges in a polygon pattern. The eggs also have aeropyles between the sculpturing on the chorion. Dennis (1979) also reported that *H. fusca* has hexagonal sculpturing on its chorion).

Candan et al. (2004) said that forest edge species such as *H. calva* and *H. abdominalis* have simple ovipositors and drop their eggs at random. They also indicated that the “eggs deposited in this manner are frequently, but temporarily, inundated with water.” The sculptured chorions with aeropyles facilitates gas exchange by trapping gas bubbles on their surface.

The air temperatures at the heights of the *H. calva* ovipositions ranged from 21.3-27.8ºC with an average of 24.6ºC.

Grooming behavior

Robber fly grooming behavior is often associated with other behaviors such as those following the completion of feeding, mating, and ovipositing. There are also

species-specific variations in the sequence and frequency of grooming. Like other robber flies, *H. calva* always use the fore legs to groom their heads, and the hind legs to groom their wings, abdomen, and genitalia while resting and during feeding and mating. They never were observed grooming the thorax.

Holcocephala calva groom their faces more often within 2 hours after sunrise, after feeding on large prey, and at the completion of feeding after pushing prey off the proboscis with the fore tarsi. The face is groomed with the fore tarsi and tibiae, often while alternately moving each side of the head up and down. After grooming the face, the fore tarsi and tibiae are often rubbed together. This sequence of grooming is often repeated a number of times for up to 13 minutes after feeding. One individual also groomed its eyes with the fore tibiae after capturing a prey.

After mating, female *H. calva* groom their abdomens more than do males. The hind tarsi are typically first rubbed together and then the posterior 2/3 of the abdomen is groomed from anterior to posterior. During grooming the abdomen is either straight or slightly curved down. After grooming the hind tarsi and tibiae may be rubbed together. The hind tarsi are also often rubbed together when an individual is feeding.

Following grooming of the abdomen, the wings are often groomed from anterior to posterior or outward, with the hind tibiae and tarsi. One individual groomed the wings using only the hind tibiae. The tops and bottoms of the wings are groomed with the wings closed or open to a 30-45º angle to the body.

One female groomed the posterior part of her abdomen with her hind tarsi after ovipositing. She then rubbed her hind tarsi together.

Throughout the day the most common grooming is when the hind tarsi and tibiae are rubbed together and when one or both hind tarsi and tibiae are used to stroke the sides of the abdomen. As exhibited by *H. abdominalis* (Johnson, 1976) and *H. fusca* (Dennis, 1979), *H. calva* spent more time grooming its wings, abdomen and hind tarsi than its face and fore tarsi.

Daily rhythm of activity

Holcocephala calva exhibit a daily rhythm of activity with peak periods of foraging, male searching flights for females with which to mate, mating, and ovipositing between 6:00-10:00 AM (Fig. 6). The number of feeding individuals had a slight peak between 8:00-9:00 AM with a steady number (61.6%) feeding between 7:00 AM-12:00 noon. The majority (87.7%) of *H. calva* observed fed between 6:00 AM-3:00 PM.

Male searching flights peak (62.9%) between 8:00-9:00 AM, with the majority (88.6%) between 7:00-10:00 AM. Matings peak (85.8%) between 6:00-9:00 AM. Ovipositing females have two peaks between 6:00-7:00 AM and 9:00-10:00 AM, with 50% each during these time periods.

Light levels and vision

Depending on cloud cover, parts or all of the Moses Creek habitat are in the sun from approximately 8:30 AM-1:45 PM. The tributary to Moses Creek and Hidden

Creek habitats has intermittent periods (approximately 15-minute periods) of sun from 9:00 AM-4:00 PM and 8:15 AM-2:00 PM, respectively. When the habitats are in the sun, light levels vary from 8,300-100,000 lux (average 31,635; n=8); when parts of the habitats are in the shade or under a light cloud cover/haze, light levels vary from 1,034-5,967 lux (average 3,800 lux; n=22). These levels can be compared with typical full daylight, but not in direct sun light, levels that vary from 10,000-25,000 lux and direct sunlight levels that range from 32,000-100,000 lux (Lackey, 2016 January 28). Light levels in shade or under an overcast sky vary from 1,000-5,000 lux.

Holcocephala calva began feeding and mating early in the morning when light levels were low. At that time the individuals could barely be seen and appeared as dark objects. The first feeding and mating (6:20 AM and 6:13 AM, respectively) took place before sunrise which varied from 6:24-6:26 AM when light levels were 84.9-96.8 lux. These light levels are similar to levels measured for twilight (10.8 lux) and/or to a very dark day (107 lux) (Engineering ToolBox, 2004). Feeding also continued into the evening (last observed feeding, 8:06 PM with sunset at 8:23-8:30 PM) when light levels were 598.6-583.3 lux or levels for a very dark day to an overcast day (1,075 lux; Engineering ToolBox, 2004). The last orientation/investigatory flight observed, occurred at 8:42 PM when light levels were 8.1-8.4 lux and the temperature was 24.8ºC. The individual flew 2.5-5.0 cm in front of and to the side of its perch for 7 seconds before relanding on its perch for the night. Johnson (1976) observed *H. abdominalis* with its first and last prey at 6:27 AM and 8:17 PM, respectively.

The first oviposition also occurred early in the morning at 6:47 AM, shortly after sunrise. At that time the light levels varied from 1,193-1,217 lux.

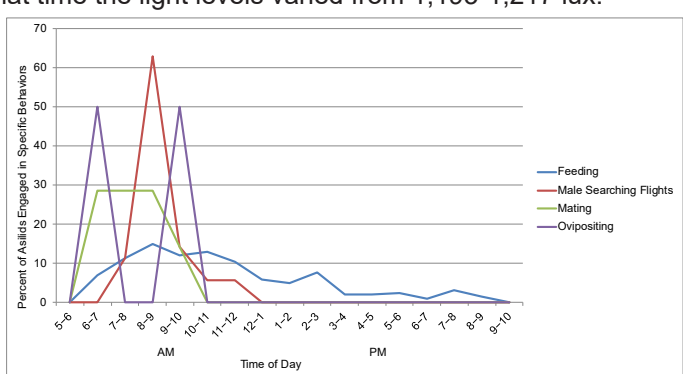


Fig. 6. Daily rhythm of activity of *Holcocephala calva* based on 326, 35, 7, and 4 observations for feeding, male searching flights, mating, and ovipositing, respectively.

Holcocephala calva continuing to be active when light levels are low indicates that they have good visual acuity and can see objects well even under low light conditions. Nation (2008) wrote that, “Insect compound eyes perform poorly in very dim light, but insects that are active at night or at dusk have special adaptations for vision in dim light. These may include wider facets and wider rhabdoms that increase sensitivity by up to 1-2 log units.”

The eyes of *H. calva* and *H. abdominalis* were examined and they both have mediofrontal enlarged facets (frontal enlarged-facet zones (EFZ)) similar to those reported for *H. fusca* (Douglass & Wehling, 2016; Wardill et al., 2017). Land (1997) said that many insects (including robber flies and dragonflies) have acute zones (localized areas of high resolution) that are forward or upward pointing that are specialized for predation. Douglass & Wehling (2016) indicated that the *H. fusca* frontal EFZ are associated with acute zones that represent a specialization of the frontal visual field. Wardill et al. (2017) commented that the behavioral, anatomical and optical data for *H. fusca* eyes show extremely specialized visual capabilities. They also wrote, "The extremely enlarged frontal ommatidia have facet lenses with extended focal lengths that focus incident light into unusually slender rhabdomeres... These specializations are known to optimize the spatial resolution of fly eyes, thus creating an area of high acuity, a fovea."

Predators and parasites

Lavigne, Dennis & Gowen (2000) indicated that cannibalism is often reported for robber flies. However, it was not observed for *H. calva* during this study or by Scarbrough (1982). Cannibalism also has not been observed for *H. abdominalis* (Johnson, 1976; Scarbrough, 1982), *H. fusca* (Dennis, 1979), or *H. oculata* (LaPierre, 2000).

A female of the robber fly *Ommatius floridensis* Bullington & Lavigne, 1984 captured one female *H. calva*.

While resting and foraging, *H. calva* individuals were disturbed by: (1) slender twig ants (Formicidae, *Pseudomyrmex gracilis* (Fab., 1804)); (2) blue dasher (Libellulidae: *Pachydiplax longipennis* (Burmeister, 1839)) and great blue skimmer (Libellulidae: *Libellula vibrans* (Fab., 1793)) dragonflies; (3) bumblebees (Apidae: *Bombus* sp.); (4) and honeybees (Apidae: *Apis mellifera* L., 1758). When disturbed the individuals would usually back down the twig 2.5-7.5 cm they were on and then resume their position on the twig tip after the intruder had left. A few individuals flew to a nearby twig tip when disturbed.

The author has observed mites on robber flies, in particular on the thorax. However, none were found on *H. calva*.

Conclusions

Holcocephala calva exhibits behavior similar to other species of Nearctic (*H. abdominalis* and *H.* fusca) and Neotropical (*H. oculata*) *Holcocephala*. This species rests on and forages primarily from dead twig tips. They often spend the night on the same twig tips. All prey are captured in flight and consists of Hymenoptera (42.3%), Diptera (33.1%), Coleoptera (6.7%), Hemiptera and Psocoptera (each 6.2%), Lepidoptera (3.1%), Araneae (1.8%), and Blattodea (Isoptera; 0.6%). During feeding, prey (mostly Hymenoptera) may be manipulated with all six tarsi in a hover next to the feeding site. Mating occurs in the tail-to-tail position and females oviposit by dropping single eggs onto the ground. This species exhibits a daily rhythm of activity with peak

feeding, male searching flights for females with which to mate, mating, and ovipositing early in the morning from 6:00-10:00 AM. Grooming behavior resembles that described for other species of Asilidae. *Holcocephala calva* are disturbed by slender twig ants, dragonflies, bumblebees, and honeybees and usually move backwards down the twig that they are on.

ACKNOWLEDGMENTS

The author thanks the staff of the St. Johns River Water Management District for the issuance of the Special Use Authorization that allows the study of robber flies in the Moses Creek Conservation Area (MCCA) and for their interest in the research.

I also thank the anonymous reviewers for their excellent and constructive comments on the manuscript.

REFERENCES

Back, E.A. (1909). The robber flies of America north of Mexico, belonging to the subfamilies Leptogastrinae and Dasypogoninae. *Transactions of the American Entomological Society*, 35, 137- 400.

Baker, N.T. & Fischer, R.L. (1975). A taxonomic and ecologic study of the Asilidae of Michigan. *The Great Lakes Entomologist*, 8(2), 31-91.

Blanton, F.S. (1939). Collecting notes on the family Asilidae (Diptera). *Bulletin of the Brooklyn Entomological Society*, 34(5), 229-235.

Brimley, C.S. (1922). List of the robberflies (Asilidae, Diptera) of North Carolina. *Entomological News*, 33, 294-298.

Bromley, S.W. (1931). A preliminary annotated list of the robber flies of Ohio (Diptera: Asilidae). *Ohio State University Museum Science Bulletin*, 1, 3-19.

Bromley, S.W. (1946). Guide to the insects of Connecticut. Part VI. The Diptera or true flies of Connecticut. Third fascicle Asilidae. *Connecticut State Geological and Natural History Survey Bulletin No. 69*, 1-48.

Bromley, S.W. (1950). Florida Asilidae (Diptera) with description of one new species. *Annals of the Entomological Society of America*, 43(2), 227-239.

Candan, S., Suludere, Z., Hasbenli, A., Çağıran, N., Lavigne, R. & Scarbrough, A. (2004). Ultrastructure of the chorion of *Dioctria flavipennis* Meigen, 1820 (Diptera: Asilidae: Stenopogoninae) compared with those of fourteen asilid species from the mid-Atlantic region of North American. *Proceedings of the Entomological Society of Washington*, 106(4), 811-825.

Carrera, M. (1958). Sobre o genero *Dasyllis* Loew, 1851 (Diptera, Asilidae) *Papéis avulsos do Departamento de Zoologia*, 13, 213-220.

Dennis, D.S. (1979). Ethology of *Holcocephala fusca* in Virginia (Diptera: Asilidae). *Proceedings of the Entomological Society of Washington,* 81(3), 366-378.

Dennis, D.S. (2014). Ethology of Holopogon phaeonotus Loew, 1874 (Diptera: Asilidae) in Northeastern Florida, U.S.A. *Journal of the Entomological Research Society*, 16(2), 141-158.

Dennis, D.S. (2016). Ethology of *Promachus bastardii* (Macquart, 1838) (Diptera: Asilidae) in Northeastern Florida, U.S.A. *Journal of the Entomological Research Society*, 18(3), 69-92.

Dennis, D.S. (2018). Ethology of *Holopogon snowi* Back, 1909 (Diptera: Asilidae) in Northeastern Florida, U.S.A. *Journal of the Entomological Research Society*, 20(1), 95-112.

Dennis, D.S. (2020). Ethology of *Proctacanthus gracilis* Bromley, 1928 (Diptera: Asilidae) in Northeastern Florida, U.S.A. *Journal of the Entomological Research Society*, 22(3), 255-273.

Dennis, D.S. & Lavigne, R.J. (1975). Comparative behavior of Wyoming robber flies II (Diptera: Asilidae). *Agricultural Experiment Station University of Wyoming-Laramie Science Monograph* No. 30, 68 pp.

Dennis, D.S., Lavigne, R.J., & Dennis, J.G. (2012). Spiders (Araneae) as prey of robber flies (Diptera: Asilidae). *Journal of the Entomological Research Society*, 14(1), 65-76.

Douglass, J.K. & Wehling, M.F. (2016). Rapid mapping of compound eye visual sampling parameters with FACETS, a highly automated wide-field goniometer. *Journal of Comparative Physiology A*, 202 (12), 839-851.

Drukewitz, S.H., Fuhrmann, N., Undheim, E.A.B., Blanke, A., Giribaldi, J., Mary, R., Laconde, G., Dutertre, S. & von Reumont, B.M. (2018). A Dipteran's novel sucker punch: evolution of arthropod atypical venom with a neurotoxic component in robber flies (Diptera, Asilidae). *Toxins*, 10(1), 1-23.

Engineering ToolBox (2004). Illuminance-Recommended light level. Retrieved from https://www.engineeringtoolbox.com/light-level-rooms-d_708.html

Fabian, S.T., Sumner, M.E., Wardill, T.J., Rossoni, S., & Gonzalez-Bellido, P.T. (2018). Interception by two predatory fly species is explained by a proportional navigation feedback controller. *Journal of the Royal Society Interface*, 15, 20180466.

Fisher, E.M. (2009). Chapter 45. Asilidae (robber flies, assassin flies, moscas cazadoras, moscas ladronas). In Brown, A. Borkent, J.M. Cumming, D.M. Wood, N.E. Woodley, & Zumbado (Eds.). *Manual of Central American Diptera* (pp. 585-632). National Research Council of Canada, Research Press, Monograph Publishing Program.

Fisher, E.M. & Wilcox, J. (1997, unpublished). Catalog of the robber flies (Diptera: Asilidae) of the Nearctic Region. California Department of Food & Agriculture; Sacramento.

Geller-Grimm, F. (2021, January 4). Robber flies (Asilidae), database, catalog of species. Retrieved from http://www.geller-grimm.de/catalog.species.htm

Hull, F.M. (1962). Robber flies of the world. The genera of the family Asilidae. *U.S. National Museum Bulletin*, 224(1), 63-64.

Johnson, P.E. (1976). *A preliminary investigation of the behavior of adult Holcocephala abdominalis (Say) (Diptera: Asilidae) in Putnam County, Indiana; with a description of the larvae and the eggs*. M.S. Thesis, DePauw University, Greencastle, Indiana.

Lackey, R. (2016, January 28). Shedding Light on Lumens, Lux and Latitude. Retrieved from https://www.cined.com/shedding-light-lumens-lux-latitude/

Land, M.F. (1997). Visual acuity in insects. *Annual Review of Entomology*, 42, 147-177.

LaPierre, L.M. (2000). Prey selection and diurnal activity of *Holcocephala oculata* (F.) (Diptera: Asilidae) in Costa Rica. *Proceedings of the Entomological Society of Washington*, 102(3), 643-651.

Lavigne, R.J., Dennis, D.S., & Gowen, J.A. (2000). Asilid literature update 1956-1976 including a brief review of robber fly biology (Diptera: Asilidae). *Agricultural Experiment Station University of Wyoming Science Monograph* 36, 93 pp.

Lavigne, R.J. & Holland, F.R. (1969). Comparative behavior of eleven species of Wyoming robber flies (Diptera: Asilidae). *Agricultural Experiment Station University of Wyoming Laramie Science Monograph* No. 18, 61 pp.

Lehr, P.A. (1958). On the biology and behavior of robber flies (Asilidae-Diptera). *Trudy Instituta Zoologii, Akademiya Nauk Kazakhstan. SSR*, 8, 173-196. (In Russian).

Martin, C.H. & Wilcox, J. (1965). Family Asilidae. In A., Stone, C.W., Sabrosky, W.W., Wirth, R.H., Foote, & J.R. Coulson, *A Catalog of the Diptera of America North of Mexico* (pp. 360-401). U.S. Department of Agriculture, Agricultural Research Service, Agriculture Handbook No. 276.

McAtee, W.L. & Banks, N. (1920). District of Columbia Diptera: Asilidae. *Proceedings of the Entomological Society of Washington*, 22(1-2), 13-33.

McGravy, K.W. & Baxa, K.A. (2011). Diversity, seasonal activity and habitat associations of robber flies (Diptera: Asilidae) in west-central Illinois. *American Midland Naturalist*, 166, 85-97.

Morgan, K.R. & Shelly, T.E. (1988). Body temperature regulation in desert robber flies (Diptera: Asilidae). *Ecological Entomology*, 13, 419-428.

Morgan, K.R., Shelly, T.E., & Kimsey, L.S. (1985). Body temperature regulation, energy metabolism, and wing loading in light-seeking and shade-seeking robber flies. *Journal of Comparative Physiology*, 155, 561-570.

Musso, J.J. (1968). Digestion extra-intestinale chez *Stenopogon sabaudus* F. et *Machimus pilipes* Meig. (Dipt. Asilidae). *Bulletin de la Société Zoologique de France*, 93(3), 487-497.

Nation, J.L. (2008). Eyes and vision. In Capinera, J.L. (Ed.) *Encyclopedia of Entomology* (pp. 1381-1392), 2nd Edition. Springer Netherlands.

Scarbrough, A.G. (1974). A faunistic study of Asilidae (Diptera) at three locations in northern Baltimore County, Maryland: incidence, relative abundance and seasonal distribution. *Proceedings of the Entomological Society of Washington*, 76(4), 385-396.

Scarbrough, A.G. (1982). Coexistence in two species of *Holcocephala* (Diptera: Asilidae) in a Maryland habitat: predatory behavior. *Proceedings of the Entomological Society of Washington*, 84(2), 349-365.

Wardill, T.J., Fabian, S.T., Pettigrew, A.C., Stavenga, D.G., Nordström, K., & Gonzalez-Bellido, P.T. (2017). A novel interception strategy in a miniature robber fly with extreme visual acuity. *Current Biology*, 27, 854-859.

Received: April 15, 2021 *Accepted: September 08, 2021*

J. Entomol. Res. Soc., 23(3): 279-286, 2021 *Research Article*
DOI: 10.51963/jers.v23i3.2069 *Online ISSN:2651-3579*

Physiological and Biochemical Effects of *Klebsiella oxytoca* Infection on Model Organism *Galleria mellonella* L.

Serkan SUGEÇTİ[1*] Cihat ÇELİK[2] Ender BÜYÜKGÜZEL[3]
Kemal BÜYÜKGÜZEL[4]

[1]Zonguldak Bülent Ecevit University, Çaycuma Food and Agriculture Vocational School, Department of Veterinary Medicine, Zonguldak, TURKEY
[2]Zonguldak Bülent Ecevit University, Çaycuma Food and Agriculture Vocational School, Department of Laboratory Technology, Zonguldak, TURKEY
[3]Zonguldak Bülent Ecevit University, Science and Art Faculty, Department of Molecular Biology and Genetics, Zonguldak, TURKEY
[4] Zonguldak Bülent Ecevit University, Science and Art Faculty, Department of Biology, Zonguldak, TURKEY
e-mails: [1]*serkan.sugecti@hotmail.com, [2]cihat.celik@beun.edu.tr, [3]endericen@hotmail.com, [4]buyukguzelk@hotmail.com
ORCID IDs: [1]0000-0003-3412-2367, [2]0000-0002-2400-3412, [3]0000-0002-4442-5081, [4]0000-0002-9589-9383

ABSTRACT

In this study, the physiological and biochemical response of model insect *Galleria mellonella* due to *Klebsiella oxytoca* infection was investigated. Changes in metabolic enzyme activities such as aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), amylase (AMYL) and gamma glutamyl transferase (GGT) occurred due to *K. oxytoca* infection. In addition, amounts of non-enzymatic antioxidant such as uric acid (UA), bilirubin (BIL) and albumin (ALB) were altered in *G. mellonella* hemolymph due to infection. The results obtained from this study showed that *G. mellonella* can be used as a model organism in the evaluation of the physiological effects of *K. oxytoca* infection on the host.

Key words: Galleria mellonella, model insect, antioxidant responses, infection.

Sugeçti, S., Çelik, C., Büyükgüzel, E., & Büyükgüzel, K. (2021). Physiological and biochemical effects of *Klebsiella oxytoca* infection on model organism *Galleria mellonella* L. *Journal of the Entomological Research Society,* 23(3), 279-286.

INTRODUCTION

Klebsiella species are nosocomial pathogens with high virulence. Although they usually infect immunocompromised individuals, these isolates make treatment difficult because they are resistant to many antibiotics (Bassetti et al, 2018; Rønning et al, 2019). *Klebsiella oxytoca* is a gram-negative bacteria associated with nosocomial infections. In recent years, the high resistance of *K. oxytoca* against antibiotics used especially in urinary tract infections has increased the interest of researchers in this pathogen (Tominaga 2018; Hu, Wei, Feng, Xie, & Zong, 2019).

In recent years, invertebrate experimental animals have been used to study bacterial infection and host interactions (Shaik, Mishra, Sehadová, & Kodrík, 2020; Lapointe, McCarthy, Dunphy, & Mandato, 2020). In addition, invertebrate infection models are frequently used in the fields of medicine, pharmacy and veterinary medicine (Matsumoto, Ishii, Shimizu, Kawamoto, & Sekimizu, 2017; Ishii, Matsumoto, Yamada, Abe, & Sekimizu, 2017; Singulani, Scorzoni, De Oliveira, Marcos, & Assato, 2018). In particular, *Galleria mellonella* (Lepidoptera: Pyralidae) larvae have been used as model organisms in many studies on bacterial pathogenicity or the efficacy of antibiotic treatments (Rossoni et al, 2017; Shaik et al, 2020; Lapointe, McCarthy, Dunphy, & Mandato, 2020). The large surface area of *G. mellonella* larvae and the easy isolation of hemolymph provide advantages for physiological and biochemical studies (Andrejko, Zdybicka-Barabas, & Cytryńska, 2014; Tsai, Loh, & Proft, 2016).

Many studies have reported that biological and chemical agents negatively affect metabolic and immune responses in insects (Sugeçti, Büyükgüzel, & Büyükgüzel, 2016; Kastamonuluoğlu, Büyükgüzel, & Büyükgüzel, 2020). Aspartate transferase (AST) and alanine transferase (ALT) activities significantly increase in insects, especially in cellular damage due to oxidative stress (Sertçelik, Sugeçti, Büyükgüzel, Necefoğlu, & Büyükgüzel, 2018). On the other hand, alkaline phosphatase (ALP) and amylase (AMYL) activities have important roles in regulating energy metabolism in insects against oxidative damage (Sugeçti & Büyükgüzel, 2018). The other metabolic enzyme, gamma glutamyl transferase (GGT), increases the availability of amino acids, mainly cysteine, for intracellular glutathione synthesis and plays an important role in maintaining non-enzymatic antioxidant defense against oxidative stress in organisms (Ndrepepa, Colleran, & Kastrati, 2018). In addition, oxidative damage to insect tissues is prevented by enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and non-enzymatic antioxidants such as amount of uric acid (UA), bilirubin (BIL) and albumin (ALB) (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018; Alp & Coskun 2018). It is known that biochemical parameters such as total protein (TP) and urea are negatively affected as a result of oxidative damage in insects (Etebari, Bizhannia, Sorati, & Matindoost, 2007; Sertçelik et al, 2018).

In this study, the changes in the activity of metabolic enzymes such as AST, ALT, ALP, AMYL, GGT and amount of biochemical parameters such as ALB, BILD, BILT, TP, UA and UREA in hemolymph of *G. mellonella* larvae after infection with *K. oxytoca* were investigated.

MATERIALS AND METHODS

Insect culture

G. mellonella culture was obtained from the Insect Culture Laboratory in Biology department of Zonguldak Bülent Ecevit University. Insect culture was continued in an incubator set at 28 ± 2°C and 65 ± 5% humidity under laboratory conditions. Insects were reared in artificial diet until the seventh larval stage of *G. mellonella*. Artificial diet was composed of 420 g of bran, 150 ml of filtered honey, 150 ml of glycerol, 20 g of ground old dark honeycomb, and 30 ml of distilled water (Bronskill, 1961).

Experimental design

K. oxytoca (ATCC 8724) was obtained from local company. *K. oxytoca* was prepared in a brain heart solution according to the 0.5 McFarland standard. The solution (10µl) prepared according to this standard (about $1x10^7$ bacteria) was injected through the abdomen of the insect. Hemolymph was collected at 2, 4, 6 and 8 hours after injection. As the control group, only 10 µl brain heart solution was injected and hemolymph was collected 8 hours after the injection. Before the hemolymph was collected, the larvae were kept on ice for about 5 minutes and their surfaces were disinfected with ethyl alcohol. Samples were stored at -80°C until analyses. The experiments were repeated four times.

Biochemical analysis

The collected hemolymph was placed in homogenization buffer (w/v 1.15% potassium chloride (KCl), 25 mM dipotassium hydrogen phosphate (K_2HPO_4), 5 mM ethylen-diaminetetraacetic acid (EDTA), 2 mM phenylmethylsulphonil fluoride (PMSF), 2 mM dithiothreitol (DTT), pH 7.0). Extracts of hemolymph were prepared at 4°C by an ultrasonic homogenizer (Bandelin Sonoplus, HD2070, Germany). Then, solution was centrifuged at 10.000 g for 10 min at 4°C. The analyses were performed with the Roche Hitachi Cobas c501 instrument (Roche, Germany) using appropriate kits. ALT (Kit no: 46051001), AST (Kit no: 46004201), GGT (Kit no: 44686801), ALP (Kit No: 44211201), AMYL (Kit No: 45142401). ALB (Kit no: 44430501), BILD (Kit no: 46042501), BILT (Kit no: 43808801), UA (Kit no: 44535001), TP (Kit no: 44686201) and UREA (Kit no: 45141501).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze data on cell damage indicators, metabolic enzymes non-enzymatic antioxidants and biochemical parameters of *G. mellonella*. Tukey's HSD test was used to determine the significance of the difference between the mean. All analyses were performed in SPSS v.15.0 (SPSS, Chicago, IL, USA). A probability level of 0.05 was used to check the significance of the difference between the averages. (SPSS, 2006).

RESULTS

Effect of *K. oxytoca* infection on metabolic enzymes activities in hemolymph of *G. mellonella* larvae

In the present study, AST activity statistically increased 8 hours after with *K. oxytoca* infection when compared the control group ($F_{4,15}$: 311.346, p<0.05). Similarly, ALT activity significantly increased in hemolymph of *G. mellonella* larvae at 8 hours after injection ($F_{4,15}$: 229.497, p<0.05). ALP activity decreased approximately 3-fold 8 hours after injection when compared to the control group ($F_{4,15}$: 16.545, p<0.05). AMYL activity significantly decreased from 12.75 U / L to 13.75 U / L 8 hours after with *K. oxytoca* infection ($F_{4,15}$: 227.019, p<0.05). Other metabolic enzyme GGT activity was statistically increased 8 hours after exposure to *K. oxytoca* ($F_{4,15}$: 24.468, p<0.05) (Table 1).

Table 1. Effect of *K. oxytoca* infection on metabolic enzymes activities in hemolymph of *G. mellonella* larvae.

Time	AST (U/L) (Mean*± S.E)†	ALT (U/L) (Mean*± S.E)†	ALP (U/L) (Mean*± S.E)†	AMYL (U/L) (Mean*± S.E)†	GGT (U/L) (Mean*± S.E)†
Control	59.25 ± 4.42a	9.50 ± 0.75a	9.75 ± 0.64a	12.75 ± 5.23a	4.27 ± 1.10a
2	68.50 ± 7.29a	30.50 ± 2.94b	4.85 ± 0.82b	12.50 ± 0.43a	2.55 ± 0.51a
4	58.50 ± 9.83a	33.50 ± 6.17b	5.00 ± 1.11b	95.00 ± 2.69b	2.75 ± 0.32a
6	55.00 ± 0.50a	72.50 ± 6.72c	1.15 ± 0.14c	29.75 ± 1.90c	2.00 ± 0.22a
8	333.01 ± 2.59b	217.01 ± 4.55d	3.30 ± 0.38bc	13.75 ± 0.86ad	14.9 ± 2.21b

* Mean of four replicates per treatment with 20 larvae per replicate

† Means within a column followed by the same lowercase letter are not significantly different (Tukey's HSD test: p > 0.05)

ϒ Control: 8 hours after brain-heart solution injection.

Effect of *K. oxytoca* infection on biochemical parameters in hemolymph of *G. mellonella* larvae

The amount of ALB decreased approximately 4-fold 8 hours after *K. oxytoca* infection ($F_{4,15}$: 58.160, p<0.05). The amount of BILD increased from 0.29 ± 0.02 mg / dL to 0.48 ± 0.05 mg / dL 8 hours after infection. However, this increase in the amount of BILD was not statistically significant ($F_{4,15}$: 3.153, p>0.05). The amount of BILT increased statistically 8 hours after *K. oxytoca* infection ($F_{4,15}$: 4.137, p<0.05). The amount of TP in hemolymph of *G. mellonella* larvae significantly decreased 8 hours after infection ($F_{4,15}$: 90.254, p<0.05). The amount of UA decreased from 0.58 ± 0.01 mg / dL to 0.26 ± 0.05 mg / dL 8 hours *K. oxytoca* infection ($F_{4,15}$: 6.852, p<0.05). The amount of UREA increased approximately 8-fold 8 hours after with *K. oxytoca* infection when compered the control group ($F_{4,15}$: 13.461, p<0.05). (Table 2).

DISCUSSION

In this study, the pathophysiological effects of the pathogen *K. oxytoca* on the invertebrate model organism *G. mellonella* were investigated. Biochemical parameters in

hemolymph of *G. mellonella* were affected negatively. In addition, changes in the amount of non-enzymatic antioxidants occurred in *G. mellonella* hemolymph due to the infection.

Table 2. Effect of *K. oxytoca* infection on biochemical parameters in hemolymph of *G. mellonella* larvae.

Time	ALB (g/dL) (Mean*± S.E)†	BILD (mg/dL) (Mean*± S.E)†	BILT (mg/dL) (Mean*± S.E)†	TP (g/dL) (Mean*± S.E)†	UA (mg/dL) (Mean*± S.E)†	UREA (mg/dL) (Mean*± S.E)†
Control$^{\gamma}$	2.15 ± 0.11a	0.29 ± 0.02a	0.39 ± 0.02a	6.72 ± 0.25a	0.58 ± 0.01a	2.55 ± 0.50a
2	1.55 ± 0.12b	0.41 ± 0.09a	0.62 ± 0.12b	4.71 ± 0.41b	0.64 ± 0.04a	16.20 ± 1.55b
4	1.85 ± 0.04ab	0.66 ± 0.15b	1.07 ± 0.25c	5.49 ± 0.08b	0.77 ± 0.15a	12.86 ± 3.22b
6	0.51 ± 0.05c	0.16 ± 0.03c	0.25 ± 0.03a	1.35 ± 0.02c	0.25 ± 0.02ab	5.05 ± 0.28a
8	0.50 ± 0.08c	0.48 ± 0.05a	0.71 ± 0.04b	1.42 ± 0.015c	0.26 ± 0.05ab	18.30 ± 1.19b

Mean of four replicates per treatment with 20 larvae per replicate

† Means within a column followed by the same lowercase letter are not significantly different (Tukey's HSD test: $p > 0.05$)

γ Control: 8 hours after brain-heart solution injection

Biological and chemical agents are known to disrupt the energy metabolism of insects, cause cell damage and alter the antioxidant system (İçen, Armutçu, Büyükgüzel, & Gürel, 2005; Sertçelik et al, 2018; Sugeçti & Büyükgüzel, 2018; Farahani, Bandani, Alizadeh, Goldansaz, & Whyard, 2020). In this study, it was determined that the cell damage indicators AST and ALT enzyme activities were increased in the hemolymph of *G. mellonella* larvae exposed to *K. oxytoca* for 8 hours. The reason for this increase may be cell damage due to the high virulence effect of the bacteria. In addition, ALP activity from metabolic enzymes was found to be decreased 8 hours after injection. The reason for this reduces may be increased oxidative damage due to the pathogenic effect of the bacteria. Studies have reported that ALP activity increases in insect tissues to regulate energy metabolism, which is disrupted by oxidative damage (Etebari et al, 2007; Sertçelik et al, 2018). In this study, it was determined that other metabolic GGT activity increased 8 hours after injection. GGT is an important metabolic enzyme that catalyzes the extracellular degradation of glutathione by hydrolyzing cysteine and γ glutamyl bond extracellularly. The amount of cysteine and glutamyl enters the cell and the synthesis of glutathione, an important antioxidant, increases (Sugeçti & Büyükgüzel, 2018). In this study, the increase in GGT activity may be physiological adaptation to increase the insect's antioxidant defense system. In another study, the physiological effects of an anthelmintic drug, oxfendazole, on the hemolymph of *G. mellonella* larvae were investigated. It has been determined that oxfendazole significantly increases the activity of cell damage indicators AST and ALT and the metabolic enzymes ALP, AMYL, CK and GGT (Sugeçti and Büyükgüzel 2018). In another study with similar results, the metal complex was injected into *G. mellonella* larvae and changes in metabolic enzymes activities were investigated. AST, ALT, LDH, CK, ALP and GGT activity increased significantly in the hemolymph of *G. mellonella* larvae 8 hours after injection of the metal complex (Sertçelik et al, 2018). In another study, transferase enzymes such as AST, ALT and GGT were increased in the hemolymph of great wax moth larvae due to *E.coli* infection (Sugeçti, 2021a).

Grizanova, Krytsyna, Surcova, & Dubovskiy (2019) reported that esterase activity was significantly reduced in *G. mellonella* midgut tissue due to *Bacillus thuringiensis* infection (Grizanova, Krytsyna, Surcova, & Dubovskiy, 2019). Wand, McCowen, Nugent, & Sutton (2013) reported that due to *K. pneumoniae* infection, LDH enzyme activity (cell damage indicator) increased in hemolymph of *G. mellonella* larvae and cell damage occurred (Wand, McCowen, Nugent, & Sutton, 2013). In another study, it was reported that cell damage was increased in *G. mellonella* larvae due to *K. pneumoniae* infection. It was determined that AST, ALT and LDH activities increased due to *K. pneumoniae* infection, and AMYL, ALP and CK enzyme activities also increased for the regulation of energy metabolism (Sugeçti, 2021b). These studies on the same insect show that biological and chemical agents increase the insect's metabolic enzymes and support our study.

Insects protect themselves against chemicals, especially insecticides and pathogenic bacteria infections with their antioxidant defense system (Dubovskii et al, 2010; Çelik & Sak, 2020). Antioxidant defense system is provided with enzymatic antioxidants such as CAT, SOD and GPx or non-enzymatic antioxidants such as ALB, UA and BIL. In this study, 8 hours after *K. oxytoca* injection, it was determined that the amount of non-enzymatic antioxidants BILT significantly increased. This increase may be due to adaptation to the oxidative damage caused by the pathogen. The amount of ALB and UA significantly decreased due to the infection. It has been reported in various studies that antioxidative levels increase in insects against the oxidative effects of pathogenic bacteria. In another study, it was reported that *G. mellonella* antioxidant effect increased due to *Bacillus thuringiensis* infection (Dubovskii et al, 2010). In another study, antioxidant enzymes such as SOD and GST levels were increased in *G. mellonella* larvae after infection with *B. thuringiensis* (Dubovskii, Olifirenko, & Glupov, 2005). Altuntaş & Duman (2017) reported that the antioxidant response in hemolymph of *G. mellonella* larvae to the entomopathogenic fungus *Purpureocillium lilacinus* infection altered as a physiological adaptation (Altuntaş & Duman 2017).

In this study, the amount of UREA in hemolymph of *G. mellonella* larvae significantly increased depending on the exposure time to the pathogen. The amount of TP decreased significantly due to *K. oxytoca* infection. It is known that especially the amount of protein increases in the regulation of energy metabolism against oxidative damage (Sertçelik et al, 2018; Çelik, Büyükgüzel, & Büyükgüzel, 2019). In this study, the reason for the decrease in the amount of TP may be damage due to infection. In particular, the increase in cell damage indicators AST and ALT activities supported this hypothesis. In a study, it has been reported that the chemical agent terbinafine (0.1 %) increased protein oxidation and causes protein damage in the midgut of *G. mellonella* larvae (Kastamonuluoğlu, Büyükgüzel, & Büyükgüzel, 2020). In another study, it was reported that the amount of TP increased in *G. mellonella* larvae exposed to different concentrations (0.003, 0.03 and 0.3%) of oxyclozanide (Çelik, Büyükgüzel, & Büyükgüzel, 2019). Etebari et al (2007) reported that the amount of TP and UREA in *Bombyx mori* L. (Lepidoptera: Bombycidae) larvae exposed to pyriproxyfen increased after 120 hours (Etebari et al, 2007). In another study, it was reported that biochemical parameters such as TP, UREA, cholesterol, ions and amount of non-enzymatic

antioxidants such as ALB, BIL and UA were altered due to 8 hours after *K. pneumoniae* infection in the hemolymph of last instar *G. mellonella* larvae (Sugeçti, 2021b).

The results obtained from this study showed that *G. mellonella* can be used as a model organism in the evaluation of the physiological effects of *K. oxytoca* infection on the host. As a result, this study showed that *G. mellonella* is an important experimental model in the investigation of biochemical interaction between pathogenic bacteria and host.

REFERENCES

Alp, E. & Coskun, M. (2018). Effects of the organophosphate insecticide fenthion on the antioxidant defense system and lipid peroxidation of *Galleria mellonella* L. *Fresenius Environmental Bulletin* 27, 8280-8285.

Altuntaş, H. & Duman, E. (2017). Toxicological effects of the entomopathogenic *Purpureocillium lilacinus* on the model organism, *Galleria mellonella*. *Biological Diversity and Conservation,* 10(1), 153-159.

Andrejko, M., Zdybicka-Barabas, A., & Cytryńska, M. (2014). Diverse effects of *Galleria mellonella* infection with entomopathogenic and clinical strains of *Pseudomonas aeruginosa*. *Journal of Invertebrate Pathology*, 115, 14-25.

Bassetti, M., Giacobbe D.R., Giamarellou, H., Viscoli, C., Daikos, G.L., Dimopoulos, G., & Karaiskos, I. (2018). Management of KPC-producing *Klebsiella pneumoniae* infections. *Clinical Microbiology and Infection*, 24, 133-144.

Bronskill, J. (1961). A cage to simplify the rearing of the greater wax moth, *Galleria mellonella* (Pyralidae)*, Journal of the Lepidopterists' Society,* 15, 102-104.

Çelik, C., Büyükgüzel, K., & Büyükgüzel, E. (2019). The effects of oxyclozanide on survival, development and total protein of *Galleria mellonella* L. (Lepidoptera: Pyralidae), *Journal of the Entomological Research Society*, 21, 95-108.

Çelik, E. & Sak, O. (2020). Effects of kinetin on biological parameters and hemocytes of *Achroia grisella* (Lepidoptera: Pyralidae). *Archives of Biological Sciences*, 72, 181-192.

Dubovskii, I. M., Olifirenko, O. A., & Glupov, V. V. (2005). Level and activities of antioxidants in intestine of larvae *Galleria mellonella* L. (Lepidoptera, Pyralidae) at peroral infestation by bacteria *Bacillus thuringiensis* ssp. galleriae. *Journal of Evolutionary Biochemistry and Physiology*, 41, 20-25.

Dubovskii, I.M., Grizanova, E.V., Chertkova, E.A., Slepneva, I.A., Komarov, D.A., Vorontsova, Y.L., & Glupov, V.V. (2010). Generation of reactive oxygen species and activity of antioxidants in hemolymph of the moth larvae *Galleria mellonella* (L.) (Lepidoptera: Piralidae) at development of the process of encapsulation. *Journal of Evolutionary Biochemistry and Physiology,* 46, 35-43.

Etebari, K., Bizhannia, A.R., Sorati, R., & Matindoost, L. (2007). Biochemical changes in haemolymph of silkworm larvae due to pyriproxyfen residue. *Pesticide Biochemistry and Physiology*, 88, 14-19.

Farahani, S., Bandani, A.R., Alizadeh, H., Goldansaz, S.H., & Whyard, S. (2020). Differential expression of heat shock proteins and antioxidant enzymes in response to temperature, starvation, and parasitism in the Carob moth larvae, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae), *PloS One*, 15, e0228104.

Grizanova, E. V., Krytsyna, T. I., Surcova, V. S., & Dubovskiy, I. M. (2019). The role of midgut nonspecific esterase in the susceptibility of *Galleria mellonella* larvae to *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, *166*, 107208.

Hu, Y., Wei, L., Feng, Y., Xie, Y., & Zong, Z. (2019). *Klebsiella huaxiensis* sp. nov., recovered from human urine. *International Journal of Systematic and Evolutionary Microbiology*, 69, 333-336.

İçen, E., Armutçu, F., Büyükgüzel, K., & Gürel, A. (2005). Biochemical stress indicators of greater wax moth exposure to organophosphorus insecticides. *Journal of Economic Entomology*, 98, 358-366.

Ishii, M., Matsumoto, Y., Yamada, T., Abe, S., & Sekimizu K. (2017). An invertebrate infection model for evaluating anti-fungal agents against dermatophytosis, *Scientific Reports*, 7, 1-11.

Kastamonuluoğlu, S., Büyükgüzel, K., & Büyükgüzel, E. (2020). The use of dietary antifungal agent terbinafine in artificial diet and its effects on some biological and biochemical parameters of the model organism *Galleria mellonella* (Lepidoptera: Pyralidae). *Journal of Economic Entomology*, 113, 1110-1117.

Lapointe, J.F., McCarthy, C.D., Dunphy, G.B., & Mandato, C.A. (2020). Physiological evidence of integrin-antibody reactive proteins influencing the innate cellular immune responses of larval *Galleria mellonella* hemocytes. *Insect Science*, 27, 239-255.

Matsumoto, Y., Ishii, M., Shimizu, K., Kawamoto, S., & Sekimizu, K. (2017). A silkworm infection model to evaluate antifungal drugs for cryptococcosis, *Medical Mycology Journal*, 58, 131-137.

Mirończuk-Chodakowska, I., Witkowska, A.M., & Zujko, M.E. (2018). Endogenous non-enzymatic antioxidants in the human body, *Advances in Medical Sciences*, 63, 68-78.

Ndrepepa, G., Colleran, R., & Kastrati, A. (2018). Gamma-glutamyl transferase and the risk of atherosclerosis and coronary heart disease, *Clinica Chimica Acta*, 476, 130-138.

Rønning, T.G., Aas, C.G., Støen, R., Bergh, K., Afset, J.E., Holte, M.S., & Radtke, A. (2019). Investigation of an outbreak caused by antibiotic-susceptible *Klebsiella oxytoca* in a neonatal intensive care unit in Norway, *Acta Paediatrica*, 108, 76-82.

Rossoni, R.D., Fuchs, B.B., De Barros, P.P., Velloso, M.D.S., Jorge, A.O.C., Junqueira, J.C., & Mylonakis, E. (2017). *Lactobacillus paracasei* modulates the immune system of *Galleria mellonella* and protects against *Candida albicans* infection. *Plos One*, *12*, e0173332.

Sertçelik, M., Sugeçti, S., Büyükgüzel, E., Necefoğlu, H., & Büyükgüzel, K. (2018). Toxicological and physiological effects of diaquabis (N, N-diethylnicotinamide-x N1) bis (4-formylbenzoato-x O) cobalt (II) complex on *Galleria mellonella* L. *Karaelmas Science and Engineering Journal*, 8, 359-364.

Shaik, H.A., Mishra, A., Sehadová, H., & Kodrík, D. (2020). Responses of sericotropin to toxic and pathogenic challenges: possible role in defense of the wax moth *Galleria mellonella. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 227, 108633.

Singulani, J.L., Scorzoni, L., De Oliveira, H.C., Marcos, C.M., & Assato, P.A. (2018). Fusco-Almeida, A.M. and Mendes-Giannini, M.J.S., Applications of invertebrate animal models to dimorphic fungal infections, *Journal of Fungi*, 4, 118.

SPSS (2006). *User's manual, Version 15. SPSS*, Chicago. IL.

Sugeçti, S., Büyükgüzel, E., & Büyükgüzel, K. (2016). Laboratory assays of the effects of oxfendazole on biological parameters of *Galleria mellonella* (Lepidoptera: Pyralidae), *Journal of Entomological Science*, 51, 129-137.

Sugeçti, S. & Büyükgüzel. K. (2018). Effects of oxfendazole on metabolic enzymes in hemolymph of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae reared on artificial diet. *Karaelmas Science and Engineering Journal*, 8, 590-594.

Sugeçti, S. (2021a). Biochemical and immune responses of model organism *Galleria mellonella* after infection with *Escherichia coli*. *Entomologia Experimentalis et Applicata*, 169, 911-917.

Sugeçti, S. (2021b). Pathophysiological effects of *Klebsiella pneumoniae* infection on *Galleria mellonella* as an invertebrate model organism. *Archives of Microbiology*, 203, 3509-3517.

Tominaga, T. (2018). Rapid detection of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Raoultella ornithinolytica* and other related bacteria in food by lateral-flow test strip immunoassays. *Journal of Microbiological Methods*, 147, 43-49.

Tsai, C.J.Y., Loh, J.M.S., & Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence*, 7, 214-229.

Wand, M.E., McCowen, J.W., Nugent, P.G., & Sutton, J.M. (2013). Complex interactions of *Klebsiella pneumoniae* with the host immune system in a *Galleria mellonella* infection model. *Journal of Medical Microbiology*, 62, 1790-1798.

Received: May 08, 2021 *Accepted: October 15, 2021*

J. Entomol. Res. Soc., 23(3): 287-304, 2021 *Review Article*
Doi:10.51963/jers.v23i3.2090 *Online ISSN:2651-3579*

Reproductive Biology of Thrips Insect Species and Their Reproductive Manipulators

Wondimagegn Atilaw WOLDEMELAK

Department of Entomology, Hungarian University of Agriculture and Life Sciences, 1118 Budapest, HUNGARY
e-mail: Wondimagegn.Atilaw.Woldemelak@hallgato.uni-szie.hu
ORCID ID: 0000-0002-9167-0100

ABSTRACT

Some thrips species are considered as model insect organisms to study reproductive biology since some closely related (sub) species were mainly reproducing by arrhenotoky, thelytoky and in some rare cases as deuterotoky. As other insect species, thrips are associated with endosymbiotic bacteria and this bacteria has led to manipulating their reproductive biology. The main goal of this paper is to review the reproductive modes, potential reproductive manipulators and their strategies to change the reproductive biology of thrips species. Thrips species are reproducing through, arrhenotoky, thelytoky and deuterotoky. *Wolbachia, Cardinium, Rickettsia* and *Spiroplasma* have been detected in the reproductive organs of some thrips species and the most common insect reproductive manipulators by causing cytoplasmic incompatibility, thelytokous parthenogenesis, male killing and feminization.

Keywords: Apomictic, Automictic, Endosymbionts, Thrips and *Wolbachia*.

Woldemelak, W.A. (2021). Reproductive biology of thrips insect species and their reproductive manipulators. *Journal of the Entomological Research Society*, 23(3), 287-304.

INTRODUCTION

The insect order Thysanoptera is composed of tiny insect species that are widespread throughout the world and in habitats ranging from forests, grasslands and scrublands to cultivated crops and gardens. The majority of thrips including almost all species of economic importance belong to the families Thripidae and Phlaeothripdae (Lewis, 1973).

Insect species in the order Thysanoptera exhibit haplodiploid sex determination mechanisms in the form of: (1) arrhenotoky (where virgin female produce haploid males and mated female produce diploid females). However, diploid male and polyploid female have been reported in *Thrips tabaci* (Lindeman) thrips species (Jacobson et al, 2013; Jacobson, Nault, Vargo, & Kennedy, 2016) and diploid males and triploid females were reported in *Heliothrips haemorrhoidalis* (Bouche, 1833) (Nguyen, Spooner-Hart, & Riegler, 2015) the parthenogenetic development of females, has independently evolved in several insect orders yet the study of its mechanisms has so far mostly focussed on haplodiploid Hymenoptera, while alternative mechanisms of thelytoky such as polyploidy are far less understood. In haplodiploid insects, thelytoky can be encoded in their genomes, or induced by maternally inherited bacteria such as *Wolbachia* or *Cardinium*. Microbially facilitated thelytoky usually results in complete homozygosity due to gamete duplication and can be reverted into arrhenotoky, the parthenogenetic development of males, through treatment with antibiotics. In contrast, genetically encoded thelytoky cannot be removed and may result in conservation of heterozygosity due to gamete fusion (Bouche, 1833).

Genetically inherited sex determinations such as automictic and apomictic thelytoky have displayed in the parthenogenetic thelytokous reproduction biology of insect species. (1) Automictic thelytokoy sex determination follows a meiotic cell division process. In this system, the new diploid female gamete has resulted from a single meiotically dividing cell, crossing over during meiosis retained (producing recombinant chromosomes) and diploid eggs produced by the fusion of sister or non-sister nuclei. (2) Apomictic thelytoky sex determination follows only a mitotic cell division process and it does not require the fusion of meiotic products, crossing over during meiosis not retained. The formation of eggs through a complete suppression of meiosis causes genetically identical progeny to their mother (Mogie, 1986; Engelsta, 2008; Heimpel & Boer, 2008). These two sex determination mechanisms are most likely common in the Hymenoptera insect species such as Sawfly *Strongylogaster maculata*, cynipid *Neoretus baccarum* and ant *Oecophylla longinoda* and ant *Wasmannia auropunctata*, Micromalthus, cecidomyid midges and cynipid wasps (Stenberg & Saura, 2009; Van Wilgenburg, Driessen, & Beukeboom, 2006; Rabeling & Kronauer, 2013). Although, these two sex determination has not been reported in the Thysanoptera insect species.

Endosymbionts are common in arthropod insect species and particularly known to occur in some thrips species (Table 2) (Stouthamer, Luck, & Hamilton, 1990; Arakaki, Miyoshi, & Noda, 2001). They are reproducing and detecting in the reproductive organs of their hosts. Research reports revealed that sexual distortion in arthropod has caused by endosymbiotic bacteria, nematode, and viruses (Kageyama, Narita, Watanabe, 2012). *Wolbachia, Cardinium, Rickettsia*, and *Spiroplasma* are the common

reproductive manipulators and have a potential to alter the reproductive modes of their hosts (Hendry, Hunter, & Baltrus, 2014; Ma, Vavre, & Beukeboom, 2014). Four major reproductive manipulation types are distinguished: cytoplasmic incompatibility, thelytokous parthenogenesis, male killing, and feminization. In this review, the effects of these manipulation types and how they interfere with arthropod sex determination in terms of host developmental timing, alteration of sex determination, and modification of sexual differentiation pathways are summarized. Transitions between different manipulation types occur frequently which suggests that they are based on similar molecular processes. It is also discussed how mechanisms of reproductive manipulation and host sex determination can be informative on each other, with a special focus on haplodiploidy. Future directions on how the study of endosymbiotic manipulation of host reproduction can be key to further studies of arthropod sex determination are shown including cytoplasmic incompatibility, male-killing, feminization, and parthenogenetic development, and can provide host protection against some viruses and other pathogens. Wolbachia differ from many other primary endosymbionts in arthropods because they undergo frequent horizontal transmission between hosts and are well known for an abundance of mobile elements and relatively high recombination rates. Some of the endosymbionts have stimulated thelytokous reproduction mode and enforced to produce female progenies by causing cytoplasmic incompatibility, thelytokous parthenogenesis, male killing and feminization (Arakaki et al, 2001; Nguyen, 2015). Reproductive biology of thrips species in the same field changed across years, which suggesting external factor that caused to manipulate the reproductive modes of thrips. To address the latter, this external factor might be the endosymbiotic bacteria, environmental mediated reproductive mode or genetic disorder of the female.

The main goal of this paper is to provide information from research literature about the reproductive biology of thrips species, potential reproductive manipulators and their strategies to change the reproductive biology of thrips species.

Reproductive modes of Thysanoptera

Arrhenotokous and thelytokous reproductive modes are a common phenomenon in many thrips species in the world (Table 1). However, deuterotokous reproductive mode is a relatively uncommon phenomenon for thrips species since it has been reported only for *A. apteris* (Strauss & Karban, 1994) and *T. tabaci* (Nault et al, 2006; Woldemelak, 2020).

Genetically inherited reproductive modes in Thysanoptera

Genetically inherited sex determination has become incredibly diversified and causing rapid change during evolution. According to Werren & Beukeboom (1998) report genetic conflict has played a vital role as the driving force of this diversity and turnover.

There are two basic forms of genetic conflict. Intragenomic conflict involves conflicting selective pressures between different genetic elements within an individual organism such as between cytoplasmic genes and autosomal genes. Intergenomic conflict occurs between genetic elements in different individuals that interact over

a particular phenotype. The genetic conflict occurred at a time of cell division while different parts of genetic systems are subjecting to line up in opposite directions. This conflict might be happening between paternal and maternal or within the genomes such as between cytoplasmic and nuclear genes or sex chromosomes and autosomes (Partridge & Hurst, 2003). Sex chromosomes are chromosomes needed for sex determination (male and female) and autosomes are all the rest of the chromosomes that are not needed for sex determination. Normark (2003) stated that three major classes of insect genetic sex determination systems. These are: a) diploid males (diplodiploidy), b) haploid males (haplodiploidy), and c) thelytoky.

Table 1. The reproductive modes of some Thysanopteran insect species.

***Thrips* species**	**Reproductive biology**	**Authors and years**
Aeolothrips vittatus	Thelytoky	(Morison, 1947)
Akainothrips citritarsus	Arrhenotoky	(Wrensch & Ebbert, 1993)
Anaphothrips obscurus	Thelytoky	(Morison, 1947)
Anaphothrips striatus	Arrhenotoky	(Risler & Kempter, 1961)
Anaphothrips sudanensis	Arrhenotoky	(Tikader, 1966)
Anthothrips niger	Arrhenotoky	(Risler & Kempter, 1961)
Apterothrips apteris	Arrhenotoky Thelytoky Deuterotoky	(Strauss & Karban, 1994)
Aptinothrips rufus	Arrhenotoky	(Sharga, 1933; Morison, 1947)
Caliothrips indicus	Thelytoky	(Ananthakrishnan, 1990)
Caliothrips fasciatus	Arrhenotoky	(Rugman-Jones et al, 2012; Lewis, 1973)
Chirothrips manicatus	Arrhenotoky	(Risler & Kempter, 1961)
Chirothrips mexicanus	Arrhenotoky	(Balu & Daniel, 1987; Ananthakrishnan & Daniel, 1981)
Chaetanaphothrips orchidii	Arrhenotokoy Thelytoky	(Pelikan, 1954; Lewis, 1993)
Echinothrips americanus	Arrhenotoky	(Li et al, 2012)
Euthrips tritici	Arrhenotoky	(Risler & Kempter, 1961)
Frankliniella fusca	Arrhenotoky	(Newsom, 1953)
Frankliniella insularis	Arrhenotoky	(Davidson & Bald, 1931)
Frankliniella oxidentalis	Arrhenotoky	(Wang et al, 2014)
Frankliniella schultzei	Arrhenotoky Thelytoky	(Gikonyo et al, 2016)
Frankliniella tritici	Arrhenotoky	(Ananthakrishnan, 1990)
Gynaikothrips ficorum	Arrhenotoky	(Wrensch & Ebbert, 1993)
Haplothrips niger	Arrhenotoky	(Loan & Holdaway, 1955)
Haplothrips subtilissimus	Thelytoky	(Putman, 1942)
Haplothrips niger	Arrhenotoky Thelytoky	(Ananthakrishnan, 1990)
Haplothrips simplex	Arrhenotoky	(Paccagnini et al, 2006)
Haplothrips statices	Arrhenotoky	(Ananthakrishnan, 1990)
Haplothrips statices	Arrhenotoky	(Risler & Kempter, 1961)
Haplothrips tritici	Thelytoky	(Ananthakrishnan, 1990)

Table 1. Continued.

***Thrips* species**	**Reproductive biology**	**Authors and years**
Haplothrips verbasci	Arrhenotoky	(Lewis, 1973)
Heliothrips haemorrhoidalis	Thelytoky Arrhenotoky	(Morison, 1947; Lewis, 1973; Han et al, 2011; Hamilton, 1967)
Helionothrips errans	Thelytoky	(Lewis, 1993)
Hercinothrips femoralis	Thelytoky	(Kumm & Moritz, 2008)
Hoplothrips pedicularius	Arrhenotoky	(Otte, 1979; Wrensch & Ebbert, 1993)
Hoplothrips fungi	Arrhenotoky	Wrensch & Ebbert, 1993)
Hoplothrips ulmi	Arrhenotoky	(Otte, 1979)
Katothrips tityrus	Arrhenotoky	(Wrensch & Ebbert, 1993)
Kladothrips rugosis	Arrhenotoky	(Wrensch & Ebbert, 1993)
Koptothrips dyskritus	Arrhenotoky	(Wrensch & Ebbert, 1993)
Koptothrips flavicornis	Arrhenotoky	(Wrensch & Ebbert, 1993)
Kurtomathrips morilli	Thelytoky	(Ananthakrishnan, 1990)
Leucothrips nigripennis	Thelytoky	(Lewis, 1993)
Limothrips cerealium	Arrhenotoky	(Sharga, 1933)
Limothrips denticornis	Arrhenotoky	(Wrensch & Ebbert, 1993; Hamilton, 1967)
Microcephalothrips abdominalis	Arrhenotoky	(Ananthakrishnan, 1990)
Onychothrips arotrum	Arrhenotoky	(Wrensch & Ebbert, 1993)
Oncothrips tepperi	Arrhenotoky	(Wrensch & Ebbert, 1993)
Onychothrips tepperi	Arrhenotoky	(Wrensch & Ebbert, 1993)
Parthenothrips dracaenae	Arrhenotoky Thelytoky	(Ananthakrishnan, 1990; Morison, 1947)
Pseudoarticulella obscurus	Thelytoky	(Morison, 1947)
Rhipiphorothrips cruentatus	Arrhenotoky	(Ananthakrishnan, 1990)
Rhopalothripoides froggatti	Arrhenotoky	(Wrensch & Ebbert, 1993)
Sciothrips cardamom	Arrhenotoky	(Ananthakrishnan, 1990)
Scirtothrips bispinoslls	Arrhenotoky	(Ananthakrishnan, 1990)
Scirtothrips citri	Arrhenotoky	(Lewis, 1973)
Scirtothrips dorsalis	Arrhenotoky	(Ananthakrishnan, 1990)
Scirtothrips longipennis	Thelytoky	(Lewis, 1993)
Scirtothrips perseae	Arrhenotoky	(Hoddle, 2002)
Scolothrips sexmaculatus	Arrhenotoky	(Coville & Allen, 1977)
Taeniothrips vulgatissimus	Arrhenothrips	(Lewis, 1993)
Taeniothrips inconsequens	Arrhenotoky	(Davidson & Bald, 1931)
Thrips linarius	Arrhenotoky	(Lewis, 1973)

Table 1. Continued.

***Thrips* species**	**Reproductive biology**	**Authors and years**
Thrips nigropilosus	Arrhenotoky Thelytoky	(Morison, 1947; Nakao & Yabu, 1998)
Thrips calcaratus	Thelytoky	(Parker et al, 1995)
Thrips tabaci	Arrhenotoky Thelytoky Deuterotoky	(Jacobson et al, 2013; Li et al, 2014; 2015 Nault et al, 2006; Woldemelak, 2020)
Warithrips maelzeri	Arrhenotoky	(Wrensch & Ebbert, 1993)
Xaniothrips leukandrus	Arrhenotoky	(Wrensch & Ebbert, 1993)
Xaniothrips xantes	Arrhenotoky	(Wrensch & Ebbert, 1993)
Zaniothrips ricini	Arrhenotokous	(Ananthakrishnan, 1990)

Diplodiploidy sex determination

In this system, the full cycle of meiosis and a fusion of two cells is involved in each generation. Both the father and mother give a haploid set of chromosomes to each offspring and therefore, the maternally and paternally derived chromosome regions have equal probabilities of being presented (Normark, 2003). Every male progeny genetically shares one haploid genome from his mother and one haploid genome from his father by default. Thus, all the offspring carry a diploid genome. Diplodiploidy sex determination mechanism has been reported in the large majority of insect orders except Thysanoptera and Hymenoptera (Normark, 2003). Diploid male have been reported in the arrhenotokous *T. tabaci* (Jacobson et al, 2016). However, this report has failed to explain the ploidy level of the next generation. It did not give an answer whether these diploid males are fertile or not. Thus, it is difficult to conclude that Thysanoptera insect species have diplodiploidy genome size.

Haplodiploid sex determination

Haplodiploidy is a genetically inherited sex determination system and the well-known form of haplodiploidy is arrhenotokous haplodiploidy. Insect species in the order of Thysanoptera usually have haplodiploidy reproductive mode, since the majority of the species are arrhenotoky (Ananthakrishnan, 1990). However, there are some few thrips species, which produce only female progeny by thelytoky reproduction mode. In this sex determination system, the male genes do not carry the paternal genome and all of the parental sperm cell allowed to transfer an identical band of maternal chromosomes to the offspring (Haig, 1993; Kuijper & Pen, 2010). The haploid male progeny that produced from the mated female did not pass through the meiosis cell division process (Brown 1963; Goldstein, 1994). The haplodiploid sons do not carry traits from their fathers but the maternal grandfathers are their closest male progenitors (Filia et al. 2015). The following sex determination models has reported in some of the Insecta orders.

Arrhenotokous haplodiploidy (Arrhenotoky): Every male produced from an unfertilized egg and has only a haploid genome inherited from his mother (Ananthakrishnan, 1990). It is not only found in Thysanoptera, but also in Hymenoptera, Hemiptera, and Coleoptera (Heimpel & Boer, 2008) in which diploid females develop

from fertilized eggs and haploid males develop from unfertilized eggs. We discuss recent progress in the understanding of the genetic and cytoplasmic mechanisms that make arrhenotoky possible. The best-understood mode of sex determination in the Hymenoptera is complementary sex determination (CSD). Hartl & Brown (1970) the factor is assumed to be an environmental variable; in the other model the factor is a newly mutated gene. In both cases there follows a population in which haploid males and diploid males coexist.

Paternal genome elimination (PGE): “it is a subset of haplodiploidy in which females and males develop from fertilized eggs, then subsequently the paternal genome set is eliminated” (Heimpel and Boer 2008) in which diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs. The best-understood mode of sex determination in the Hymenoptera is complementary sex determination (CSD). Lastly the male carries only haploid genes from his mother (Filia, Bain, & Ross, 2015). It is reported mainly in Hemiptera, Coleoptera, and Diptera insect orders (Heimpel & Boer, 2008) in which diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs. However, it did not get research attention from Thysanoptera insect species. In haplodiploid species, three types of paternal genome elimination are reported (Filia et al; 2015). Such as a) (N) haploid male in which the male is produced from an unfertilized egg. b) (2N) in which the zygote is formed from the half-paternal and half maternal genome, yet during cleavage the paternal genomes are eliminated and only the maternal genome is transmitted through male sperm. c) (2N*) where the paternal genome has transcriptionally silenced in somatic cells and N for embryonic PGE.

Thelytokous

It is a type of parthenogenetic reproduction mode. The females transmit only maternal genes and produce only daughters. In another word, it favours the unfertilized eggs that develop into females. In this reproduction mode, there is a complete lack of males. Either genetically inherited or intracellular bacteria determine this reproductive mode. The major features of thelytoky reproduction biology are: (a) reproductive efficiency, (b) there is no energy wastage for mating and (c) a lack of recombination between the genomes of different individuals. In thelytokous reproduction system the individual ploidy level is varied. The thelytokous *T. tabaci* strain comprises of a diploid and tetraploid genome (Jacobson et al, 2013; 2016). The polyploidy genome might be favouring the thelytokous reproduction mode (Nguyen et al, 2015). In haplodiploid insects, thelytoky can be encoded in their genomes, or induced by maternally inherited bacteria such as *Wolbachia* or *Cardinium*. Microbially facilitated thelytoky usually results in complete homozygosity due to gamete duplication and can be reverted into arrhenotoky, the parthenogenetic development of males, through treatment with antibiotics. In contrast, genetically encoded thelytoky cannot be removed and may result in conservation of heterozygosity due to gamete fusion. There are two major cytoplasmic mechanisms to induce thelytokous parthenogenesis and both resulting diploid individuals, such as automictic and apomictic thelytokous.

Automictic thelytoky: It follows a meiosis cell division process where a new progeny obtained from a product of single meioticaly dividing cell, crossing over during meiosis retained, and diploid eggs formed by the fusion of sister or non-sister nuclei containing recombinant chromosomes (Mogie, 1986; Heimpel & Boer, 2008). Gamete duplication goes homozygous after one generation. Likely, recombination does not have any impression on the distribution of the numbers of deleterious mutations per individual (Haccou & Schneider, 2003) only a few specific reproductive modes are considered in the search of explanations for the maintenance of sex. There are, however, many alternatives. Including these may give radically different conclusions. The theory on deterministic deleterious mutations states that in large populations segregation and recombination may lead to a lower load of deleterious mutations, provided that there are synergistic interactions. Empirical research suggests that effects of deleterious mutations are often multiplicative. Such situations have largely been ignored in the literature, since recombination and segregation have no effect on mutation load in the absence of epistasis. However, this is true only when clonal reproduction and sexual reproduction with equal male and female ploidy are considered. We consider several alternative reproductive modes that are all known to occur in insects: arrhenotoky, paternal genome elimination, apomictic thelytoky, and automictic thelytoky with different cytological mechanisms to restore diploidy (Haccou & Schneider, 2003). Diploidy is regenerated during or after meiosis and chromosome number may be doubled during the first mitotic division following meiosis and giving rise to diploid eggs that are homozygous at all loci (Engelsta, 2008). Mostly automictic thelytoky is common in Hymenoptra insects and the diploid female is obtained by terminal fusion, central fusion and gamete duplication (Stenberg & Saura, 2009; Rabeling & Kronauer, 2013).

Apomictic thelytoky: It is a mitotic parthenogenetic reproduction, thus it does not require full meiosis cell division and fusion of meiotic cell products. The eggs are formed through complete suppression of meiosis and the developed progenies are genetically identical to their maternal (Engelsta, 2008). The egg cell is produced through mitotic and consequently the progenies consist of identical genomes to their mother (Rabeling & Kronauer, 2013). Exclusively one cell division mastered during the mitosis process and the number of chromosomes has not reduced. The diploid females obtained from the true clones of the mother due to the lack of genetic recombination. Over time, mutations are thought to accumulate independently in the two alleles at any given locus, leading to genetic divergence and high levels of heterozygosity in ancient apomictic lineages (Welch & Meselson, 2000). However, ameiotic recombination in apomictic lineages can lead to the loss of heterozygosity, which can have a substantially stronger effect on genome evolution than the accumulation of mutations can (Omilian, Cristescu, Dudycha, & Lynch, 2006). It is common in aphids, the beetle *Micromalthus*, cecidomyid midges and cynipid wasps (Stenberg & Saura, 2009).

Sex determinant endosymbionts

Endosymbionts are living microorganisms, which inhabit within the body and cells of living organisms. The interaction between hosts and intracellular endosymbionts

is commonly found in many arthropods species (Douglas, 1998; Newton et al, 2016). Endosymbionts are reproducing within the generative cells such as sperm and ovaries of the host and persist mutually for a prolonged period of time (Weinert, Araujo-Jnr, Ahmed, & Welch, 2015). The interaction between endosymbionts and host insect species induce reproductive changes of the host (Weeks, Velten, & Stouthamer, 2003; Kageyama, Narita, & Watanabe, 2012). Recent evidence suggests that another intracellular bacterium, a Cytophaga-like organism (CLO) and improve the survival rate and evolution of the hosts (Moran & Telang, 1998). Some of the endosymbionts induce parthenogenetic thelytokous reproduction (Arakaki et al, 2001; Huigens, De Almeida, Boons, Luck, & Stouthamer, 2004; Nguyen, 2015). Occasionally, these symbiotic bacteria manipulate the reproductive biology of the hosts to enhance their own transmission and reduce the gene flow between populations and they allow genetic divergence for locally adaptive strains (Engelsta & Telschow, 2009). In spider mites, the prevalence of endosymbiotic bacteria is significantly more common in females than males, suggesting it might be behaving as a sex-ratio distorter (Duron, Hurst, Hornett, Josling, & Engelstädter, 2008).

The major parthenogenesis inducing endosymbionts

Wolbachia, Cardinium, Rickettsia, and *Spiroplasma* are the common parthenogenesis inducing endosymbionts for various insects and completely ascertain the reproductive biology of the hosts (Hendry, Hunter, & Baltrus, 2014; Ma et al, 2014). Endosymbionts, such as *Wolbachia*, *Cardinium*, *Rickettsia*, and *Spiroplasma*, can manipulate host reproduction. Four major reproductive manipulation types are distinguished: cytoplasmic incompatibility, thelytokous parthenogenesis, male killing, and feminization. In this review, the effects of these manipulation types and how they interfere with arthropod sex determination in terms of host developmental timing, alteration of sex determination, and modification of sexual differentiation pathways are summarized. Transitions between different manipulation types occur frequently which suggests that they are based on similar molecular processes. It is also discussed how mechanisms of reproductive manipulation and host sex determination can be informative on each other, with a special focus on haplodiploidy including cytoplasmic incompatibility, male-killing, feminization, and parthenogenetic development, and can provide host protection against some viruses and other pathogens. Wolbachia differ from many other primary endosymbionts in arthropods because they undergo frequent horizontal transmission between hosts and are well known for an abundance of mobile elements and relatively high recombination rates.

Wolbachia is a member of Alphaprotoeobacteria class and the most desirable and abundant parthenogenesis-inducing endosymbiont that determines the reproductive biology of its hosts (Yen & Barr, 1971). There are two main kinds of *Wolbachia* (A and B) that diverged from 58 to 67 million years before present based upon synonymous substitution rates (Werren, Zhang, & Guo, 1995).

The parthenogenesis-inducing bacteria favour female-biased offspring (Engelsta & Telschow, 2009). When Wolbachia-infected male sperm fertilized the uninfected

female egg: (1) it caused to fail the first division of the new embryo, (2) the male chromosomes never completely condensed to proceed meiosis cell division and (3) after all, it caused to divide the male chromosome improperly (Nguyen, 2015). It seems that sperm from infected male hold a factor to interfere with male cell divisions and resulting early death of male embryos and only allows the cell division to produce a parthenogenetic female. Similarly, the Wolbachia-free female coupled with infected male fails to develop male progeny (Yishay, 2009).

Most likely, a parthenogenetic female sex manipulator endosymbionts are common in the major insect orders such as Hymenoptera, Coleoptera, Diptera, Hemiptera, Lepidoptera, and Orthoptera. However, the overall extent of Wolbachia prevalence is higher in Hymenoptera insect order than in the others (Werren et al, 1995). A recent report showed that Wolbachia bacteria has induced the reproduction biology of Thysanoptera insect order (Nguyen, Morrow, Spooner-Hart, & Riegler, 2017)yet interactions between both bacterial endosymbionts are rarely studied.

Table 2. Reproductive manipulating endosymbionts in thrips species.

Thrips species	Types of bacteria evolved	Author and year
Aptinothrips rufus	Wolbachia	(Van der Kooi & Schwander, 2014)
Caliothrips fasciatus	Wolbachia	(Rugman-Jones et al, 2012)
Echinothrips americanus	Wolbachia	(Kumm & Moritz, 2008)
Franklinothrips vespiformis	Wolbachia	(Arakaki et al, 2001)
Gynaikothrips ficorum	Wolbachia	(Kumm & Moritz, 2008)
Heliothrips haemorrhoidalis	Wolbachia	(Koivisto & Braig, 2003)
Hercinothrips femoralis	Wolbachia	(Kumm & Moritz, 2008)
Parthenothrips dracaenae	Wolbachia	(Kumm & Moritz, 2008)
Pezothrips kellyanus	Cardinium	(Nguyen et al, 2017)
Suocerathrips linguis	Wolbachia	(Kumm & Moritz, 2008)
Sciothrips cardamomi	Wolbachia	(Jacob et al, 2014)
Thrips palmi	Wolbachia	(Saurav et al, 2016)
Thrips tabaci	Wolbachia	(Gawande et al, 2019)

Endosymbionts strategies to manipulate the reproduction biology of their hosts

The endosymbionts are using various strategies to distort the reproduction biology of their hosts and enhance their own transmission to the next generation. Among them cytoplasmic incompatibility, thelytokous parthenogenesis, male killing and feminization are the most common strategies (Neill, Giordano, Colbert, Timothy, & Robertson, 1992; Schilthuizen et al, 1992; Cook & Butcher, 1999; Bandi, Dunn, Hurst, & Rigaud, 2001; Koivisto & Braig, 2003; Dale & Moran, 2006; Hendry et al, 2014; Ma et al, 2014; Newton et al, 2016) such as female-biased sex ratios, parthenogenesis, and sterility of crosses either between infected males and uninfected females or between infected individuals (Weeks et al, 2003; Zabalou et al, 2004; Yishay, 2016). These strategies are providing

them either to produce more females or to cause uninfected females reproductively incompatible with infected males (Nguyen et al, 2017) yet interactions between both bacterial endosymbionts are rarely studied.

Cytoplasmic Incompatibility (CI)

CI is the process that causes the mating of male and female ineffective to develop the feasible progeny. It results from modification in the gamete cells caused by intracellular endosymbiotic bacteria. It has been reported for six insect orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera) and terrestrial crustaceans and mites (Ma et al, 2014). The co-infect of *Wolbachia* and *Cardinium* causes CI in Thysanoptera insect species (Nguyen et al, 2017).

Endosymbiotic bacteria induce CI in two ways, such as unidirectional and bidirectional. In the first hand, unidirectional cytoplasmic incompatibility is resulted from crossing between infected male and uninfected female. Unidirectional CI has led to the death of embryos while the infected male mate with the uninfected female (Breeuwer & Werren, 1990; O'neill & Karr, 1990). On the other hand, bidirectional cytoplasmic incompatibility occurred while the infected male mates with a female infected by a different strain of endosymbiont (Merqot, Liorente, Jacques, Atlan, & Montchamp-Moreau, 1995; Perrot-Minnot, Guo, & Werren, 1996). Cytological studies reported that during incompatible crosses: the paternal chromosomes do not condense, harmed and eventually are lost during the first mitotic divisions (Callaini, Dallai, & Riparbelli, 1997; Tram, Sullivan, Tram, & Sullivan, 2002). However, viable offspring can produce from both unidirectional and bidirectional cytoplasmic incompatibility if both parents genes are infected by the same endosymbiotic bacterial strain. Cytoplasmically incompatible genes have no biological involvement for the male development; rather it enhances male evolutionary dead-end (Hurst & Parks, 1993). Thus, a bacteria permits its host to develop female-biased offspring (Partridge and Hurst 2003).

The endosymbiotic bacteria has caused delayed the paternal chromatin condensation and allowed the maternal chromosomes to anaphase cell division process. Among these bacterial species, *Wolbachia* bacteria is one of the most common genus that leads to disruption of the condensation of crossing the cytoplasmically incompatible strains of parental chromatin compliments. This effect has been well studied in the wasp *Nasonia vitripennis* and *Drosophila simulans* (Ryan & Saul, 1968; O'Neill & Karr, 1990; Callaini et al, 1997). For normal cell division, both the maternal and paternal chromosomes line up on the middle of the equator and both chromosomes would be passed to the anaphase stage. Whereas, the infected paternal chromosomes have condensed improperly and delayed at the metaphase plate, but female chromatids have accomplished anaphase plate and migrated to the opposite poles of the spindle. This delay at the anaphase stage has led to dramatic errors in paternal chromatin inheritance.

Thelytokous parthenogenesis

Reversible or microbe-induced and irreversible thelytoky are the two parthenogenetic thelytokous that induced by endosymbiotic microbes (Stouthamerff & Kazmert,

1994). High temperature did not affect sex allocation ratios in either thelytokous or male-producing *T. tabaci* populations (Nault et al, 2006). In the reversible thelytoky, male produced after the removal of microbes via high temperature or antibiotic treatment. Whereas, the irreversible thelytoky endosymbiotic bacteria is not detected and neither high temperature nor antibiotic treatment induces the production of males (Stouthamer, Luck, & Hamilton, 1990).

Male killing

Maternally inherited endosymbionts has been reported to distort the male embryo in a broad range of insect host species (Hurst & Jiggins, 2000). They can distort the sex ratio of their hosts by killing the infected male offspring (Groenenboom & Hogeweg, 2002). Hurst (1991) stated that there are two types of male-killing cytoplasmic genes characterized by their time of activity. (1) Early male killer:- it affects the embryo and kill the male gene at embryo stage. Male killing at embryo stage has been reported in Diptera, Hymenoptera, Coleoptera, Lepidoptera, and Hemiptera insect orders. However, there is no research report for thrips species. (2) Late male killer: - the male dies at the fourth instar larval developmental stage and most commonly observed only in mosquitoes.

Hurst & Majerus (1993) reviewed three evolutionary advantages why maternally inherited microorganisms do kill the male. First, killing males may produce enough food resources to sibling females that bear a clonal relative of the bacterium. Such an enhancement in resources availability might be either direct, from permitting the consumption of the soma of male eggs, or indirect, from the reduction of competition suffered by sisters of such individuals. Such resource reallocation conceived to increase the fitness of the daughters, the line through which the microbe may pass itself vertically. Second, avoidance of inbreeding, females, which bear the microbe, will have few if any brothers, and are thus less likely to inbreed. Their daughters are thus less likely to suffer from inbreeding depression. Third, male's death may be a mechanism to produce the horizontal transfer of the microbe out of the male lineage, through which it cannot passes vertically, into other organisms (Higashiura, Ishihara, & Schaefer, 1999).

The endosymbiotic bacteria is transmitting to its host insect through vertical and horizontal pathways (Huigens et al, 2004). The vertical transmission pathway displays the major way of endosymbiotic transmission from mother to offspring (Hoffmann et al, 1990; Bandi et al, 2001). The hereditary bacteria infect the offspring at the time of embryogenesis process and the progeny emerged with cytoplasmic inducing bacteria. Whereas in the horizontal mode of transmission the infection of endosymbionts happens between interspecies and the infection might be happening during feeding.

CONCLUSION AND FUTURE RESEARCH REMARKS

Thysanoptera insect species prominently reproduced by arrhenotoky, thelytoky and occasionally deuterotoky. Arrhenotokous (male produced from the unfertilized eggs), thelytokous (virgin females produce only female progenies or female progenies are

producing from unfertilized eggs) and deuterotoky (virgin female produce both male and female). Thysanoptera order insect species usually pointed out haplodiploid sex determination.

Endosymbionts exhibit a vital role to change the evolutionary biology of their hosts and trigger parthenogenetically thelytokous reproduction mode. *Wolbachia, Cardinium, Rickettsia* and *Spiroplasma* are the most common parthenogenic endosymbionts and have a potential to change the reproduction biology of some thrips species. Most likely, *Wolbachia* bacteria exhibit the highest percentages to infect the arthropod insect species and induces to produce female sex-biased offspring. These endosymbionts have used reproductive manipulating strategies such as cytoplasmic incompatibility, thelytokous parthenogenesis, male killing, and feminization. Apomictic and automictic thelytokous reproduction might manipulate the sex of thrips species. The role of these two genetically thelytokous inducing and the role ploidy levels in the sex determination of thrips species would be the future research area.

REFERENCES

Ananthakrishnan, T. (1990). *Reproductive biology of thrips*. Indira Publishing House USA, pp 4-16.

Ananthakrishnan, T. & Daniel, A. (1981). Behavioural components in feeding, reproduction and dispersal of the grass seed-feeeding Thrips chirthrips mexicanus crawfolrd. *Current Science*, 50, 733-735.

Arakaki, N., Miyoshi, T., & Noda, H. (2001). *Wolbachia*-mediated parthenogenesis in the predatory thrips Franklinothrips vespiformis (Thysanoptera: Insecta). *Proceedings of the Royal Society B: Biological Sciences*, 268(1471), 1011-1016.

Balu, A. & Daniel, M. (1987). Eco-behavioural and biological studies on two species of seed infesting thrips (Thyasanoptera: Insecta). *Proceedings: Animal Sciences*, 96(1), 23-32

Bandi, C., Dunn, A.M., Hurst, G.D.D., & Rigaud, T. (2001). Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends in Parasitology*, 17(2), 88-94

Breeuwer, J.A.J., & Werren, J.H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between insect species. *Nature,* 346, 558-560.

Brown, S. (1963). Automatic frequency response in the evolution of male haploidy and other coccid chromosome systems. *Genetics*, 49,797-817.

Callaini, G., Dallai, R., & Riparbelli, M.G. (1997). *Wolbachia*-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans*. *Journal of Cell Science*, 110(2), 271-280.

Cook, J.M., Butcher, R.D.J. (1999). The transmission and effects of *Wolbachia* bacteria in parasitoids. *Researches on Population Ecology*, 41(1), 15-28.

Coville, P.L. & Allen, W.W. (1977). Life table and feeding habits of *Scolothrips sexmaculatus* (Thysanoptera: Thripidae). *Annals of the Entomological Society of America*, 70(1), 11-16.

Dale, C. & Moran, N.A. (2006). Molecular interactions between bacterial symbionts and their Hosts. *Cell*, 126(3), 453-465. https://doi.org/10.1016/j.cell.2006.07.014

Davidson, J. & Bald, J. (1931). Sex determination in *Frankliniella insularis* (Thysanoptera). *The Australian Journal of Experimental Biology and Medical Scienc*e, 8(2), 139-142.

Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbiosis: Aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology*, 43(1), 17-37.

Duron, O., Hurst, G.D.D., Hornett, E.A., Josling, J.A., & Engelstädter, J. (2008). High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Molecular Ecology,* 17(6), 1427-1437.

Engelsta, J. (2008). Constraints on the evolution of asexual reproduction. *Bio Essays*, 30(11-12), 1138-1150. https://doi.org/10.1002/bies.20833

Engelsta, J. & Telschow, A. (2009). Cytoplasmic incompatibility and host population structure. *Heredity,* 103(3), 196-207.

Engelstädter, J. & Hurst, G. D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology Evolution and Systematics*, 40, 127-149.

Filia, G.D., Bain, S.A., & Ross, L. (2015). Haplodiploidy and the reproductive ecology of arthropods. *Current Opinion in Insect Science*, 9, 36-43.

Gawande, S.J., Anandhan, S., Ingle, A., Roylawar, P., Khandagale, K., Gawai, T., & Singh, M. (2019). Microbiome profiling of the onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae). *PloS one*, 14(9), e0223281.

Gikonyo, M.W., Niassy, S., Moritz, G.B., Khamis, F.M., Magiri, E., & Subramanian, S. (2016). Resolving the taxonomic status of *Frankliniella schultzei* (Thysanoptera: Thripidae) colour forms in Kenya a morphological-, biological-, molecular- and ecological-based approach. *International Journal of Tropical Insect Science*, 37(02), 57-70. Doi: 10.1017/s1742758416000126

Goldstein, D.B. (1994). Deleterious mutations and the evolution of male haploidy. *The American Naturalist,* 144(1), 176-183.

Groenenboom, M.A.C. & Hogeweg, P. (2002). Space and the persistence of male-killing endosymbionts in insect populations. *Proceedings of the Royal Society B: Biological Sciences,* 269(1509), 2509-2518. doi:10.1098/rspb.2002.2197.

Haccou, P. & Schneider, M.V. (2003). Modes of reproduction and the accumulation of deleterious mutations with multiplicative fitness effects. *Genetics*, 166(2), 1093-1104. https://doi.org/10.1534/genetics.166.2.1093

Haig, D. (1993). The evolution of unusual chromosomal systems in coccoids: extraordinary sex ratios revisited. *Journal of Evolutionary Biology*, 6(1), 69-77.

Hamilton, W.D. (1967). Extraordinary sex ratios. *Science*, 156(3774), 477-488.

Han, F., Liu, X.X., Tian, J.C., Zhang, Q.W., & Shelton, A. (2011). A new source of cabbage host plant resistance to the diamondback moth (Lepidoptera: Plutellidae). *Florida Entomologist*, 94(3), 711-714

Hartl, D. & Brown, S. (1970). The origin of male haploid genetic systems and their expected sex ratio. *Theoretical Population Biology,* 1, 165-190.

Heimpel, G.E. & De Boer, J.G. (2008). Sex determination in the Hymenoptera. *Annual Review of Entomology*, 53(1), 209-230.

Hendry, T.A., Hunter, M.S., & Baltrus, D.A. (2014). The facultative symbiont *Rickettsia* protects an invasive whitefly against entomopathogenic *Pseudomonas* syringae strains. *Applied and Environmental Microbiology,* 80, 7161-7168.

Higashiura, Y., Ishihara, M., & Schaefer, P.W. (1999). Sex ratio distortion and severe inbreeding depression in the gypsy moth *Lymantria dispar* L. in Hokkaido Japan. *Heredity*, 83(3), 290-297.

Hoddle, M.S. (2002). Developmental and reproductive biology of *Scirtothrips perseae* (Thysanoptera: Thripidae): a new avocado pest in California. *Bulletin of Entomological Research*, 92(4), 279-285.

Hoffmann, A.A., Turellit, M., & Harshman, L.G. (1990). Factors affecting the distribution of cytoplasmic incompatibility in. *Genetics Society of America*, 126, 933-948.

Huigens, M.E., De Almeida, R.P., Boons, P.A.H., Luck, R.F., & Stouthamer,R. (2004). Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proceedings of the Royal Society B: Biological Sciences*, 271(1538), 509-515.

Hurst, G.D. & Majerus, M.E. (1993). Why do maternally inherited microorganisms kill males? *Heredity,* 71(1), 81.

Hurst, G.D. & Jiggins, F.M. (2000). Male-killing bacteria in insects: mechanisms, incidence, and implications. *Emerging Infectious Diseases,* 6(4), 329.

Hurst, L.D. & Parks, S. (1993) The incidences. Mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biological Reviews*, 68(1), 121-194.

Hurst, L.D. (1991). The incidences and evolution of cytoplasmic male killers. *Proceedings of the Royal Society B: Biological Sciences,* 244(1310), 91-99.

Hurst, L.D.(1993). The incidences. Mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biological Reviews*, 68(1), 121-194

Jacob, T.K.D., Silva, S., Senthil Kumar, C.M., Devasahayam, S., Rajalakshmi, V., Sujeesh, E.S., & Abraham, S. (2015). Single strain infection of adult and larval cardamom thrips (*Sciothrips cardamomi*) by *Wolbachia* subgroup Con belonging to super group B in India. *Invertebrate Reproduction and Development*, 59(1), 1-8.

Jacobson, A.L., Johnston, J.S., Rotenberg, D., Whitfield, A.E., Booth, W., Vargo, E.L., & Kennedy, G.G. (2013). Genome size and ploidy of Thysanoptera. *Insect Molecular Biology*, 22(1), 12-17.

Jacobson, A.L., Nault, B.A., Vargo, E.L., & Kennedy, G.G. (2016). Restricted gene flow among lineages of *Thrips tabaci* supports genetic divergence among cryptic species groups. *Plos One*, 11(19), e0163882.

Kageyama, D., Narita, S., & Watanabe, M. (2012). Insect sex determination manipulated by their endosymbionts: Incidences, mechanisms and implications. *Insects*, 3(1), 161-199. https://doi.org/10.3390/insects3010161

Koivisto, R.K. & Braig, H.R. (2003). Intraclonal genetic variation: ecological and evolutionary aspects. In H.D., Loxdale, G., Lushai (Eds.). Microorganisms and parthenogenesis. *Biological Journal of the Linnean Society,* 79(1), 43-58.

Kuijper, B. & Pen, I. (2010). The evolution of haplodiploidy by male-killing endosymbionts: importance of population structure and endosymbiont mutualisms. *Journal of Evolutionary Biology*, 23(1), 40-52.

Kumm, S. & Moritz, G. (2008). First detection of *Wolbachia* in arrhenotokous populations of Thrips Species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. *Environmental Entomology*, 37(6), 1422-1428.

Lewis, T., (1973). *Thrips their biology, ecology and economic importance* (pp. 36-50). London, UK: Academic Press Inc.

Li, X.W., Fail, J., Wang, P., Feng., J.N., & Shelton, A.M. (2014). Performance of arrhenotokous and thelytokous *Thrips tabaci* (Thysanoptera: Thripidae) on onion and cabbage and its implications on evolution and pest management. *Journal of Economic Entomology,* 107(4), 1526-1534. Doi: 10.1603/ec14070

Li, X.W., Wang, P., Fail, J., & Shelton, A.M. (2015). Detection of gene flow from sexual to asexual lineages in *Thrips tabaci* (Thysanoptera: Thripidae). *PLOS ONE,* 10(9), 1-13

Li, X.W., Zhang, X.C., Jiang, H.X., & Feng, J.N. (2012). Comparisons of developmental and reproductive biology between parthenogenetic and sexual *Echinothrips americanus* (Thysanoptera: Thripidae). *Environmental Entomology,* 41(3), 706-713. Doi: 10.1603/en11325

Loan, H.F.G. (1955). Biology of the red clover thrips, *Haplothrips niger* (Osborn) (Thysanoptera: Phloeothripidae). *The Canadian Entomologist,* 87(5), 210-219

Ma, W.J., Vavre, F., & Beukeboom, L.W. (2014). Manipulation of arthropod sex determination by endosymbionts: Diversity and molecular mechanisms. *Sexual Development,* 8(1-3), 59-73. Doi: 10.1159/000357024

Merqot, H., Liorente, B., Jacques, M., Atlan, A., & Montchamp-Moreau, C. (1995). Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans. Genetics*, 141(3), 1015-1023.

Mogie, M. (1986). Automixis: its distribution and status. *Biological Journal of the Linnean Society*, 28(3), 321-329

Moran, N.A. & Telang, A. (1998). Bacteriocyte-associated symbionts of insects. *Bioscience*, 48(4), 295-304

Morison, G.D. (1947). Thysanoptera of the London area. *London Naturalist*, 59, 1-131

Nakao, S. & Yabu, S. (1998). Genetics of wing polymorphism in the arrhenotokous *Thrips nigropilosus* Uzel. *Japan Journal of Entomology* (N. S.) 1, 9-19

Nault, B.A., Shelton, A.M., Gangloff-Kaufmann, J.L., Clark, M.E., Werren, J.L., Cabrera-la Rosa, J.C., & Kennedy, G.G (2006). Reproductive modes in onion thrips (Thysanoptera: Thripidae) populations from New York onion Fields. *Environmental Entomology,* 35(5), 1264-1271

Neill, S.L.O., Giordano, R., Colbert, A.M.E., Timothy, L., & Robertson, H.M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences,* 89(7), 2699-2702

Newsom, L.D. (1953). The tobacco thrips: its seasonal history and status as a cotton pest. *LSU Agricultural Experiment Station Reports*, 632.

Newton, I.L.G., Clark, M.E., Kent, B.N., Bordenstein, S.R., Qu, J., Richards, S., & Werren, J.H. (2016). Comparative genomics of two closely related *Wolbachia* with different reproductive effects on hosts. *Genome Biology and Evolution*, 8(5), 1526-1542. Doi: 10.1093/gbe/evw096

Nguyen, D.T. (2015). Genetic diversity, reproductive systems and endosymbionts of two invasive thrips species. PhD thesis, University of Western Sydney, pp 2-12.

Nguyen, D.T., Morrow, J.L., Spooner-Hart, R.N., & Riegler, M. (2017). Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont co-infections in haplodiploid thrips populations. *Evolution*, 71(4), 995-1008. Doi:10.1111/evo.13197

Nguyen, D.T., Spooner-Hart, R.N., & Riegler, M. (2015). Polyploidy versus endosymbionts in obligately thelytokous thrips. *BMC Evolutionary Biology,* 15(1), 23. Doi: 10.1186/s12862-015-0304-6

Normark, B. (2003). The evolution of alternative genetic systems in insects. *Annual Review of Entomology*, 48(1), 397-423.

Omilian, A.R., Cristescu, M.E., Dudycha, J.L., & Lynch, M. (2006). Ameiotic recombination in asexual lineages of *Daphnia*. *Proceedings of the National Academy of Sciences,* 103(49), 18638-18643

O'Neill, S.L. & Karr, T.L. (1990). Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature*, 348(6297), 178

Otte, D. (1979). *Historical Development of sexual selection theory, sexual selection and reproductive competion in insects*. *Academic press* INC. New York, pp 76

Paccagnini, E., De Marzo, L., Giusti, F., & Dallai, R. (2006). The aberrant spermatogenesis of the *Haplothrips simplex* (Buffa) (Thysanoptera): Ultrastructural study. *Tissue and Cell,* 38(3), 177-186.

Parker, B.L. & Skinner, M. (1995). *Thrips biology and management*. Lewis T. (Ed.). Doi:10.1007/978-1-4899-1409-5

Partridge, L. & Hurst, L.D. (2003). Sex and conflict. *Science,* 281(5385), 2003-2008.

Pelikan, J. (1954). Remarks on the orchid thrips *Chaetanaphothrips orchidii* (M.). *Folia Zoologica Entomologica,* 3, 3-12

Perrot-Minnot, M.J., Guo, L.R., & Werren, J.H. (1996). Single and double infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis* effects on compatibility. *Genetics*, 143(2), 961-972

Putman, W.L. (1942). Notes on the predaceous thrips *Haplothrips subtilissimus* hal. and A*eolothrips melaleucus* hal. *The Canadian Entomologis*t, 74(3), 37-43

Rabeling, C. & Kronauer, D.J.C. (2013). Thelytokous parthenogenesis in Eusocial Hymenoptera. *Annual Review of Entomology*, 58(1), 273-292.

Risler, H. & Kempter. E. (1961). Die haploidie der männchen und die endopolyploidie in einigen geweben von haplothrips (Thysanoptera). *Chromosoma*, 12(1), 351-361

Rugman-Jones, J., Hoddle, M., Amrich, R., Heraty, J., Stouthamer-Igel, C. & Stouthamer, R. (2012). Phylogeographic structure, outbreeding depression , and reluctant virgin oviposition in the bean thrips, *Caliothrips fasciatus* (Pergande) (Thysanoptera: Thripidae), in California. *Bulletin of Entomological Research,* 102(06), 698-709

Ryan, S.L. & Saul, G. (1968). Post-fertilization effect of incompatibility factors in *Mormoniella*. *Molecular and General Genetics*, MGG 103(1), 29-36

Saurav, G.K., Daimei, G., Rana, V.S., Popli, S., & Rajagopal, R. (2016). Detection and localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae). *Indian Journal of Microbiology*, 56(2), 167-171

Schilthuizen, M., Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., & Solignac, M. (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings of the Royal Society B: Biological Sciences,* 250(1328), 91-98. https://doi.org/10.1098/rspb.1992.0135

Sharga, U. (1933). Biology and life history of *Limothrips cerealium* haliday and *Aptinothrips rufus* gmelin feeding on Gramineae. *Annals of Applied Biology*, 20(2), 308-326. https://doi.org/10.1111/j.1744-7348.1933.tb07768.x

Sogo, K., Miura, K., Aizawa, M., Watanabe, T., & Stouthamer, R. (2014). Genetic structure in relation to reproduction mode in *Thrips tabaci* (Insecta: Thysanoptera). *Applied Entomology and Zoology*, 50(1), 73-77

Stenberg, P. & Saura, A. (2009). *Cytology of asexual animals* (pp. 63-74). In lost sex. Springer Dordrecht Heidelberg London New York.

Stouthamerff, R. & Kazmert, D.J. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*, 73, 317-327.

Stouthamer, R., Luck, R.F., & Hamilton, W.D. (1990). Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. *Proceedings of the National Academy of Sciences*, 87(7), 2424-2427

Strauss, S.Y. & Karban, R. (1994). The significance of outcrossing in an intimate plant-herbivore relationship. II. Does outcrossing pose a problem for thrips adapted to the host-plant clone? *Evolution*, 48(2): 465-476. https://doi.org/10.2307/241010

Tikader, B.K. (1966). On the seasonal fluctuation and biology of *Anaphothrips flavicinctus* (Karny) on *Panicum maximum* in Madras. *Journal of the Bombay Natural History Society*, 65(1), 243-248.

Tram, U., Sullivan, W., Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia* -Induced Cytoplasmic Incompatibility. *Science,* 296(5570), 1124-1126.

Van der, K.C.J. & Schwander, T. (2014). Evolution of asexuality via different mechanisms in grass thrips (Thysanoptera: Aptinothrips). *Evolution,* 68(7), 1883-1893. doi:10.1111/evo.12402.

Van Wilgenburg, E., Driessen, G., & Beukeboom, L. W. (2006). Single locus complementary sex determination in Hymenoptera: an" unintelligent" design? *Frontiers in Zoology*, 3(1), 1-15.

Wang, J.C., Zhang, B., Li, H., Wang, J.P., & Zheng, C. (2014). Effects of exposure to high temperature on *Frankliniella occidentalis* (Thysanoptera: Thripidae), under arrhenotoky and sexual reproduction conditions. *Florida Entomologist*, 97(2), 504-510

Weeks, A.R., Velten, R., & Stouthamer, R. (2003). Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proceedings. Biological the Royal Society B: Biological sciences*, 270(1526),1857-65

Weinert, L.A., Araujo-Jnr, E.V., Ahmed, M.Z., & Welch, J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, 282(1807), 1-6

Welch, D.B.M. & Meselson, M. (2000). Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. S*cience*, 288(5469), 1211-1215

Werren, J.H. & Beukeboom, L.W. (1998). Sex determination, sex ratios, and genetic conflict. *Annual Review of Ecology and Systematics,* 29(1), 233-261

Werren, J.H., Zhang, W., & Guo, L.R. (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proceedings of the Royal Society B: Biological Sciences,* 261(1360), 55-63

Woldemelak, W.A. (2020). The existence of deuterotokous reproduction mode in the *T. tabaci* (Thysanoptera: Thripidae) cryptic species complex. *Journal of Horticultural Research*, 28(1), 21-28.

Wrensch, D., Ebbert, M. (1993). *Evolution and diversity of sex ratio in insects and mites* (pp 56). Chapman and Hall, New York, USA.

Yishay, R. (2016). Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology*, 13(7), 2009-2016.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., & Bourtzis, K. (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences*, 101(42), 15042-15045.

Received: February 16, 2021 *Accepted: August 27, 2021*

J. Entomol. Res. Soc., 23(3): 305-319, 2021 *Research Article*
Doi: 10.51963/jers.v23i3.2021 *Online ISSN:2651-3579*

Molecular Characterization of the *Vairimorpha (Nosema) ceranae* Infection from *Bombus terrestris* (Linnaeus, 1758) (Hymenoptera: Apidae) in Turkey

Onur TOSUN[1]* Çağrı BEKİRCAN[2]

[1]Karadeniz Technical University, Maçka Vocational School, Department of Veterinary Medicine, Trabzon, TURKEY
[2]Selçuk University, Sarayönü Vocational School, Department of Veterinary Medicine, Konya, TURKEY
e-mails: [1]*onrtsn61@hotmail.com, [2]cagribekircan@hotmail.com
ORCID IDs: [1]*0000-0002-6763-5671, [2]0000-0002-5968-7359

ABSTRACT

The current study aimed to determine the natural Microsporidium pathogen of the *Bombus terrestris* L. (Hymenoptera: Apidae) in Turkey (Mersin, Antalya, Muğla, İzmir, Aydın). During 2019 and 2020, the commercial and wild populations of *B. terrestris* were investigated in this survey. In the studies, natural microsporidiosis was detected in commercial *B. terrestris* populations. Fresh oval spores were measured as 4.91 ± 0.48 (6.12 - 3.73) µm in length and 2.54 ± 0.31 (3.27 - 1.88) µm in width (n=60). Both SSU rRNA and RPB1 gene sequences of the current microsporidium were top hits with the *Vairimorpha (Nosema) ceranae* isolates. While the SSU rRNA gene sequence matched with the *Vairimorpha ceranae* clone NCS44 (LC510228) isolated from the *Apis cerana japonica* at 99.24% identity (100% coverage), the RPB1 gene sequence was matched with the *Vairimorpha ceranae* isolate 1994 (KJ473287) at 99.02% identity (100% coverage). Based on the light microscopy and molecular phylogeny the current microsporidium was a new isolate of the *V. ceranae* and named here in as *Vairimorpha ceranae* Tr-07.

Key words: *Bombus terrestris*, microsporidiosis, RPB1, SSU rRNA, *Vairimorpha ceranae*.

Tosun, O. & Bekircan, Ç. (2021). Molecular characterization of the *Vairimorpha* (*Nosema*) *ceranae* infection from *Bombus terrestris* (Linnaeus, 1758) (Hymenoptera: Apidae) in Turkey. *Journal of the Entomological Research Society*, 32(3), 305-319.

INTRODUCTION

Bees are infected by lots of pathogens and parasites that cause abnormalities in their metabolism, immune system, behavior and perception (Antúnez et al, 2009; Gómez-Moracho, Heeb, & Lihoreau, 2017; Li, Chen & Cook, 2018). As a result of these infections, bee individuals and colonies lose their fitness. Undoubtedly, *Vairimorpha* (*Nosema) ceranae* is the most common pathogen in bee species. The infection caused by *V. ceranae*, show different symptoms on their hosts at the physiological levels as changing gene expression in the brain, inhibiting the apoptosis of epithelial cells and deregulating immune responses (Holt, Aronstein, & Grozinger, 2013; Martín-Hernández et al, 2017; 2018) and behavioral levels as starting foraging earlier in life, exhibiting more frequent but shorter foraging flights, reducing homing abilities and lowering olfactory learning performances (Wolf et al, 2014; Dosselli, Grassl, Carson, Simmons, & Baer, 2016; Perry, Søvik, Myerscough, & Barron, 2016; Gage et al, 2018).

In recent years*, V. ceranae* has been frequently identified in wild bee species, especially bumblebees (*Bombus* spp.) (Plischuk et al, 2009; Li et al, 2012; Graystock, Yates, Darvill, Goulson, & Hughes, 2013). The *V. ceranae* infection in bumblebees causes reduced foraging performance of all colonies and damages their cognitive skills (Piiroinen & Goulson, 2016).

Numerical declines and local extinctions in bumblebee species have been reported in recent years. Studies have shown that four species have begun to disappear in Europe, and two species have been completely extinct in the British Isles (Goulson, Lye, & Darvill, 2008). Also, significant decreases were found in the populations of different bumblebees in North America, England and Ireland (Fitzpatrick et al, 2007; Grixti, Wong, Cameron, & Favret, 2009; Williams & Osborne, 2009). it has been stated that one of the important causes of these losses is parasites and pathogens (Cox-Foster et al, 2007; Cameron et al, 2011).

Bumblebees, which are important in pollination was determined about a hundred years ago, have been mass-produced for the past 25 years and are widely used as pollinators in greenhouse cultivation (Güler, Aytekin, & Dikmen, 2011; Argun Karslı & Gürel, 2015). More than one million bumblebees are commercially produced annually in the world, and more than 90% of this is *Bombus terrestris* L. (Hymenoptera: Apidae) (Velthius & Doorn, 2006). Parasites and pathogens in bumblebees must be accurately and rapidly identified to prevent damage to native species and to safely carry out commercial bumblebee colony breeding. For this aim, the present study tries to determine the natural pathogen and parasites of the *Bombus terrestris* L. in Turkey.

MATERIALS AND METHODS

Specimen collection and Light microscopical observation

In this study, commercially produced and wild-type members of the *B. terrestris* were collected and were examined for parasites and pathogens. While the commercially produced members were collected from greenhouses in five different provinces

(Mersin, Antalya, Muğla, İzmir, Aydın), the wild type adult members of the *B. terrestris* were collected from the north-east part of Turkey (Artvin, Trabzon, Rize, Giresun, Ordu) in 2019-2020. During the field study, due to the low population densities of the wild type, the sample numbers were less than expected. Samples were caught with sweep nets and live samples were transported immediately to the laboratory for further examinations. On the other hand, the samples which were collected commercially produced members from greenhouses lands or hives, had been found dead when collected. Members of *B. terrestris* morphologically identified according to Mauss (1994) and for wet mount preparation samples were dissected with Ringer's solution and examined under the light microscope (Tosun, 2020; Yıldırım & Bekircan, 2020). Infection positive slides were photographed using Zeiss AXIO microscope equipped with an Axicam ERc5s digital camera. The necessary measurements and analysis were made using ZEN 2.3 Blue Edition imaging software. While some infection-positive remaining tissues were preserved in 95% ethanol for molecular studies, others were preserved in 2.5% glutaraldehyde in PBS for transmission electron microscopy.

DNA extraction, Amplification and Molecular analysis

Ethanol-fixed infected tissues were washed with distilled water 3 times (15 min) to remove ethanol. The genomic DNA was extracted using the QIAGEN DNA Isolation Kit, No: 69504 according to the manufacturer's instructions. To amplify the SSU rRNA gene, the QIAGEN Multiplex PCR Kit (No. 206143) and 18F/1537R primer set was used (Baki & Bekircan, 2018). Amplification processes were performed according to the kit's protocol in a 50 µl reaction system. Amplification conditions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 60°C for 90 s, elongation at 72 °C for 2 min and a final extension at 72 °C for 10 min. Also, in the present study, the largest subunit of RNA polymerase II (RPB1) gene alignments were amplified as in the SSU rRNA gene. To amplify, the QIAGEN Multiplex PCR Kit (No. 206143) and primer set were used (Tosun, 2020; Yıldırım, 2021). The base sequences of the SSU rRNA and RPB1 gene were determined in the Macrogen Inc. Company, The Netherlands.

The sequences fragments were assembled using BioEdit and obtained consensus sequences (Hall, 1999)"mendeley":{"formattedCitation":"(Hall, 1999. Sequences with high similarity were determined according to the BLAST search and those of our interest were retrieved from the NCBI GenBank database and the literature (Table 1). The new combinations were used as re-assigned in 2020 by Tokarev et al, in the phylogenetic analysis (Tokarev et al, 2020). In the analysis, all sequences were aligned with CLUSTAL_W. Pairwise genetic distances were determined using the Kimura-2 parameter. Phylogenetic analyses were conducted using the maximum likelihood (ML) method in MEGA 10. Bootstrap confidence values were calculated with 1000 repetitions and the optimal evolutionary model was determined as GTR +I + G.

Table 1. Small subunit (SSU) ribosomal RNA and RNA polymerase II largest subunit (RPB1) gene sequences used for phylogenetic analyses.

	Accession No	Organism name	Host	Order	Family
SSU rRNA	**MW396669**	***Vairimorpha* (*Nosema*) *cerenae*Tr-07**	***Bombus terrestris***	**Hymenoptera**	**Apidae**
	LC510251	*Vairimorpha ceranae* (Japan)	*Apis cerana*	Hymenoptera	Apidae
	LC510228	*Vairimorpha ceranae* (Japan)	*Apis cerana*	Hymenoptera	Apidae
	DQ673615	*Vairimorpha ceranae* (Switzerland)	*Apis mellifera*	Hymenoptera	Apidae
	DQ329034	*Vairimorpha ceranae* (Spain)	*Apis mellifera*	Hymenoptera	Apidae
	KU937104	*Vairimorpha ceranae* (India)	*Apis mellifera*	Hymenoptera	Apidae
	KC680654	*Vairimorpha ceranae* (Thailand)	*Apis mellifera*	Hymenoptera	Apidae
	KC680650	*Vairimorpha ceranae* (Thailand)	*Bombus sp.*	Hymenoptera	Apidae
	JN872261	*Vairimorpha ceranae* (China)	*Bombus sp.*	Hymenoptera	Apidae
	DQ235446	*Vairimorpha apis* (Spain)	*Apis mellifera*	Hymenoptera	Apidae
	FJ789796	*Vairimorpha apis* (Australia)	*Apis mellifera*	Hymenoptera	Apidae
	U11047	*Vairimorpha vespula*	*Vespula vulgaris*	Hymenoptera	Vespidae
	Y00266	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
	HM370543	*Nosema bombi* (Russia)	*Bombus lucorum*	Hymenoptera	Apidae
	KF002566	*Nosema bombi* (Mexico)	*Bombus ephippiatus*	Hymenoptera	Apidae
	JN872231	*Nosema bombi* (China)	*Bombus sp.*	Hymenoptera	Apidae
	MF776532	*Nosema bombi* (Thailand)	*Bombus sp.*	Hymenoptera	Apidae
	AY741105	*Nosema bombi* (Ireland)	*Bombus pascuorum*	Hymenoptera	Apidae
	KF916504	*Nosema bombi* (Turkey)	*Bombus sp.*	Hymenoptera	Apidae
	D85503	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
	KT020736	*Nosema fumiferanae*	*Epiphyas postvittana*	Lepidoptera	Tortricidae
	L39109	*Endoreticulatus schubergi*	*Cholistoneura fumiferana*	Lepidoptera	Tortricidae
RPB1	**MW415412**	***Vairimorpha* (*Nosema*) *cerenae*Tr-07**	***Bombus terrestris***	**Hymenoptera**	**Apidae**
	KJ473287	*Vairimorpha ceranae* (Chile)	*Apis mellifera*	Hymenoptera	Apidae
	KM001627	*Vairimorpha ceranae* (China)	*Apis ceranae*	Hymenoptera	Apidae
	DQ996230	*Vairimorpha apis*	*Apis mellifera*	Hymenoptera	Apidae
	AF060234	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
	DQ996236	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
	JX213749	*Vairimorpha lymantriae*	*Lymantria dispar*	Lepidoptera	Lymantria
	JX239748	*Vairimorpha disparis*	*Lymantria dispar*	Lepidoptera	Erebidae
	MT461295	*Nosema fumiferanae* TY61	*Apomyelois (Ectomyelois) ceratoniae*	Lepidoptera	Pyralidae

Table 1. Continued.

	Accession No	Organism name	Host	Order	Family
	HQ457435	*Nosema fumiferanae*	*Choristoneura fumiferana*	Lepidoptera	Tortricidae
	HQ457436	*Nosema sp.*	*Choristoneura occidentalis*	Lepidoptera	Tortricidae
	AJ278948	*Nosema tyriae*	*Tyria jacobaeae*	Lepidoptera	Arctiidae
	DQ996231	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
	DQ996234	*Nosema trichoplusiae*	*Trichoplusia ni*	Lepidoptera	Noctuidae
	HQ457438	*Nosema disstriae*	*Malacasoma disstria*	Lepidoptera	Lasiocampidae
	DQ996232	*Nosema empoascae*	*Empoasca fabae*	Homoptera	Cicadellidae
	DQ996233	*Nosema granulosis*	*Gammarus duebeni*	Amphipoda	Gammaridae
	XM 014708712	*Ordospora colligata*	*Daphnia magna*	Cladocera	Daphniidae

RESULTS

Light microscopy

In the present study, the commercially produced members of the *B. terrestris* were collected from greenhouses where tomato production was carried out in five different provinces: Mersin, Antalya, Muğla, İzmir and Aydın. In this survey, 547 samples were collected from greenhouses and examined during 2019-2020. As a result of the examinations, 51 samples were infected by the microsporidian pathogen (infection rate: 9.32%). Determined fresh oval spores were measured as 4.91 ± 0.48 (6.12 - 3.73) µm in length and 2.54 ± 0.31 (3.27 - 1.88) µm in width (n=60). Infected members gut systems fully filled with the oval mature spores (Fig. 1). In addition, during this study 171 wild members were collected from the provinces (Artvin, Rize, Trabzon, Giresun and Ordu) where those of determined before. As a result of the examinations, no microsporidiosis was found in the smears prepared from wild members (Table 2). Therefore, this group was not included in subsequent statistical analysis.

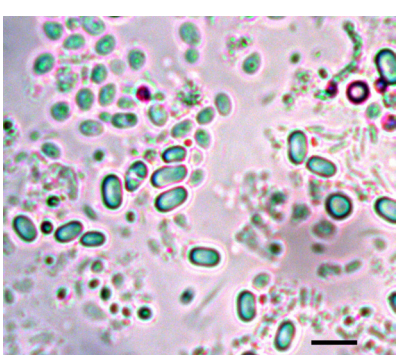


Fig. 1. The light micrograph of the *V. ceranae* Tr-07 fresh oval spores, bar: 5µm.

The infection prevalence was calculated based on the rate of bumblebees determined to be microsporidiosis positive with the microscopic examination in this study. After the analysis, infection prevalence based on provinces was determined as 14.28% in Mersin, 15.74% in Antalya, 14.28% in Muğla, respectively. No infection was detected in İzmir and Aydın. When comparing the infection rates on a year and month basis it was seen that the infection rate was 9.81% in 2019 and 8.86% in 2020 where in months, these rates were a range from 9.65% in May, 10.16% in June and 8.15% in July, respectively (Table 2).

Molecular phylogeny

The molecular phylogeny of the current microsporidium which isolated from infected *B. terrestris* tissues was based on the partial SSU rRNA and RPB1 gene. An 1177 nucleotide section of the SSU rRNA and 674 nucleotides of the RPB1 were obtained with 35.9% and 32.5% GC content after the sequencing. And these sequences of the current microsporidium were deposited in GenBank with MW396669 and MW415412 accession codes. Each sequence was subjected to BLAST analysis that matched only microsporidian records. Both SSU rRNA and RPB1 gene sequences of the current microsporidium were top hits with the *V. ceranae* isolates. While the SSU rRNA gene sequence matched with the *Vairimorpha ceranae* clone NCS44 (LC510228) isolated from the *Apis cerana japonica* at 99.24% identity (100% coverage), the RPB1 gene sequence was matched with the *Vairimorpha ceranae* isolate 1994 (KJ473287) at 99.02% identity (100% coverage).

The pairwise distance analysis that carried for the SSU rRNA gene sequence, was conducted with 22 microsporidian sequences. Pairwise phylogenetic distances between the current microsporidium and other species ranged from 0.010 to 0.505. The distance between the current microsporidium and the type species of the genus, *Vairimorpha necatrix* (Pilley, 1976) was determined as 0.068 (Table 3). Also, it was differentiating from the *Nosema bombycis* (Nägeli, 1857), the type species of *Nosema* genus, with 0.243 difference.

For RPB1 gene sequence, 18 microsporidian sequences were used in the pairwise phylogenetic distance analysis. And the distances were ranged from 0.010 to 0.388. In the analysis made on the RPB1 gene, they gave results that support the results of the analysis made with the SSU rRNA gene. And, the current microsporidium was more closely related to the *Vairimorpha* species (Table 3).

In conclusion, based on the morphological and molecular information, the current microsporidium isolated from *B. terrestris* was a new isolate of *Vairimorpha ceranae*.

Table 2. *Vairimorpha* (*Nosema*) *cerenae* infection in *B. terrestris* from the different sampling localities and months.

	Sample type	Province	Months	Dissected samples	Total	Infected samples	Infection rate (%)	Total infection rate (%)
2019	Commercial	Mersin	May	15	48	4	26.67	14.58
			June	20		3	15	
			July	13		-	-	
		Antalya	May	20	62	3	15	19.35
			June	22		5	22.73	
			July	20		4	20	
		Muğla	May	15	52	2	13.33	13.46
			June	17		3	17.64	
			July	20		2	10	
		İzmir	May	15	55	-	-	-
			June	20		-	-	
			July	20		-	-	
		Aydın	May	17	48	-	-	-
			June	13		-	-	
			July	18		-	-	
	Wild	Artvin	April	9	18	-	-	-
			May	6		-	-	
			June	3		-	-	
		Rize	April	6	15	-	-	-
			May	4		-	-	
			June	5		-	-	
		Trabzon	April	10	21	-	-	-
			May	7		-	-	
			June	4		-	-	
		Giresun	April	5	14	-	-	-
			May	3		-	-	
			June	6		-	-	
		Ordu	April	3	13	-	-	-
			May	5		-	-	
			June	5		-	-	

Table 2. Continued.

	Sample type	Province	Months	Dissected samples	Total	Infected samples	Infection rate (%)	Total infection rate (%)
2020	Commercial	Mersin	May	22	57	3	13.63	14.03
			June	20		2	10	
			July	15		3	20	
		Antalya	May	18	65	3	16.66	12.30
			June	22		2	9.09	
			July	25		3	12	
		Muğla	May	20	60	2	10	13.33
			June	18		2	11.11	
			July	22		4	18.18	
		İzmir	May	22	55	-	-	-
			June	18		-	-	
			July	15		-	-	
		Aydın	May	14	45	-	-	-
			June	13		-	-	
			July	18		-	-	
	Wild	Artvin	May	4	13	-	-	-
			June	6		-	-	
			July	3		-	-	
		Rize	May	9	19	-	-	-
			June	5		-	-	
			July	5		-	-	
		Trabzon	May	11	25	-	-	-
			June	6		-	-	
			July	8		-	-	
		Giresun	May	5	15	-	-	-
			June	6		-	-	
			July	4		-	-	
		Ordu	May	6	18	-	-	-
			June	8		-	-	
			July	4		-	-	
			GENERAL TOTAL		**718**			**7.10%**

Table 3. Comparison of current microsporidium and other related microsporidia based on the small subunit ribosomal RNA gene (SSU rRNA) and the largest subunit of RNA polymerase II (RPB1) gene by query cover, by nucleotide identity, by Pairwise distance analysis, and GC% content.

SSU rRNA	MW396669	*Vairimorpha* (*Nosema*) *cerenae*Tr-07	Query cover %	Pairwise distances	GC content (35.9%)
	LC510251	*Vairimorpha ceranae* (Japan)	99	0.01046	36.3
	LC510228	*Vairimorpha ceranae* (Japan)	99	0.01046	38.1
	DQ673615	*Vairimorpha ceranae* (Switzerland)	98	0.01752	38.8
	DQ329034	*Vairimorpha ceranae* (Spain)	98	0.01752	36.1
	KU937104	*Vairimorpha ceranae* (India)	99	0.01046	40
	KC680654	*Vairimorpha ceranae* (Thailand)	92	0.01046	36.1
	KC680650	*Vairimorpha ceranae* (Thailand)	92	0.01046	37.3
	JN872261	*Vairimorpha ceranae* (China)	42	0.01046	41.3
	DQ235446	*Vairimorpha apis* (Spain)	99	0.09585	38.7
	FJ789796	*Vairimorpha apis* (Australia)	92	0.09585	38.5
	U11047	*Vairimorpha vespula*	99	0.03184	36.8
	Y00266	*Vairimorpha necatrix*	99	0.06882	37.4
	HM370543	*Nosema bombi* (Russia)	58	0.06883	35.8
	KF002566	*Nosema bombi* (Mexico)	38	0.06883	35.8
	JN872231	*Nosema bombi* (China)	40	0.07266	35.7
	MF776532	*Nosema bombi* (Thailand)	22	0.07065	33.8
	AY741105	*Nosema bombi* (Ireland)	97	0.06550	35.9
	KF916504	*Nosema bombi* (Turkey)	25	0.07266	36.3
	D85503	*Nosema bombycis*	93	0.24367	34.1
	KT020736	*Nosema fumiferanae*	94	0.24862	32.3
	L39109	*Endoreticulatus schubergi*	71	0.50510	51
RPB1	**MW415412**	*Vairimorpha(Nosema)cerenae*Tr-07	Query cover %	Pairwise distances	GC content (32.5%)
	KJ473287	*Vairimorpha ceranae*	100	0.01077	32.4
	KM001627	*Vairimorpha ceranae*	100	0.01319	32.2
	DQ996230	*Vairimorpha apis*	98	0.22293	31.2
	AF060234	*Vairimorpha necatrix*	98	0.23869	32.5
	DQ996236	*Vairimorpha necatrix*	98	0.23869	30.9
	JX213749	*Vairimorpha lymantriae*	93	0.22134	36
	JX239748	*Vairimorpha disparis*	94	0.23456	36.4
	MT461295	*Nosema fumiferanae* TY61	96	0.27543	36.2

Table 3. Continued.

	MW396669	*Vairimorpha* (*Nosema*) *cerenae*Tr-07	Query cover %	Pairwise distances	GC content (35.9%)
RPB1	HQ457435	*Nosema fumiferanae*	94	0.27867	36.4
	HQ457436	*Nosema sp.*	94	0.26972	36.8
	AJ278948	*Nosema tyriae*	98	0.26806	36.7
	DQ996231	*Nosema bombycis*	98	0.26759	36.6
	DQ996234	*Nosema trichoplusiae*	98	0.27150	36.7
	HQ457438	*Nosema disstriae*	96	0.27711	
	DQ996232	*Nosema empoascae*	95	0.34913	43.6
	DQ996233	*Nosema granulosis*	94	0.29770	42.9
	XM 014708712	*Ordospora colligata*	89	0.38837	43.3

"-"No significant similarity found.

DISCUSSION

This survey of pathogens of the *B. terrestris* from different provinces of Turkey showed that while the microsporidiosis originated from *V. ceranae* was commonly occur at commercial bumblebee populations in Turkey, no infection was found in wild populations. If it was necessary to make a self-criticism of the study here, it can be said that the reason for the no determination of any infection in wild populations was due to the low sample count. Because recent studies showed that the wild *Bombus* species were frequently infected with the microsporidian species like a *V. ceranae* (Li et al, 2012; Plischuk & Lange, 2016; Sinpoo, Disayathanoowat, Williams, & Chantawannakul, 2019).

The current microsporidium detected from commercial members of the *B. terrestris* was determined to be the first *V. ceranae* isolate of Turkey as a result of both microscopical and molecular examinations. The microsporidial taxonomy was constructed based on light microscopy and measurements (Kudo, 1924; Weiser, 1977; Sprague, Becnel, & Hazard, 1992). In the examinations made in this context, it was determined that the current microsporidium spores had similar features to the data presented by numerous studies previously conducted for the definition and detection of *V. ceranae* (Fries, Feng, da Silva, Slemenda, & Pieniazek, 1996; Chen et al, 2009). Especially in the last quarter, the microsporidial taxonomy has been constructed with the molecular phylogeny and species identifications are made on this basis (Baker, Vossbrinck, Maddox, & Undeen, 1994; Baker, Vossbrinck, Didier, Maddox, & Shadduck, 1995; Huang, Tsai, Lo, Soichi & Wang 2004; Bekircan, 2020; Tokarev et al, 2020; Tosun, 2020) 1968 (Microsporidia: Nosematidae. Therefore, in this study partial sequences of SSU rRNA and RPB1 genes of the current microsporidium were analyzed. In the BLAST analysis conducted with partial sequences of the SSU rRNA and RPB1 genes, the current microsporidium showed high similarities with *V.*

ceranae isolates (Table 3). According to SSU rRNA pairwise distance analysis, the current microsporidium was differentiating with only 0.010 from *V. ceranae* Thailand (KC680650) and China (JN872261) isolates, which were isolated from *Bombus* species (Sinpoo et al, 2019). The phylogenetic trees, which were constructed with SSU rRNA and RPB1 gene sequences, displayed two distinct clades: *Nosema* and *Vairimorpha* (Fig. 2). In both trees, the current microsporidium was grouping with the type species (*V. necatrix*) of the *Vairimorpha* genus and branched with the *V. ceranae* isolates. In the SSU rRNA tree, the current microsporidium settled the same node with the Switzerland (DQ673615) and Spain (DQ329034) isolates of the *V. ceranae* which were isolated from the *Apis mellifera* (Higes, García-Palencia, Martín-Hernández, & Meana, 2007; Martín-Hernández et al, 2007) (Table 3). And the distances between the current microsporidium and these isolates were determined as the same (0.017). Also, in the RPB1 tree, the current microsporidium settled the same node with the *V. ceranae* isolates (KJ473287 and KM001627) as in the SSU rRNA tree (Fig. 2).

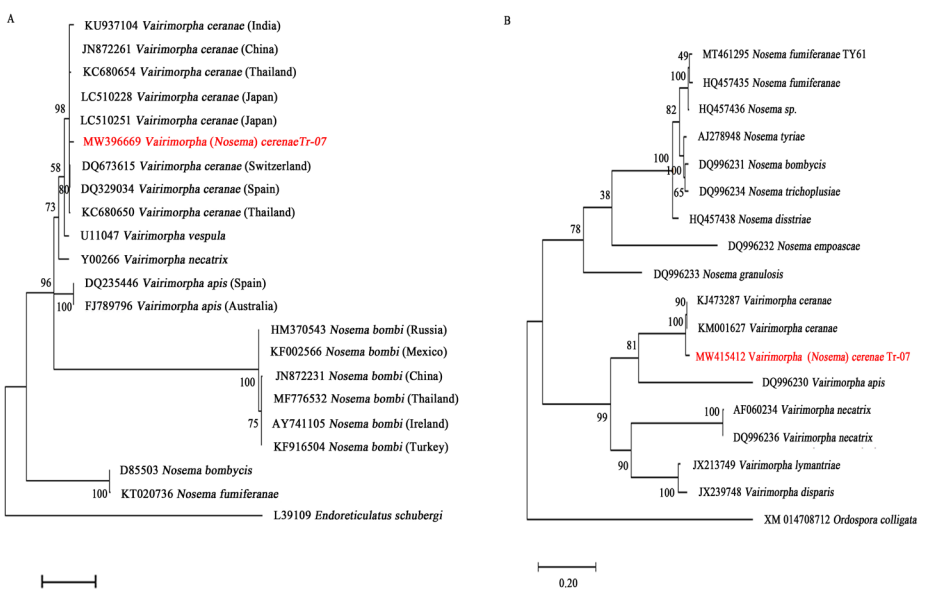


Fig. 2. Phylogenetic trees constructed by maximum likelihood (ML) revealed that the current microsporidium whose sequences were obtained in the present study was most closely related to the *V. ceranae* isolates. *Endoreticulatus schubergi* (L39109) and *Ordospora colligata* (XM014708712) were used as outgroups. The analysis was done on 1000 bootstrapped data sets. Bootstrap values were shown at each node. The scale bar represented substitutions per nucleotide site. A: 16S SSU rRNA tree B: RPB1 tree.

In conclusion, phylogenetical analysis showed that the current microsporidium from *B. terrestris* was almost identical to *V. ceranae* isolates. So, based on the light microscopy and phylogenetical status the current microsporidium was a new isolate of the *V. ceranae* and named herein as *Vairimorpha ceranae* Tr-07 (MW396669).

In addition, the prevalence of the *V. ceranae* Tr-07 infection from commercial *B. terrestris* members was evaluated in this study. Infection was detected in three of the five provinces where the samples were collected, and the province where the disease was most common was determined as Antalya (15.74%). When assessed the prevalence according to the months, June was the month that the infection was peaked (10.16%) (Table 2). Although greenhouses are areas where controlled air conditions are provided, this situation is eliminated in order to reduce costs in summer months and natural weather conditions are valid in these areas. And in the greenhouses where samples were collected, natural climatic conditions prevailed. There are many studies revealing the variability of *V. ceranae* infection according to weather conditions and months (Gisder et al, 2010; Tosun, 2012; Özgör, Güzerin, & Keskin, 2015). In 2015, Özgör et al, determined that *V. ceranae* formation in Turkey was directly affected by the temperature and humidity. Similarly, in the current study, the peak point was determined in June which the average data of the temperature and humidity were high relatively. Although the three provinces where *V. ceranae* infection was detected are geographically relatively close to each other, the infection was most frequently detected in Antalya. This situation can be explained due to the variability of the artificial diets of businesses as stated in Gómez-Moracho, Durand, Pasquaretta, Heeb, & Lihoreau in 2021.

Finally, the current study revealed the first *V. ceranae* infection at the *B. terrestris* in Turkey and its current status.

ACKNOWLEDGEMENTS

We are thankful to Beekeeper Mehmet TURKUT for making this study possible. All authors designed the project, provided samples and contributed to the preparation of the manuscript.

REFERENCES

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., & Higes, M. (2009). Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). *Environmental Microbiology*, 11(9), 2284-2290. https://doi.org/10.1111/j.1462-2920.2009.01953.x

Argun Karslı, B. & Gürel, F. (2015). Identification of some important Bumblebee (*Bombus terrestris* L.) parasites by molecular methods. *Uludağ Arıcılık Dergisi*, 14(2), 88-98. http://dergipark.gov.tr/uluaricilik/issue/34048/376896

Baker, M.D., Vossbrinck, C.R., Maddox, J.V., & Undeen, A.H. (1994). Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. *Journal of Invertebrate Pathology*, 64(2), 100-106. https://doi.org/10.1006/jipa.1994.1077

Baker, M.D., Vossbrinck, C.R., Didier, E.S., Maddox, J.V., & Shadduck, J.A. (1995). Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. *The Journal of Eukaryotic Microbiology*, 42(5), 564-570. https://doi.org/10.1111/j.1550-7408.1995.tb05906.x

Baki, H. & Bekircan, Ç. (2018). A new microsporidium, *Vairimorpha subcoccinellae* n. sp. (Microsporidia: Burenellidae), isolated from *Subcoccinella vigintiquatuorpunctata* L. (Coleoptera: Coccinellidae). *Journal of Invertebrate Pathology*, 151, 182-190. https://doi.org/10.1016/j.jip.2017.12.004

Bekircan, Ç. (2020). Assignment of *Vairimorpha leptinotarsae* comb. nov. on the basis of molecular characterization of *Nosema leptinotarsae* Lipa, 1968 (Microsporidia: Nosematidae). *Parasitology*, 147(9), 1019-1025. https://doi.org/10.1017/S0031182020000669

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., & Griswold, T.L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences of the United States of America*, 108(2), 662-667. https://doi.org/10.1073/pnas.1014743108

Chen, Y.P., Evans, J.D., Murphy, C., Gutell, R., Zuker, M., Gundensen-Rindal, D., & Pettis, J.S. (2009). Morphological, molecular, and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. *Journal of Eukaryotic Microbiology*, 56(2), 142-147. https://doi.org/10.1111/j.1550-7408.2008.00374.x

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., VanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Lipkin, W.I. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, 318(5848), 283-287. https://doi.org/10.1126/science.1146498

Dosselli, R., Grassl, J., Carson, A., Simmons, L.W., & Baer, B. (2016). Flight behaviour of honey bee (*Apis mellifera*) workers is altered by initial infections of the fungal parasite *Nosema apis*. *Scientific Reports*, 6, 1-11. https://doi.org/10.1038/srep36649

Fitzpatrick, Ú., Murray, T.E., Paxton, R.J., Breen, J., Cotton, D., Santorum, V., & Brown, M.J.F. (2007). Rarity and decline in bumblebees - A test of causes and correlates in the Irish fauna. *Biological Conservation*, 136(2), 185-194. https://doi.org/10.1016/j.biocon.2006.11.012

Fries, I., Feng, F., da Silva, A., Slemenda, S.B., & Pieniazek, N.J. (1996). *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). *European Journal of Protistology*, 32(3), 356-365. https://doi.org/10.1016/S0932-4739(96)80059-9

Gage, S.L., Kramer, C., Calle, S., Carroll, M., Heien, M., & DeGrandi-Hoffman, G. (2018). *Nosema ceranae* parasitism impacts olfactory learning and memory and neurochemistry in honey bees (*Apis mellifera*). *Journal of Experimental Biology*, 221(4), 1-11. https://doi.org/10.1242/jeb.161489

Gisder, S., Hedtke, K., Möckel, N., Frielitz, M.C., Linde, A., & Genersch, E. (2010). Five-year cohort study of *Nosema* spp. in Germany: Does climate shape virulence and assertiveness of *Nosema ceranae*? *Applied and Environmental Microbiology*, 76(9), 3032-3038. https://doi.org/10.1128/AEM.03097-09

Gómez-Moracho, T., Heeb, P., & Lihoreau, M. (2017). Effects of parasites and pathogens on bee cognition. *Ecological Entomology*, 42, 51-64. https://doi.org/10.1111/een.12434

Gómez-Moracho, T., Durand, T., Pasquaretta, C., Heeb, P., & Lihoreau, M. (2021). Artificial diets modulate infection rates by *Nosema ceranae* in bumblebees. *Microorganisms*, 9(1), 1-18. https://doi.org/10.3390/microorganisms9010158

Goulson, D., Lye, G.C., & Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology*, 53, 191-208. https://doi.org/10.1146/annurev.ento.53.103106.093454

Graystock, P., Yates, K., Darvill, B., Goulson, D., & Hughes, W.O.H. (2013). Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. *Journal of Invertebrate Pathology*, 114(2), 114-119. https://doi.org/10.1016/j.jip.2013.06.005

Grixti, J.C., Wong, L.T., Cameron, S.A., & Favret, C. (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation*, 142(1), 75-84. https://doi.org/10.1016/j.biocon.2008.09.027

Güler, Y., Aytekin, A.M., & Dikmen F. (2011). Bombini and Halictidae (Hymenoptera: Apoidae) fauna of Afyonkarahisar province of Turkey. *Journal of the Entomological Research Society*, 13(1), 1-22. https://doi.org/10.51963/jers.v23i2

Hall, T.A. (1999). PdfBioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Higes, M., García-Palencia, P., Martín-Hernández, R., & Meana, A. (2007). Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). *Journal of Invertebrate Pathology*, 94(3), 211-217. https://doi.org/10.1016/j.jip.2006.11.001

Holt, H.L., Aronstein, K.A., & Grozinger, C.M. (2013). Chronic parasitization by *Nosema microsporidia* causes global expression changes in core nutritional, metabolic and behavioral pathways in honey bee workers (Apis mellifera). *BMC Genomics*, 14(1). https://doi.org/10.1186/1471-2164-14-799

Huang, W.F., Tsai, S.J., Lo, C.F., Soichi, Y., & Wang, C.H. (2004). The novel organization and complete sequence of the ribosomal RNA gene of *Nosema bombycis*. *Fungal Genetics and Biology*, 41(5), 473-481. https://doi.org/10.1016/j.fgb.2003.12.005

Kudo, R. (1924). A biologic and taxonomic study of the Microsporidia. *Illinois Biological Monographs*, 9, 1-268.

Li, J., Chen, W., Wu, J., Peng, W., An, J., Schmid-Hempel, P., & Schmid-Hempel, R. (2012). Diversity of nosema associated with bumblebees (*Bombus* spp.) from China. *International Journal for Parasitology*, 42(1), 49-61. https://doi.org/10.1016/j.ijpara.2011.10.005

Li, W., Chen, Y., & Cook, S.C. (2018). Chronic *Nosema ceranae* infection inflicts comprehensive and persistent immunosuppression and accelerated lipid loss in host *Apis mellifera* honey bees. *International Journal for Parasitology*, 48(6), 433-444. https://doi.org/10.1016/j.ijpara.2017.11.004

Martín-Hernández, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailón, E., & Higes, M. (2007). Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Applied and Environmental Microbiology*, 73(20), 6331-6338. https://doi.org/10.1128/AEM.00270-07

Martín-Hernández, R., Higes, M., Sagastume, S., Juarranz, Á., Dias-Almeida, J., Budge, G.E., Meana, A., & Boonham, N. (2017). Microsporidia infection impacts the host cell's cycle and reduces host cell apoptosis. *PLoS ONE*, 12(2), 1-17. https://doi.org/10.1371/journal.pone.0170183

Martín-Hernández, R., Bartolomé, C., Chejanovsky, N., Le Conte, Y., Dalmon, A., Dussaubat, C., García-Palencia, P., Meana, A., Pinto, M.A., Soroker, V., & Higes, M. (2018). *Nosema ceranae* in *Apis mellifera*: a 12 years postdetection perspective. *Environmental Microbiology*, 20(4), 1302-1329. https://doi.org/10.1111/1462-2920.14103

Mauss, V. (1994). *Bestimmungsschlüssel für Hummeln*. Deutscher Jugendbund für Naturbeobachtungen (DJN), 55 s.

Nägeli, K.W. (1857). Überdie neue Krankheit der Seidenraupe und verwandte Organismen. *Botanische Zeitung*, 15, 60-761.

Özgör, E., Güzerin, E., & Keskin, N. (2015). Determination and comparison of *Nosema apis* and *Nosema ceranae* in terms of geographic and climatic factors. *Hacettepe Journal of Biology and Chemistry*, 43(1), 9-15. https://doi.org/10.15671/HJBC.20154311200

Perry, C.J., Søvik, E., Myerscough, M.R., & Barron, A.B. (2016). Rapid behavioral maturation accelerates failure of stressed honey bee colonies. *Proceedings of the National Academy of Sciences of the United States of America*, 113(30), E4431. https://doi.org/10.1073/pnas.1610243113

Piiroinen, S. & Goulson, D. (2016). Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, 283(1828). https://doi.org/10.1098/rspb.2016.0246

Pilley, B.M. (1976). A new genus, *Vairimorpha* (Protozoa: Microsporida), for *Nosema necatrix* Kramer 1965: Pathogenicity and life cycle in *Spodoptera exempta* (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology*, 28(2), 177-183. https://doi.org/10.1016/0022-2011(76)90119-1

Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A., Abrahamovich, A.H., Lange, C., & Higes, M. (2009). South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). *Environmental Microbiology Reports*, 1(2), 131-135. https://doi.org/10.1111/j.1758-2229.2009.00018.x

Plischuk, S. & Lange, C.E. (2016). *Bombus brasiliensis* Lepeletier (Hymenoptera, Apidae) infected with *Nosema ceranae* (Microsporidia). *Revista Brasileira de Entomologia*, 60(4), 347-351. https://doi.org/10.1016/j.rbe.2016.06.003

Sinpoo, C., Disayathanoowat, T., Williams, P.H., & Chantawannakul, P. (2019). Prevalence of infection by the microsporidian *Nosema* spp. in native bumblebees (*Bombus* spp.) in northern Thailand. *PLOS ONE*, 14(3), e0213171. https://doi.org/10.1371/journal.pone.0213171

Sprague, V., Becnel, J.J., & Hazard, E.I. (1992). Taxonomy of phylum microspora. *Critical Reviews in Microbiology*, 18(5-6), 285-395. https://doi.org/10.3109/10408419209113519

Tokarev, Y.S., Huang, W.F., Solter, L.F., Malysh, J.M., Becnel, J.J., & Vossbrinck, C.R. (2020). A formal redefinition of the genera *Nosema* and *Vairimorpha* (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics. *Journal of Invertebrate Pathology*, 169 (October 2019), 107279. https://doi.org/10.1016/j.jip.2019.107279

Tosun, O. (2012). *Bal Arılarında (Apis mellifera L., 1758) Nosemosis (Nosematosis) Hastalığının Doğu Karadeniz Bölgesinde Bulunan Arı Kolonilerindeki Varlığı, Dağılımı ve Hastalık Etkenlerinin Karakterizasyonu*. [Doctora tezi, Karadeniz Teknik Üniversitesi]. Fen Bilimleri Enstitüsü, 112 s.

Tosun, O. (2020). A new isolate of *Nosema fumiferanae* (Microsporidia: Nosematidae) from the date moth *Apomyelois (Ectomyelois) ceratoniae*, Zeller, 1839 (Lepidoptera: Pyralidae). *Parasitology*, 147(13), 1461-1468. https://doi.org/10.1017/S0031182020001481

Velthius, H.W. & Doorn, A. Van. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, 37, 421-451.

Weiser, J. (1977). Contribution to the classification of Microsporidia. *Vestnik Ceskoslovenske Spolecnosti Zoologicke*, 41, 308-320.

Williams, P.H. & Osborne, J.L. (2009). Bumblebee vulnerability and conservation world-wide. *Apidologie*, 40(3), 367-387. https://doi.org/10.1051/apido/2009025

Wolf, S., McMahon, D.P., Lim, K.S., Pull, C.D., Clark, S.J., Paxton, R.J., & Osborne, J.L. (2014). So near and yet so far: Harmonic radar reveals reduced homing ability of nosema infected honeybees. *PLoS ONE*, 9(8). https://doi.org/10.1371/journal.pone.0103989

Yıldırım, H. (2021). The first record of *Vairimorpha hostounsky* sp. nov. Infection in the blue shieldbug, *Zicrona caerulea* Linnaeus, 1758 (Hemiptera: Pentatomidae). *Microbial Pathogenesis,* 155, 104883. https://doi.org/10.1016/j.micpath.2021.104883

Yıldırım, H. & Bekircan, Ç. (2020). Ultrastructural and molecular characterization of *Nosema alticae* sp. nov. (Microsporidia: Nosematidae), pathogen of the flea beetle, *Altica hampei* Allard, 1867 (Coleoptera: Chrysomelidae). *Journal of Invertebrate Pathology*, 170(November 2019). https://doi.org/10.1016/j.jip.2019.107302

Received: July 14, 2021 *Accepted: September 09, 2021*

AUTHOR GUIDELINES

Journal of the Entomological Research Society (J. Entomol. Res. Soc.) accepts and publishes original research articles in the all fields of entomology. The journal publishes regular research papers and review articles. Brief and timely reports may be submitted as short communications, where articles with less detailed results and evaluations sections can be accepted as short communication. The Editors first evaluate all manuscripts. At this stage, manuscripts that fail to be original, have serious scientific flaws, have poor grammar or English language, or are outside the aims and scope of the Journal will be rejected. Those that meet the minimum criteria are passed onto at least 2 experts for review. Authors should suggest four reviewers with their names, addresses and e-mail addresses who would review their manuscript. Information on the reviewers should also be uploaded as an appendix to the manuscript. Of these four reviewers, at most two should be in the author's native country and the others will be in other countries. Two reviewers are selected from these four suggested reviewers or editors may assign other reviewers. A final decision to accept or reject the manuscript will be sent to the author along with any recommendations made by the reviewers. Reviewers make recommendations to the Editor whether to accept or reject the manuscript for publishing. The Editor reaches a final decision based on the reviewers' recommendations, as well as his/her own evaluation of the manuscript.

The manuscripts should be written in Arial with 12 type size with double spacing in Microsoft Office Word. The paragraphs should not be indented. The Manuscripts in general should not exceed 30 pages.

Heading: The title of the manuscript should be informative, but preferably not exceed twenty words. Just under the heading, please provide the title, full name(s) of author(s) (The name(s) of all authors should be start with capital letter, and surname(s) should be typed in upper case), with full address and e-mails of each author on a separate line. If a genus or species name is included in the manuscript heading, these should be written in full with no abbreviations, including the author name and date; e.g. Aphodius lividus (Olivier, 1789)

Abstract: An abstract provided at the beginning of the manuscript should indicate the main aspects of the subject, not exceed 200 words, and should be followed by 5-7 key words.

Text: The standard order of sections for original manuscripts is as follows: Introduction, Material and Methods, Results, Conclusions and Discussion, Acknowledgements, References. Sub-titles should be up to the third level and Italic format should be avoided except for species names. . The scientific names (e.g. genus- and species-group names) are the only words to be italicized. References should be cited in the text by the last name(s) of the author(s) and year of publication. Atribution in main text must be given like that (Surname, 1900a; 1900b; 1991; Surname, et al, 2000, Surname1 & Surname2, 2001). Two Authors: The surname of both authors is stated with either 'and' or an ampersand (&) between. For example: Surname1 & Surname2 (2017) state… Or …(Surname1 & Surname2, 2017). Three, Four or Five Authors: For the first cite, all names should be listed: Surname1, Surname2, & Surname3 (2017) state… Or …(Surname1, Surname2, & Surname3, 2017). Further cites can be shorted to the first author's name followed by et al: Surname1 et al (2017) state… Or …(Surname1 et al, 2017). Six or more authors: Only the first author's surname should be stated followed by et al: (Surname1 et al, 2017). Works should be cited with a, b, c etc following the date. For example: (Surname1, 2017a) or (Surname1, 2017b). If these works are by the same author, the surname is stated once followed by the dates in order chronologically. For instance: Surname (2007, 2013, 2017) Or (Surname, 2007, 2013, 2017). If a page number will be given for a quote, the page number must be given after the date, for example (Surname, 2017, p.104). This rule holds for all of the variations listed. Groups of references should be listed chronologically.
For faunistic research follow this order, Distribution:., Material examined:…, Host plant:….etc.

Example:
Sphex oxianus Gussakovskij, 1928
Distribution: Central and South West Asia, Afghanistan, Iran, Israel, Turkey (Bohart and Menke, 1976; Menke and Pulawski, 2000; Kazenas, 2001), Turkey: Artvin (De Beaumont, 1967).
Material examined: Ankara, Altındağ, Çubuk Dam Lake, 900 m, 29.06.1998, 1 ♂; Kalecik, 600 m, 24. 07. 2001, 2 ♀♀, Kalecik, 800 m, 25. 07. 2001, 3 ♀♀
Host plant: *Echinophora* sp.
Please use ♀, ♂ symbols. Please write upper genus categories with capital letters.

Illustrations: Illustrations, graphs, their caption or legends should form a separate, and a self-explanatory unit. Abbreviations in the legends should be explained but if there are too many, they should be included into a separate list. The original drawing and photographs should not be more than twice as large as when printed.

Morphological illustrations should include a scale bar. Photographs and electron micrographs should be in high-resolution JPEG file format (300 dpi). Drawings (black and white type) should be in TIFF format and their size should be no more than 10 MB. Graphs should also be in black and white and submitted in excel file format. Tables should include headings and explanations, and should be numbered consecutively. Their approximate position in the text should be indicated in the margin. Legends and titles of the graphs and tables should be in Arial with 12 type size. Please do not embed the figures, graphs and table into the text, and send them as supplementary files. In the text attribution to the figures should be given in parenthesis and must be abbreviate like this; (Fig.1).Figs. 1-10. A. marriotti sp. n. 1. Male (holotype), dorsal. 2. Female (paratype)

References:

Titles of manuscripts published in languages other than the major ones (English, German, French, Spanish, Portuguese, Turkish) should be an English translation (in parentheses) with an explanatory note at end, e.g. (in Russian). The list of references should be given at the end of the article and listed alphabetically, according to the following examples. All periodical names should be unabbreviated and italicized. In references, journal titles must be written in full (not abbreviated).

Journal Article

Beirne, B.P. & Young, D.A. (1953). The North American species of Cicadula (Homoptera, Cicadellidae). *Canadian Entomologist*, 85(1), 215-226.

Mitchell, J.A. (2017). Citation: Why is it so important. *Mendeley Journal*, 67(2), 81-95. Retrieved from https://www.mendeley.com/reference-management/reference-manager

Book

Steinmann, H.A. & Zombori, L. (1985). *An atlas of insect morphology*. (2nd ed.). Akadèmiai Kiadò, Budapest, Hungary.

Edited Book

Williams, S.T. (Ed.). (2015). *Referencing: A guide to citation rules* (3rd ed.). New York, NY: My Publisher

Edited Book Chapter

Troy, B.N. (2015). APA citation rules. In S.T, Williams (Ed.). *A guide to citation rules* (2nd ed., pp. 50-95). New York, NY: Publishers.

E-Book

Mitchell, J.A., Thomson, M., & Coyne, R.P. (2017). *A guide to citation*. Retrieved from https://www.mendeley.com/reference-management/reference-manager

Author, A. (date). *Title of book*. doi:xxxxxxxxxxxx

E-Book Chapter

Troy, B.N. (2015). APA citation rules. In S.T, Williams (Ed.). *A guide to citation rules* (2nd ed., pp. 50-95). Retrieved from https://www.mendeley.com/reference-management/reference-manager

Author, A. (date). Title of chapter. In E. Editor (Ed.). *Title of book* (pp. xx-xx). doi:xxxxxxxxxx

URLs

Mitchell, J.A. (2017, May 21). *How and when to reference*. Retrieved from https://www.howandwhentoreference.com.

Thesis

Ersoy, D. E. (2014). *Sündiken Dağlarinin (Eskişehir) Aphodiinae ve Rutelinae Alt Familyalarinin Sistematiği ve Faunasi (Coleoptera: Scarabaeidae)* (Publication No. 12345678) [Doctoral dissertation - Master's thesis, Gazi University]. Council of Higher Education Thesis center. https://tez.yok.gov.tr/UlusalTezMerkezi/

Nomenclature should be in absolute agreement with the current ICZN rules. The only acceptable type concepts are: holotype, paratype, etc. The following abbreviations should be adopted: gen. n., sp. n., stat. n. and comb. n. Journal of the Entomological Research Society uses the Open Journal Systems (OJS) platform, which will enable the journal to accept submissions online. For submitting a manuscript please go to web page http://www.entomol.org and register as author and submit your manuscript online.

Copyright form: You can download JERS copyrigth form in our web site, then sign it with all authors and send us.

URL: http://www.entomol.org

e-mails: jers@entomol.org

Address: Journal of the Entomological Research Society, P.box.110 Bahcelievler P.Isl.Mud. 06502, Ankara/TURKEY

CONTENTS