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Biodiversity and Ecology of Lepidoptera

Insights and Advances

Edited by Farzana Khan Perveen

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Meet the editor

Dr. Farzana Khan Perveen, FLS, Gold Medalist, obtained her BSc (Hons) and MSc in Zoology (Entomology) from the University of Karachi, Pakistan. She also obtained an MAS in Agriculture (Agronomy) from Nagoya University, Japan, and a Ph.D. in Toxicology from the University of Karachi. She is the founder and chairperson of the Department of Zoology and the previous controller of examinations at Shaheed Benazir Bhutto University (SBBU), Pakistan. She is also the previous founder and chairperson of the Departments of Zoology at Hazara University and Kohat University of Science and Technology, both in Pakistan. She is the author of 150 high-impact research papers, 135 abstracts, 40 books, and 9 book chapters. She has also edited nine books. She has supervised numerous undergraduate and graduate students. She has organized and participated in numerous international and national conferences and received multiple awards and fellowships. She is a member of research societies, journal editorial boards, the World Commission on Protected Areas, and the International Union for Conservation of Nature. Her fields of interest are parasitology, toxicology, forensic entomology, and zoology.

Contents

Preface

This book presents comprehensive and concise knowledge about Lepidoptera biodiversity. It includes seven chapters organized into two sections.

Section 1, “Lepidoptera: Behavioural Diversity”, consists of four chapters.

Chapter 1, “Introductory Chapter: Lepidoptera Biodiversity and Conservation in the Twenty-first Century” by Farzana Khan Perveen and Anzela Khan, is an introduction to Lepidoptera biodiversity and conservation. It describes that Lepidoptera is one of the most widespread and extensively recognizable insect orders in the world. They are a symbol of beauty and grace, but they are confronted with a multitude of challenges globally. These challenges have contributed to the deterioration of ecosystems and pose a significant threat to the environment and Lepidoptera species. In the twenty-first century, one of the most pressing challenges these insects face is biodiversity decline. Therefore, there is a need to reverse this decline and restore ecosystems for Lepidoptera survival.

Chapter 2, “Masters of Camouflage and Mimicry: Unusual World of Lepidoptera” by Farzana Khan Perveen and Anzela Khan, discusses camouflage and mimicry of Lepidoptera. Camouflage evolved from the French word camoufler, which originally meant disguise. Camoufler, derived from camouflet, means a puff of smoke that obscures visibility. As a kind of defense, Lepidoptera uses camouflage, also known as cryptic coloration, to conceal its appearance and blend in with its surroundings. This adaptation prevents Lepidoptera from being detected or recognized by other animals. Lepidoptera, in particular, uses a variety of strategies, including disruptive coloration, active camouflage, concealing coloration, disguise, mimicry, countershading, aposematic coloration, color matching, self-decoration, and blending into their surroundings. Camouflage involves animals resembling inanimate objects, while mimicry refers to the similarities between different Lepidoptera species. Nature has provided them with great weapons for survival through these amazing techniques.

Chapter 3, “Gynandromorphy Behavior of Lepidopterans” by Soumya Shephalika Dash and Satya Narayan Satapathy, explores a rare phenomenon known as gynandromorphism, in which an individual develops with a mosaic of both male and female traits. In insects, bilateral gynandromorphs are the most commonly observed, where the left and right halves of the body display different sexes. This phenomenon has been reported in 12 orders within the class Insecta. Within the order Lepidoptera (butterflies and moths), gynandromorphism has been documented in 18 families, with a greater number of cases observed in the families Papilionidae, Pieridae, and Saturniidae. Lepidopterans are known for their sexual dichromatism, primarily expressed through wing coloration. While gynandromorph specimens have been reported in various butterfly families such as Lycaenidae, Nymphalidae, Pieridae, Papilionidae, and Geometridae, there have also been documented cases in certain moth species, including Noctuidae species like *Agrotis segetum* and *Agrotis ipsilon*.

Chapter 4, "Non-Coding RNAs in Lepidoptera" by Dandan Li et al., reports that in the last few years the amount of genomic sequence data has grown exponentially. Many non-coding RNAs (ncRNAs) have been identified from organisms ranging from bacteria to humans. ncRNAs are various and multi-faced; they can regulate gene expression through chromosomal, transcriptional, post-transcriptional, and translational levels and then participate in the whole process of development in different organisms. ncRNAs were identified in Lepidoptera in the 1980s; they can play roles in growth, metamorphosis, metabolism, sex determination, reproduction, and immune response of insects. Now, the use of ncRNAs in pest control of Lepidoptera is also in process. This chapter reviews the recent advance of ncRNAs in Lepidoptera and the prospect of future studies of ncRNAs in insects.

Section 2, "Moths: Bioecology and Genetics", consists of three chapters.

Chapter, 5 "Hawkmoths (Lepidoptera, Sphingidae) Flight Potential Trajectories from Wind Systems in Atlantic Rainforest in Southeast Brazil" by Marcio D'Arrochella et al., discusses flight potential trajectories of Hawkmoths. In tropical regions, pollination is primarily carried out by hawkmoths (Lepidoptera, Sphingidae), being perceived as one of the most important groups. Lepidoptera, in general, comprises approximately 146,000 species of hawkmoths, 87% of them being nocturnal. In these regions, there are 7100 species, approximately 3100 of which are found in Brazil. The Sphingidae family is one of the most representative families when it comes to pollination, being more abundant in low-altitude environments. In Brazil, most of the studies focus on the morphology of individuals for taxonomic research, with few publications on their dispersal available. By mapping flight paths for feeding and reproduction, the occurrence of pollination is estimated, enabling the connectivity of forest fragments and ensuring gene exchange. The north/south orientation proved to be dominant, potentially connecting fragments of forests with varying sizes, shapes, and conservation states, extending from coastal areas to the mountainous regions within the southeastern part of Brazil.

Chapter 6, "Invasive Leaf-Mining Moths (Lepidoptera, Gracillariidae) in Ukraine: Ecology, Degree of Invasion" by Kyrylo Holoborodko, elucidates the bioecological characteristics of the existence of five invasive leafminer moth species of the family Gracillariidae Stainton, 1854—*Parectopa robiniella* (Clemens, 1863), *Phyllonorycter issikii* (Kumata, 1963), *Phyllonorycter platani* (Staudinger, 1870), *Macrosaccus robiniella* (Clemens, 1859), and *Cameraria ohridella* (Deschka and Dimic, 1986)—and the extent of their impact on dendroflora in Ukraine. The chapter examines features of their invasion into various ecosystems and studies the effects of Gracillariidae caterpillar feeding on host (food) plants. The caterpillars of invasive leafminer moth species affected the functional state of host plants, which was confirmed by alterations in the content of soluble proteins, activity, and isoenzyme composition of benzidine peroxidase during the growing season of the host plants. The results obtained show the activation of the enzymatic antioxidant protection system in host plants in response to the damaging effect of Gracillariidae caterpillars, which ensures the survival of the plant and completes its ontogenesis program in unfavorable conditions.

Chapter, 7 "Population Genetic Studies of Silkworm (*Bombyx mori* L.) and Phylogenetic Relationships" by Teodora Atanasova Staykova and Panomir Ivanov Tzenov, evaluates the degree of genetic variability and phylogenetic relationships between 13 breeds of mulberry silkworm (*Bombyx mori* L.) from genetic resources of Bulgaria through isozyme polymorphism. Using PAGE, the authors found intra and interbreed polymorphism among 8 of the 9 studied isoenzyme loci (Bes A, Bes B, Bes D, Bes E, Pgm A, Mdh A, Bph, and Alp A). At the Hk locus, they found interbreed polymorphism only. The number of alleles per polymorphic locus ranged from one to two. The degree of polymorphism ranged from 0% to 77.80%. Low levels of observed heterozygosity in comparison with the expected one was calculated in all breeds. The combined FIS value over all polymorphic loci was 0.3205, which reflects a substantial deficit of heterozygotes. The value of FST showed that 49.21% of the overall genetic diversity observed was among breeds. The dendrogram constructed manifested that the two breeds of Japanese origin (Daizo and Japanese 106) were genetically most distant from other breeds. The data for isoenzyme polymorphism and genetic structure of the tested breeds can be used for genetic improvement and to develop new hybrids for silk production.

This book provides a comprehensive overview of Lepidoptera biodiversity, focusing on the most important evidence of their various advantageous aspects. It is a useful resource for scientists, researchers, naturalists, conservationists, agriculturalists, biologists, professors, teachers, students, and everyone who is interested in Lepidoptera. The editor is grateful to all authors for their contributions. Special thanks go to Publishing Process Manager Miss Nina Miocevic and all the staff of IntechOpen. The experiments described in this book comply with the current laws of the country in which they were performed.

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Section 1

Lepidoptera: Behavioural Diversity

Chapter 1

Introductory Chapter: Lepidoptera Biodiversity and Conservation in the Twenty-First Century

Farzana Khan Perveen and Anzela Khan

1. Introduction

1.1 Biodiversity

Lepidoptera is one of the most widespread and extensively recognizable insect orders in the world. It is the second-largest and the most diverse order within the class Insecta. It is characterized into four distinct groups: butterflies, skippers, micromoths, and macromoths. It includes 126 families, 46 super-families, and 174,250 species. Of these, butterflies alone contribute 70,820 species. They are scattered worldwide excluding Antarctica. It signifies 10% of the total defined species of living organisms. They are a symbol of beauty and grace (**Figure 1**) [1–3].

2. Conservation

Lepidoptera species globally are confronted with a multitude of challenges, ranging from habitat loss, degradation to climate change, invasive species, intensified agriculture, afforestation, fragmentation, limited host plant availability, inadequate nectar sources, disruption of ecological interactions, unsustainable resource use, pollution, the expanding human population, overconsumption of resources, and various other anthropogenic factors. These challenges have contributed to the deterioration of ecosystems and pose a significant threat to the survival of these species. In the twenty-first century, one of the most pressing challenges they face is the reversal of biodiversity decline and the restoration of ecosystems. Recognizing the urgency, the European Agenda 2030 emphasizes the priority of biodiversity conservation. Consequently, it is essential to develop specific conversation tools, including action plans for threatened species, assessment protocols for sustainable agriculture, and models for prioritization of species and areas. In conclusion, effectively addressing the numerous challenges faced by Lepidoptera species and implementing comprehensive conservation measures are imperative to reverse the decline in biodiversity and restore ecosystems. Current policies exhibit significant gaps in protecting Lepidoptera biodiversity, emphasizing the need for urgent action and holistic approaches.

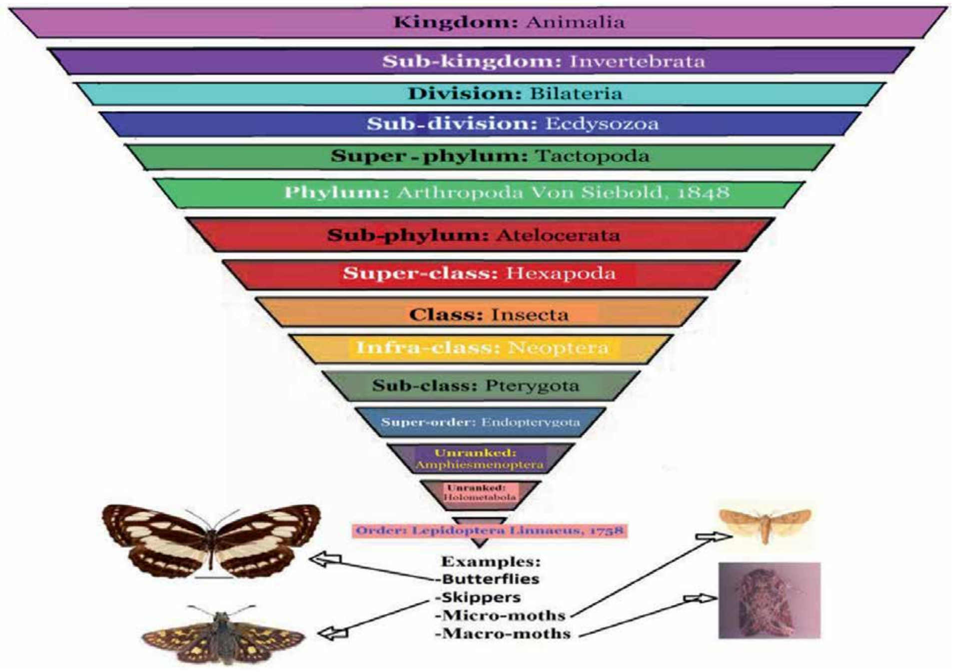

Figure 1.
General criteria of the pyramid for taxonomic rank of Lepidoptera with examples [4–6].

3. Variability

Lepidoptera have great variability in size depending on generation, latitude, altitude, or climate. There are also variations in the wing ornamentation due to seasonal and geographical variations. They show sexual dimorphism, many species have differences between male and female, often minimal differences, but which can go in some species to a totally different coloring or ornamentation, such as in the common Azure, *Polyommatus icarus* (Rottemburg, 1775) [7, 8]. The black grass-dart butterfly, *Ocybadistes knightorum* Lambkin and Donaldson, 1994, is a species of butterfly belonging to the Hesperiidae family. This species exists only in New South Wales. Throughout the power source Boambee region, its availability is extremely constrained (**Figure 2**) [11–13].

4. International Union for Conservation of Nature (IUCN)

Lepidoptera numbers are, universally, continuing to decrease [14]. The components that cause losses have been numerous although interdependent, but a lot of them remain to be entirely known [15, 16]. The Red List was created by the International Union for Conservation of Nature (IUCN) in 1964. After that, it has been utilized as a vital sign of the state of the global Lepidoptera biodiversity. The Red List can be employed as a tool to educate and inspire initiatives that might contribute toward the conservation of butterfly biodiversity, which is essential when you would like all of the species to live. They can serve as more than simply a list of Lepidoptera species and their state as a barometer of life. Governments use the Red List Index (RLI) to monitor how they are progressing toward goals all minimizing Lepidoptera biodiversity loss. The RLI displays variations in the cumulative mortality risk for Lepidoptera species.

Figure 2.
(i) Sexual dimorphism in the common Azure, Polyommatus icarus (Rottemburg, 1775); a: male; b: female; c: mating view from below, male on the left; (ii): limited distribution: d: black grass-dart butterfly, Ocybadistes knightorum Lambkin and Donaldson, 1994; (iii) geographic variability in the white admiral, Limenitis arthemis (Drury, 1773), e: subspecies, white admiral butterfly, L. a. arthemis (Drury, 1773); f: subspecies, the red-spotted purple, L. a. astyanax Butler, 1869; (iv) seasonal variability in map butterfly, Araschnia levana Linnaeus, 1758, g: spring form of A. levana; h: Prorsa summer form of A. levana; (v) the two forms of Apatura: i: the lesser purple emperor, A. Ilia Rhône 1969 form; j: purple emperor, A. clyta Rhône 1969 form; (vi) regardless of all these factors, there is always some individual variability, no two butterflies are ever the same; k: peacock butterfly, Aglais io (Linnaeus, 1758), classic specimen; l: A. io, an aberrant specimen [9, 10].

The mission and objectives of IUCN serve to promote and support communities across every continent in protecting the authenticity and variety of nature, as well as in ensuring that all uses that involve natural assets are fair and environmentally responsible. Extinct, extinct in the wild, critically endangered, endangered, vulnerable, on risk, data deficient, and not evaluated are some of the categories that consist of species on the Red List (**Table 1**). To keep track of all threatened species, the IUCN created the Red Data Book of Lepidoptera species. Through this book, the IUCN is trying to create awareness about the endangered Lepidoptera species [17–19].

5. Red List 2022

The IUCN Red List, which is annually produced, is largely acknowledged as the most complete collection of data on the threat to extinction facing Lepidoptera species around the world. According to the IUCN Red List and the World Wildlife Fund (WWF) of the UK, the fact has been included that Lepidoptera species are declining (**Tables 2** and **3**) [17–19].

6. Conservation under climate change

Lepidoptera provides a vast range of ecosystem services, including pollination of flowers, which are also outstanding indicators of environmental quality, have a

The Red List categories

Extinct (EX)	There is no possibility if the final creature is still alive
Extinct in the wild (EW)	Only documented to survive individual in cultivation, captivity, or far from its natural area
Regionally extinct (RE)	Individual vanished at the regional level
Critically endangered (CR)	Creature is having a very high chance of going extinct in the wild
Endangered (EN)	Having an extremely significant chance of going extinct in the wild
Vulnerable (VU)	Posing a high risk of mortality within wild places
Near threatened (NT)	Almost reaching, or very likely to arrive for at susceptible type soon
Least concern (LC)	It is not possible to go extinct in the near future because of the population's stability
Data deficient (DD)	There are not enough data on its quantity or dispersion to determine how likely it is to go extinct
Not applicable (NA)	The population is not relevant
Not evaluated (NE)	The population is not analyzed

Table 1.
The International Union for Conservation of Nature's (IUCN) red list categories for species [17–19].

significant esthetic value, and provide enjoyment to public around the biosphere. Lepidoptera can be affected in many ways. Utmost frequently, species can be changed in their range, in distribution, and in population size. Additionally, Lepidoptera may alter their phenology in response to climate change, for example, as a consequence of changes in the climate, some species have already changed the entire geographic distance, typically relocating to higher latitudes or elevations. Butterflies and moths emerge earlier in spring due to high temperature. There are many examples of disturbance of vital interactions with prey and food plants. Though, additional implications from the changing climate are not yet fully understood. At present, there are many species in danger and are going to become extinct than ever before, placing biodiversity at a crossroads. Because connectivity and terrain availability are frequently scarce, if they have been even noticed, it is exceedingly difficult to establish conservation measures as a result of the extent to which anthropogenic activity has been ruined, degraded, and separated the landscape. Because many species and their ecosystems are less able to respond due in large part to anthropogenic terrorizations. It is particularly challenging to build successful preservation plans according to the problem of changing the climate. Preservation schemes must contain an environmentally climatic friendly adaptability element, either right from the beginning as well as a final optional subsequent addition [20–23].

7. Conservation initiatives

Lepidoptera, predominantly butterflies and large moths, are prevalent goals for conservation efforts and serve as flagship species. They can offer assistance to expose the requirement for habitat and resource protection and the ecological value of invertebrates [24]. An alliance of participant aquariums, botanical gardens, museums, zoos, partner organizations, projects, and center was established in 2001 with the collaboration of local, federal, and state organizations to aid in the conservation

S. no.	Common and *Scientific* name, authority year	Population*	Threatened species in year**	Habitat	Geographic range	Picture
(1)	Skipper butterfly, *Abantis meru*, Evans, 1947	EN	2022	Forest	Kenya	
(2)	Arogos skipper, *Atrytone arogos*, Boisduval and Le Conte, 1834	EN	2021	Grassland	United States	
(3)	Queen Alexandra's birdwing, *Ornithoptera alexandrae*, Rothschild, 1894	EN	2021	Forests	Oro Province, eastern Papua New Guinea	
(4)	Luzon peacock swallowtail, *Papilio chikae*, Igarashi, 1965	EN	2021	Humid tropical forests, mountains	Luzon Island, Philippines	
(5)	Homerus swallowtail or Jamaican swallowtail, *Papilio homerus*, Fabricius, 1793	EN	2021	Virgin forests	Jamaica Island	
(6)	Teita glider, *Cymothoe teita*, van Someren, 1939	EN	2022	Forest	Taita Hills Kenya, part of Eastern Arc Mountains	
(7)	Brushfooted hipparchia butterfly, *Hipparchia christenseni*, Kudrna, 1977	EN	2013	Shrubland	Greece	

S. no.	Common and *Scientific* name, authority year	Population[*]	Threatened species in year[**]	Habitat	Geographic range	Picture
(8)	Madeiran speckled wood, *Pararge xiphia*, Fabricius, 1775	EN	2009	Forest	Portugal (Madeira)	
(9)	Sudeten ringlet, *Erebia sudetica*, Staudinger, 1861	VU	2009	Forest, grassland, rocky area	France, Spain	
	Balkan Heath, *Coenonympha orientalis*, Rebel, 1910	VU	2009	Terrestrial, forest, grassland	Europe	
(10)	Chios meadow brown, *Maniola chia*, Thomson, 1987	NT	2013	Shrubland	Greek islands Chios and Oinousses	
(11)	Spring ringlet, *Erebia epistygne*, Hübner, 1824	NT	2009	Grassland, rocky areas (inland cliffs and mountain peaks)	Europe Southeast France and Spain	
(12)	Woodland grayling, *Hipparchia fagi*, Scopoli, 1763	NT	2009	Grassland, rocky areas (inland cliffs and mountain peaks)	Europe, Russia	

[*] *CR: critically endangered; EN: endangered; VU: vulnerable; LC: least concern; NT: near threatened; LR: lower risk.*

[**] *Threatened species in the mentioned year.*

Table 2.
The world declining butterfly and skipper species according to the IUCN Red List [17–19].

S. no.	Common and *Scientific* name, authority year	Population*	Threatened species in year**	Habitat	Geographic range	Picture
(1)	Traca moth, *Eudarcia atlantica*, Henderickx, 1995	CR	2017	Rocky area (inland cliffs and mountain peaks), marine coastal/ supratidal	Portugal (Azores)	
(2)	Moth, *Neomariania incertella*, Rebel, 1940	CR	2017	Forest, shrubland, grassland	Portugal (Azores)	
(3)	Marion's plume moth, *Agdistis marionae*, Arenberger, Beard, Hasenfuss and Karisch, 2012	CR	2015	Marine coastal/ supratidal	Saint Helena, Ascension and Tristan da Cunha	
(4)	Fungus moth, *Tinea poecilella*, Rebel, 1940	CR	2017	Forest, artificial/ terrestrial	Portugal (Azores)	
(5)	Enigma moth, *Aenigmatinea glatzella* Kristensen and Edwards, 2015	CR	2021	Marine coastal/ supratidal	South Australia	
(6)	Agrotis *moth*, *Agrotis kuamauna*, Medeiros and Kirkpatrick, 2019	EN	2020	Rocky areas (inland cliffs and mountain peaks)	United States (Hawaiian)	
(7)	*Twirler moth*, *Brachmia infuscatella*, Rebel, 1940	EN	2017	Forest, shrubland, grassland, artificial/ terrestrial	Portugal (Azores)	

S. no.	Common and *Scientific* name, authority year	Population[*]	Threatened species in year[**]	Habitat	Geographic range	Picture
(8)	Scopulini moths, *Scopula separata*, Walker, 1875	VU	2019	Desert	Saint Helena, Ascension and Tristan da Cunha	
(9)	Midway noctuid moth, *Agrotis fasciata*, Rothschild, 1894	LR/NT	2020	Shrubland, marine coastal/super tidal	United States (Hawaiian)	

[*] CR: critically endangered; EN: endangered; VU: vulnerable; LC: least concern; concern; NT: near threatened; LR: lower risk.
[**] Threatened species in the mentioned year.

Table 3.
The world declining moths species according to the IUCN Red List [17–19].

of endangered, threatened, and vulnerable Lepidoptera species of North America. Habitats were also created to sustain them. Many of the partnerships formed through the initiative results of projects provide a chance to increase their conservation efforts. The conservation program has been developed for the benefit of Lepidoptera. Laboratories such as Special Projects Labs have been established in the McGuire Center for Lepidoptera and Biodiversity, which are the home to several butterflies' conservation projects, of which one such popular project is the Mimi blue. For the conservation projects for butterflies and moths, no more space is required and it is easy for organizations of all sizes to participate [25–27].

8. Reintroduction, translocation, and captive breeding

The terms reintroduction, translocation, and captive breeding are sometimes used interchangeably, but in fact, they have distinct meanings. Reintroduction refers to the deliberate movement and release of a species within its native range after it has vanished. Translocation refers to the relocation of living things from one location to another through human intervention. As a result, it may be a method to introduce creatures back into the wild while also encouraging them to move outside of their natural area. The production of new individuals from animals kept in captivity is known as captive breeding. As a result, it may be a method of obtaining the source of the creatures for rebirth or relocation [28–31].

9. Biodiversity heritage relicts

Numerous landscapes constitute the outcome of interactions between biological mechanisms, the economy, as well as a community's social and administrative structures. Consequently, certain landscapes could include lingeringly degraded habitats hosting evidence of former biodiversity as a result of human-induced alterations throughout history, which are known as biodiversity patrimony relicts [32].

10. Habitat restoration

The habitat restoration of Lepidoptera is a recent concept in human history. In order to ensure that native Lepidoptera and humans may continue to use the land, disturbed habitats must be identified and their native Lepidoptera must be restored. It works to restore regions where the habitat has been destroyed. It is a major contributing component in the global extinction crisis facing Lepidoptera species. The typical abundance and distribution of Lepidoptera species in the habitats are altered by its destruction. If the land is to remain productive in the future, repair of all these forms of disruptions is necessary. It is significant for a variety of reasons, including pragmatism, economics, and esthetics. Restoring habitats can facilitate the return of Lepidoptera to disturbed areas for the sake of recreational activities or for its own sake. By restoring biotic (e.g., species composition, interspecies interactions) and abiotic (e.g., disturbance, soil chemistry, and water content) elements to historical levels, it is possible to protect, manage, and restore plants, which are important elements for the restoration of butterflies and moths species. Restoration of ecosystems in the right way shows that there were once many species in the region instead of only one in monoculture. For reestablishment of flora, soil conditions are crucial, because they will decide what will grow and where. Aeration, mineral content, soil moisture, and the presence of microbes are all significant aspects to take into account for habitat restoration [33–35].

11. Citizen Science

For changing our environment, over large geographic areas, Citizen Science is proving to be an operative tool in tracking the rapid pace. It is widespread progressively attractive in regions like North America as well as in a few European nations. Public and school age participants have been engaged in gathering scientific data to promote long-term environmental monitoring. The Citizen Scientists are generally those volunteers who participate in such schemes. More recently, volunteers are used by the Irish butterfly and the bumblebee monitoring programs to collect information regarding detections involving these species. Most of the aforementioned networks as well as projects have as their major objective the gathering of information necessary for monitoring shifts within the variety and growth of Lepidoptera. In Green wave, youngsters engage in investigation-based learning scientific endeavors that are both simple and practical. Altogether, several diverse networks aid toward spreading an understanding about serious ecological problems including the decline in biodiversity as well as the impact of climate change. Whereas concurrently, the children, school pupils, and general population promote the development of scientific skills. Furthermore, they provide scientific data which have great value, by which we can track the environmental changes for improvements toward preserving the biodiversity of Lepidoptera [36–38].

12. Genetic diversity: a case study of the threatened large blue butterfly, *Maculinea arion*

In reintroductions, an imperative factor is the amount of genetic diversity captured, when introducing the individuals of Lepidoptera in the environment. When populations of Lepidoptera are introduced, at first they are very small, and thus are susceptible to stochastic events and genetic drift. The degree of genetic diversity that has been retained is important for populations' long-term survival and their ability to adapt to climate change through evolution. Many European nations have observed the loss of numerous butterfly species on a national level. The great blue butterfly, *Maculinea arion* (Linnaeus, 1758), which was once extinct in the UK in 1979, was later brought back from Sweden due to its global vulnerability. On five sites in the UK and seven sites on Oland, including the source population, the genetic diversity of the reintroduced *M. arion* over 19 generations after translocation was examined. The reintroduced and source populations showed comparable levels of genetic diversity, however, the UK and Swedish groups showed genetic differentiation. Significant genetic heterogeneity between recently arrived UK groups that were only a few kilometers apart was also discovered. Several private alleles that were not present in the source population in 2011 were present in the reintroduced groups. As a result, they already comprise a distinct subset of *M. arion*'s northwestern populations' genetic diversity. The findings demonstrated that for this species, the IUCN and other guidelines used in the 1990s for relocation and maintaining the greatest amount of

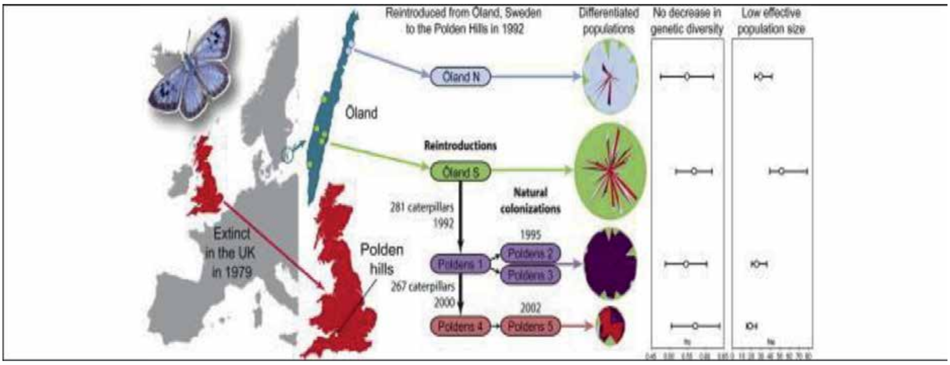

Figure 3.
Studying the genetic diversity of reintroduced and source populations to evaluate reintroduction plans: A case study of the threatened large blue butterfly, Maculinea arion (Linnaeus, 1758) [39].

genetic variety during reintroductions were essentially acceptable. It will, therefore, be helpful for guiding the expanding use of reintroductions as a tactic for the conservation of endangered Lepidoptera species (**Figure 3**) [39].

13. Conclusion

In reality, Lepidoptera species are declining worldwide. The IUCN, WWF, and other associations are trying to combat diminishing of Lepidoptera species. The intimidations and challenges to Lepidoptera conservation and recovery continue to grow. As the number of targeted species-specific recovery programs has been initiated with involving the integration of *ex-situ* and *in-situ* components. For the conservation of Lepidoptera species, great efforts have been mandated with significant commitments of funds, labor, and time. Nevertheless, it is also needed to involve the government to achieve identified recovery targets. Conservation techniques must improve the use of evaluations supported by evidence and utilize them more effectively for systematic preparation in order to accomplish the best restoration achievements. Lepidoptera species preservation remains a young as well as an underdeveloped discipline in numerous aspects. The abundance of vertebrate-based examples, successes, and—possibly most importantly—lessons experienced must be used to its advantage.

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Chapter 2

Masters of Camouflage and Mimicry: Unusual World of Lepidoptera

Farzana Khan Perveen and Anzela Khan

Abstract

Camouflage is derived from the French word "camoufler", which originally means disguise. Camoufler derives from camouflet, means a puff of smoke that obscures visibility. As a kind of defense, Lepidoptera uses camouflage, also known as cryptic coloration means to conceal its appearance and blend it with its surroundings. This adaptation prevents Lepidoptera from being detected or recognized by other animals. Lepidoptera, in particular, uses a variety of strategies, including disruptive coloration, active camouflage, concealing coloration, disguise, mimicry, counter-shading, aposematic coloration, color matching, self-decoration, and blending into their surroundings. Camouflage involves animals resembling inanimate objects, while mimicry refers to the similarities between different Lepidoptera species. Since the earliest evolutionists, these ideas have been used as examples of natural selection and adaptation. For surviving in their environments, Lepidoptera employs physical, behavioral, and structural adaptations. Such adaptations include hibernation, migration, instinct, and learning. A Lepidoptera's body has features called structural adaptations that help it for surviving, such as water retention capacity and defensive coloration. In camouflage, there are eight factors of recognition: position, shape, shadow, texture, color, tone, movement, and shine, those must be considered to ensure that the animal's location remains concealed. Nature has provided its creatures with great weapons for survival through these amazing techniques.

Keywords: adaptation, camouflage, Lepidoptera, predators, mimicry

1. Introduction

During roughly 400 million years on this planet, one million species of insects have developed with a great diversity of shape and color to protect them from predators [1]. Animals have evolved several strategies in prey-predator interactions due to selective pressures, such as mimicry and camouflage [2]. However, several fossil insects have been reported showing plant-like mimesis and debris-carrying camouflage from the Mesozoic [3]. It was reported that the earliest mimetic and defensive strategies of a stick insect from the Middle Jurassic of China, Klimpel's Hedgehog Cactus, *Aclistophasma echinulatum* (*Weidlich and Werderm)* exquisitely preserved abdominal extensions and femoral spines. The distribution of these characteristics mapped onto the phylogeny of (F: Phasmatodea) reveals that abdominal extensions and femoral spines developed multiple times during the evolution of stick insects, Spanish walking stick, *Leptynia hispanica* Nicolás Vega, indicates that the origin of abdominal extensions predates other modifications, while tergal extensions predate other expansions of the body, such as those of the sterna and pleura, as well as defensive femoral spines [4].

Antipredator defenses among insects commonly involve the interplay of two functional categories. The primary defense, also called passive defense, is the prey's avoidance of detection by the predator, usually by means of hiding or shifting periods of activity, crypsis, aposematism, or pseudaposematism. The prey's secondary defense is evading capture after the initiation of a predator's attack. Secondary defenses involve active escape, antipredator displays, flash coloration, defensive chemical secretion, and feigning death [5, 6]. The active fighting of the prey against a predator, when seized, is sometimes referred to as a separate third category [7, 8]. But, together with the previous series of behaviors, they are referred to as active defenses. Naturally, the ideal situation for any prey is to invest sufficiently in passive mechanisms of defense to avoid the chances of requiring active defense and the increased probability of death that comes when an attack has been initiated [9].

1.1 History

For well over a century, the zoologists have interested in and researching the topic of camouflage. Natural selection, a hypothesis put out by Charles Darwin in 1859, contends those traits, like camouflage, arose as a result of given specific animals a reproductive advantage that allow them to produce, on average, more offspring than other members of the same species. His research demonstrated that the pupae of the swallow-tailed moth, *Ourapteryx sambucaria* Linnaeus, 1758 [Family (F): Geometridae] were camouflaged to resemble the backgrounds on which they were raised as larvae (**Figure 1**) [11].

The avoidance of detection by predators and parasites is critical to survival. Two complex mechanisms for such avoidance are mimicry and camouflage, with fossils providing valuable insight into the evolution of these strategies. It was reported that a diverse insect assemblage exhibited these adaptations from mid-Cretaceous Kachin amber (99 million years ago), including plant mimesis in Tridactylidae (pygmy mole crickets) and debris-carrying camouflage in Gelastocoridae (toad bugs) and Psocodea (bark lice) [12].

Poulton reported that the color of animals, particularly camouflage. Different types of camouflage, such as specific defensive resemblance, where an animal mimics another object, or general aggressive resemblance, where a predator blends in with the background to approach prey, were classified by him in his book. For colors of animals, his research demonstrated that *O. sambucaria* pupae were camouflaged to blend with the environments in which they were raised as larvae. The major means of camouflage at the time was thought to be Poulton's overall protective resemblance (**Figure 1, i: a-c**) [13].

The orange-tipped butterfly, *Anthocharis cardamines* (Linnaeus, 1758) (F: Pieridae), has attractive colorations, according to Beddard (1940) that the sporadic green patches on the underside of the wings may have been intended to represent a crude drawing of the plant's miniature flowerets (an umbellifer), which would explain how they are similar to one another (**Figure 1, ii: d-g**) [14].

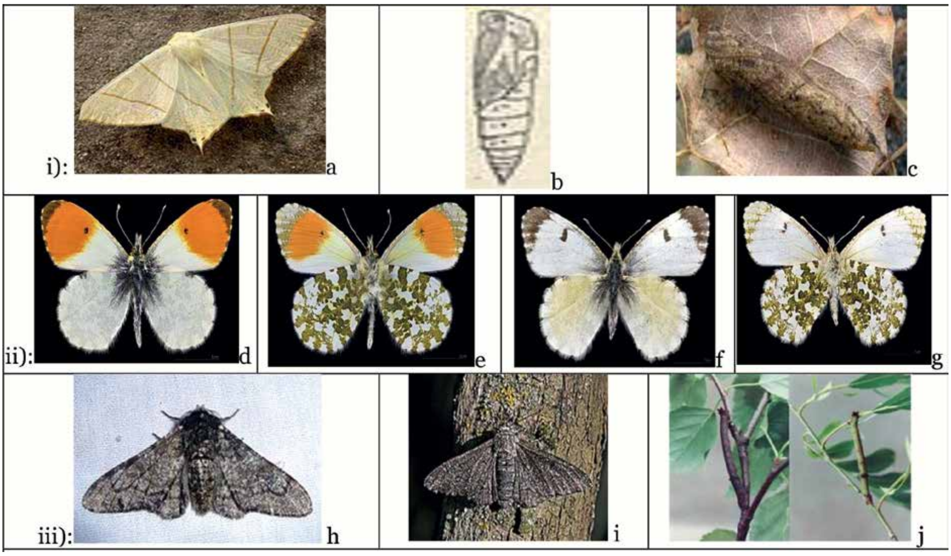

Figure 1.
(i): Swallow-tailed moth, Ourapteryx sambucaria *Linnaeus, 1758 [Family (F): Geometridae]: a; pupa showing color assumed when larva has been placed on white paper before pupation: b; pupa with usual dark color assumed in cocoon with natural size: c [2]; (ii): Camouflage, the alluring coloration in the orange tip butterfly,* Anthocharis cardamines *(Linnaeus, 1758) (F: Pieridae);* A. cardamines *♂: d;* A. cardamines *♂: e;* A. cardamines *♂: f;* A. cardamines *♂: g [3]; (iii): Peppered moth,* Biston betularia *Linnaeus, 1758 (F: Geometridae): h; its camouflage with substrate of natural background: i; a* B. betularia *caterpillar mimics a twig: j [10].*

The peppered moth, *Biston betularia* Linnaeus, 1758 (F: Geometridae), is a temperate species of night-flying moth. Natural selection and population genetics are examples of evolution in *B. betularia*. Prey animals frequently replicate their surroundings, as seen in the *B. betularia* caterpillar's imitation of a branch in **Figure 1** (**iii: h-j**) [10].

1.2 Camouflage and disguise

It is difficult to distinguish between disguise and *camouflage*, but generally speaking, camouflage is used to describe something that can blend in nicely with a natural *background* or substrate, thanks to its color, pattern, and texture. Various types of butterflies and moths have colors and patterns that blend in with each of these backdrops when they rest on a variety of substrates, such as leaf, soil, rocks, and tree trunks. On the other side, *disguise* refers to a butterfly or moth that resembles another natural *object*, like a leaf or flower [15, 16].

Numerous caterpillars are skilled twig impersonators. The geometrids (F: Geometridae) is a special huge group that goes by this name (**Figure 2a**). When disturbed, they will freeze and give the impact of heighten. They have the appearance and feel of twigs. They lack middle-body legs to make them appear like twigs, and as a result, they walk in a loop, which gives the group its name [15, 16].

To evade predators, butterflies employ a variety of strategies. Sometimes, like in the case of the green hairstreak, *Callophrys rubi* (Linnaeus, 1758) (F: Lycaenidae) resting on green foliage and the Neotropical Lysippus Metalmark, *Riodina lysippus* (Linnaeus, 1758) (F: Riodinidae) hides under leaves to avoid being seen, respectively (**Figure 2b** and **Figure 2c**). A nice example of a British butterfly that is challenging

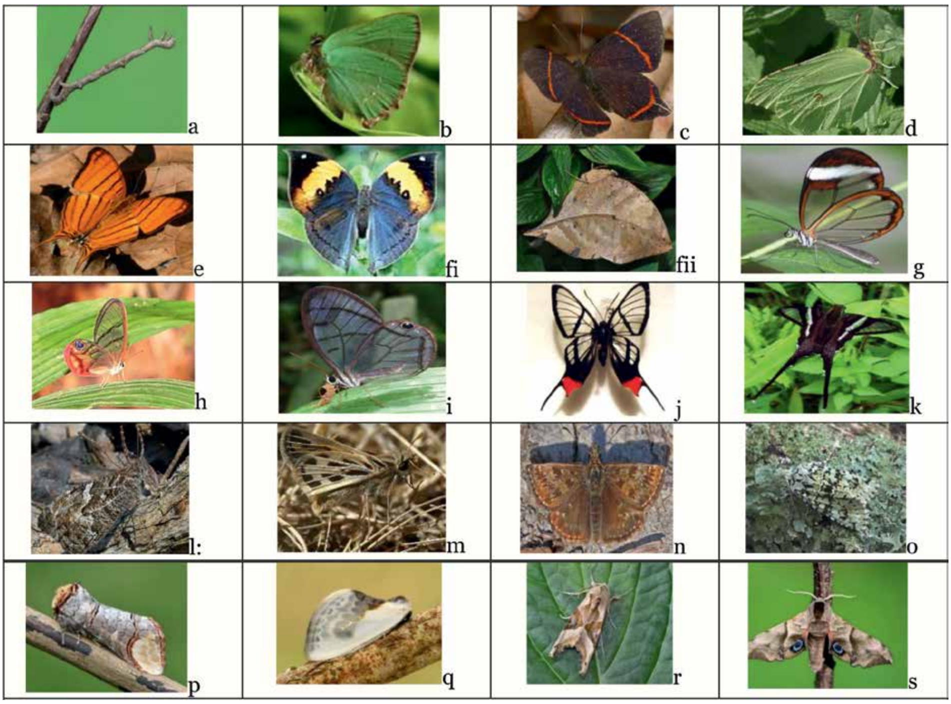

Figure 2.
Camouflage and disguise in Lepidoptera: a: caterpillar (F: Geometrids); b: green hairstreak, Callophrys rubi *(Linnaeus, 1758) (F: Lycaenidae); c: lysippus metalmark,* Riodina lysippus *(Linnaeus, 1758) (F: Riodinidae); d: brimstone,* Gonepteryx rhamni *(Linnaeus, 1758) (F: Pieridae); e: orange daggerwing,* Marpesia berania *(Hewitson 1852) (F: Nymphalidae); f: dead leaf butterfly,* Kallima inachus *(Doyère, 1840) i: dorsal surface; ii: ventral surface (F: Nymphalidae); g: Ithomiine glasswings,* Greta oto *(Hewitson, 1854) (F: Nymphalidae); h: blushing phantom,* Cithaerias pireta *(Stoll, 1780) (F: Nymphalidae); i: uncolored clearwing-satyr,* Dulcedo polita *(Hewitson, 1869) (F: Nymphalidae); j: octauius swordtail,* Chorinea octauius *(Fabricius, 1787) (F: Riodinidae); k: white dragontail,* Lamproptera curius *(Fabricius, 1787) (F: Papilionidae); l: grayling butterfly,* Hipparchia semele *(Linnaeus, 1758) (F: Nymphalidae); m: peruana skipper,* Hylephila peruana *Draudt, 1923 (Family: Hesperiidae); n: dingy skipper,* Erynnis tages *(Linnaeus, 1758) (F: Hesperiidae); o: merveille du jour,* Griposia aprilina *(Linnaeus, 1758) (F: Noctuidae); p: buff-tip,* Phalera bucephala *(Linnaeus, 1758) (F: Noctuidae); q: Chinese character,* Cilix glaucata *(Scopoli, 1763) (F: Drepanidae); r: angle shades,* Phlogophora meticulosa *(Linnaeus, 1758) (F: Noctuidae); s: eyed hawk-moth,* Smerinthus ocellatus *(Linnaeus, 1758) (F: Sphingidae) [16].*

to see when resting on white garlic mustard blooms is the orange-tipped, *Anthocharis cardamines* (F: Pieridae) (**Figure 1, ii: d, e, f, g**) [16].

The brimstone, *Gonepteryx rhamni* (Linnaeus, 1758) (F: Pieridae) (**Figure 2d**), is a great example of deception since it has the coloration, shape, and elevated veins of a living leaf. The orange dagger wing, *Marpesia berania* (Hewitson, 1852) (F: Nymphalidae) (**Figure 2e**), and the pale-spotted leafwing, *Memphis pithyusa* (Felder, 1869) (F: Nymphalidae) are two examples of the many species that may be found in the tropics those mimic decaying brown leaves [16].

In Malaysia, the dead leaf butterfly, *Kallima inachus* (Doyère, 1840) (F: Nymphalidae), is the ideal illustration of camouflage. It has wings (dorsal surface) (**Figure 2**: fi) that can tolerate rain and wetness in deep forests, allowing it to fly high and quickly. When threatened or pursued by birds, it flies erratically and swiftly, falls to the ground, and buries itself in the undergrowth by folding its wings to blend in. Its wings resemble a dry leaf with dark veins, when they are closed (ventral surface

(**Figure 2**: fii). The *K. inachus* prefers to dwell on tree trunks or the ground with fallen leaves, but it occasionally visits flowers. Its natural predators include birds, ants, and spiders, and it feeds on rotting fruits and animal excrement [17].

Butterflies that use transparency to obscure themselves comprise the Ithomiine (Tribe: Ithomiini) glasswings, *Greta oto* (Hewitson, 1854) (F: Nymphalidae) (**Figure 2g**), the Neotropical satyrines, such as the blushing phantom, *Cithaerias pireta* (Stoll, 1780) (F: Nymphalidae) (**Figure 2h**) and the uncolored clearwing-satyr, *Dulcedo polita* (Hewitson, 1869) (F: Nymphalidae) (**Figure 2i**). The Octauius swordtail, *Chorinea octauius* (Fabricius, 1787) (F: Riodinidae) (**Figure 2j**), and the white dragon tail, *Lamproptera curius* (Fabricius, 1787) (F: Papilionidae) (**Figure 2k**) are additional specimens from other families [18].

The grayling butterfly, *Hipparchia semele* (Linnaeus, 1758) (F: Nymphalidae), which is expertly disguised when resting on decaying wood, is depicted in **Figure 2**. The skipper from Peru, *Hylephila peruana* Draudt, 1923 (F: Hesperiidae) (**Figure 2m**) is exceedingly challenging to spot among the dry grasses of its Andean environment due to the disruptive patterning. In leaf litter, the dingy skipper, *Erynnis tages* (Linnaeus, 1758) (F: Hesperiidae) (**Figure 2n**) blends well [19].

Because moths are frequently eaten, they have developed a range of camouflage techniques, including delicate colors and patterns that fit in with their environment. The outcomes are frequently rather beautiful, in addition to being incredibly brilliant. They have colors and patterns to match the locations, where they rest because they are particularly vulnerable to being seen in daylight. Because they blend into the bark of branches and are difficult to spot in glooms, many moths have patterns of gray and brown on their wings.

The merveille du jour, *Griposia aprilina* (Linnaeus, 1758) (F: Noctuidae) (**Figure 2o**), is the ideal complement to bark that has been covered with lichen. The buff-tip, *Phalera bucephala* (Linnaeus, 1758) (F: Noctuidae) (**Figure 2p**), has taken things a step further by resembling branches in both color and shape. It also looks like a particular kind of birch tree twig with its distinctive silvery-colored bark. In case that was not enough, it even resembles a broken birch twig exactly [15].

The Chinese character moth, *Cilix glaucata* (Scopoli, 1763) (F: Drepanidae) (**Figure 2q**), is quite noticeable, but it looks exactly like a bird dropping, both in shape and color; consequently, it is definitely not a tempting morsel. A few moths practice camouflage not to be firm to see but to disguise themselves as something unpleasant; subsequently, those predators will not even deliberate on eating them [15].

Numerous moths have markings that obstruct their contour, making it difficult to identify their shape. The common garden moth known as the angle shades, *Phlogophora meticulosa* (Linnaeus, 1758) (F: Noctuidae) (**Figure 2r**), employs a variety of tactics. Its form is divided into less moth-like pieces by the triangle patterns. The hues of this material are also suitable for hiding among fallen leaves or on bark. Additionally, when at rest, the wings' crumpled shape resembles a dry leaf or the folds and fissures in bark [15].

A cunning combination of two techniques (camouflage and mimicry) is used by the eyed hawk-moth, *Smerinthus ocellatus* (Linnaeus, 1758) (F: Sphingidae) (**Figure 2s**). It is difficult to see against bark because, while it is resting, its forewings, which are camouflaged, conceal its hind wings. However, if it is startled, it abruptly unfolds its hind wings to reveal a flash of bright eyes sufficient to surprise and scare away a predator [15].

1.3 Mimicry

An animal or plant's defense against predators is its close external similarity to another animal, plant, or inanimate item. Unappealing butterfly patterns are frequently noticeable. According to experiments, some bird predators may memorize these patterns and learn to steer clear of preying on species with similar patterns in the future. Naturalists claim that leaf mimicry offers some of the most striking illustrations of disguise-based on camouflage. Plentiful species of Lepidoptera that bear a resemblance to leaves also have wing patterns that closely look like the irregularly shaped holes left by insect or decay damage. Notwithstanding claims that these patterns can either make them look more like damaged leaves or interfere with surface presence by giving off deceptive profundity indications. The buddha moth, *Siculodes aurorula* (Guenée, 1858) (F: Thyrididae) was used in two field trials employing artificial butterfly-like targets. It is shown in **Figure 3i** that untruthful pit characters proposition extensive subsistence profits against avian predation. Additionally, it was shown in supercomputer-built visual research experimentation that humans were similarly hindered from detecting such targets. Similar to how dark marks do not have the same effect as light ones, in fact, they increase detection. It was determined that the mechanism is the disruption of a homogeneous wing surface (surface disruptive camouflage), and because the holes resemble those occasionally present in actual leaves, the disruptive benefits are not negated by the costs associated with being noticeable [19].

1.3.1 Batesian mimicry

Batesian mimicry is that in which an edible species is protected by its resemblance to one avoided by predators. The mimic gains protection and the predator does not eat

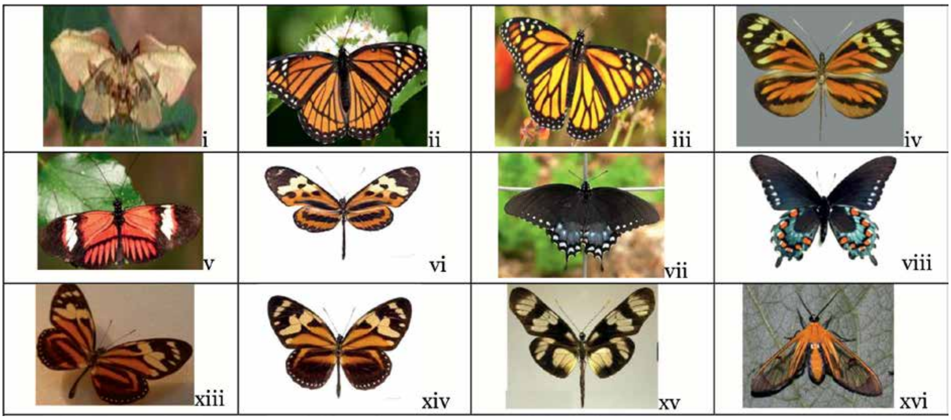

Figure 3.
Mimicry in Lepidoptera: i: buddha moth, Siculodes aurorula *Guenée, 1858 (F: Thyrididae); ii: North American viceroy,* Limenitis archippus *(Cramer, 1776) (F: Nymphalidae); iii: Monarch,* Danaus plexippus *(Linnaeus, 1758) (F: Nymphalidae); iv: tiger mimic white,* Dismorphia amphione *(Cramer, 1779) (F: Pieridae); v: postman butterfly,* Heliconius melpomene *(Linnaeus, 1758) (F: Nymphalidae); vi: milkweed butterfly, Ithomiines* mechanitis polymnia *Godman and Salvin, 1879 (F: Nymphalidae); vii: spicebush swallowtail,* Papilio troilus *(Linnaeus, 1758) (F: Papilionidae); viii: pipevine swallowtail,* Battus philenor *(Linnaeus, 1771) (F: Papilionidae); xiii: Omphale's king shoemaker,* Lycorea pasinuntia *(Stoll, 1780) (F: Nymphalidae); xiv: Tropical milkweed butterflies,* Lycorea ilione *(Cramer, 1775) (F: Nymphalidae); xv: Dismorphiine asionym,* Patia orise *(Boisduval, 1836) (F: Pieridae); xvi: tiger moths,* Cosmosoma spp. *Hübner, [1823] (F: Erebidae) [20].*

it because the predator made a mistake. For example, the palatable North American species, viceroy, *Limenitis archippus* (Cramer, 1776) (F: Nymphalidae) (**Figure 3ii**), which bears a quite remarkable resemblance to the highly toxic Monarch, *Danaus plexippus* (Linnaeus, 1758) (F: Nymphalidae) (**Figure 3iii**). However, recent studies have revealed that both of these species are unpalatable, making them Mullerian mimics. There are, however, many other instances of genuine Batesian mimicry, including the palatable, the tiger mimic white, *Dismorphia amphione* (Cramer, 1779) (F: Pieridae) (**Figure 3iv**), and the postman butterfly, *Heliconius melpomene* (Linnaeus, 1758) (F: Nymphalidae) (**Figure 3v**) species which mimic toxic milkweed butterfly, Ithomiines *Mechanitis polymnia* Godman and Salvin, 1879 (F: Nymphalidae) (**Figure 3vi**); and the palatable spicebush Swallowtail, *Papilio troilus* (Linnaeus, 1758) (F: Papilionidae) (**Figure 3vii**), which mimics the toxic pipevine swallowtail, *Battus philenor* (Linnaeus, 1771) (F: Papilionidae) (**Figure 3viii**). Bates proposed the hypothesis that occasionally, edible species created mutant forms with visual traits resembling poisonous species. Therefore, he reasoned, they would be less likely to be eaten by birds and would pass those traits on to their progeny. Bates hypothesized that the palatable species had developed to nearly resemble the dangerous species as a result of subsequent mutations over millennia [20].

1.3.2 Mullerian mimicry

Mullerian mimicry is a form of mimicry in which two or more harmful or unpalatable Lepidoptera develop similar appearances as a shared protective device. In 1879, Mullerian realized that there were also many cases, where both the mimic and the model were unpalatable. When a bird catches any one of these butterflies, either model or mimic, and realizes it is unpalatable or toxic, it quickly learns to keep away from all similarly patterned species. This type of evolutionary cooperation is referred to as Mullerian mimicry and is a very common phenomenon among the families Ithomiinae, Danainae, and Pieridae. Muller demonstrated mathematically that this form of mimicry is biased in favor of the scarcer species by a factor of the square of the ratio of species abundance. It is advantageous for there to be a large number of species involved in a Mullerian mimicry complex as it increases the power of the warning signal [21].

1.3.3 Tiger complex

The tiger complex, a collection of roughly 200 Neotropical species that all have a common pattern of orange and yellow stripes on a black background, is a well-known instance of butterfly mimicry. The complex includes many unpalatable Ithomiines such as the harmonia tiger-wing, *Tithorea harmonia* (Cramer, 1777) (F: Nymphalidae) (**Figure 3ix**); the tarricina longwing, *Tithorea tarricina* Hewitson, 1858 (F: Nymphalidae) (**Figure 3x**); the clearwing (Ithomiine) butterflies, *Melinaea marsaeus* (Hewitson, 1860) (F: Nymphalidae) (**Figure 3xi**) and the forbestra, *Forbestra equicola* (Cramer, 1780) (F: Nymphalidae) (**Figure 3xii**), the unpalatable Danaines such as Omphale's king Shoemaker, *Lycorea pasinuntia* (Stoll, 1780) (F: Nymphalidae) (**Figure 3xiii**), and several highly toxic day-flying moths from the Arctiid subfamily Pericopinae. At the end of the dry season, members of the tiger complex typically congregate in huge groups in damp gullies in the forest. Mimicry is most effective as a defense during this period, when they are very submissive and easy prey for birds. Any bird that has the bad experience of tasting one of the tiger-complex members soon learns to stay away from any species that has a similar appearance, and they might even be able to communicate their disagreeable character to other birds [22].

1.3.4 Mimicry rings

There are several more mimicry rings. The first example of mimicry ring, the glass-wing ring is a group of fairly large species with transparent wings. The butterflies that contain toxic Mullerian mimicry, mostly belong to the genera of the tribe, Ithomiine such as Methona and Thyridia, and the tribe, toxic Danaini with genera *Monarch*. The tropical milkweed butterfly, *Lycorea ilione* (Cramer, 1775) (F: Nymphalidae) (**Figure 3xiv**) shows Mullerian mimicry, and the palatable Dismorphiine basionym, *Patia orise* (Boisduval, 183) (F: Pieridae) (**Figure 3xv**) shows Batesian mimicry. The second example of mimicry ring, the orange-ring is comprised of a group of bright orange species, including the Julia butterfly, *Dryas iulia* (Fabricius, 1775) (F: Nymphalidae), the Juliette, *Eueides aliphera* (Godart, 1819) (F: Nymphalidae) and the ruddy daggerwing, *Marpesia petreus* Cramer, 1776 (F: Nymphalidae) [23].

1.3.5 Wasp mimicry

The day-flying moths (F: Sesiidae) include many species with short, translucent wings and black-and-yellow-banded bodies. They remarkably resemble wasps and hornets, and because of their likeness to these stinging insects, they almost definitely avoid predation. Numerous Arctiid moth species that belong to the *Cosmosoma* spp. Hübner, [1823] (F: Erebidae) (**Figure 3xvi**) are found in the Neotropics. These moths have brightly patterned red, orange, or yellow bodies and translucent wings. The majority of them are nocturnal in behavior and hide out among vegetation during the day, where their intimidating demeanor may prevent them from being eaten [24].

1.3.6 Transformational mimicry

Mimicry is not just limited to fully-grown butterflies and moths. Many tasty caterpillars imitate unappealing species. After molting, caterpillars frequently alter their look on regular basis. It is possible for a caterpillar to imitate different models at different instars. Additionally, the adult butterfly or moth that develops from that caterpillar could look just like a different species [25].

1.3.7 Aposematic coloration

Aposematic coloration is a term used to describe colors and patterns those act as a warning to predators that a potential prey species is unpalatable, toxic, or dangerous. Various studies have shown that all vertebrates including insectivorous birds associate greens and blues with safety and inherently regard red, orange, yellow, and white as signs of danger. It is also widely accepted that patterns incorporating stripes or spots draw attention to objects. Consequently, it is no surprise to find that toxic or unpalatable butterflies have evolved color schemes that reflect these facts in order to label themselves as being unpleasant to eat, thereby dissuading birds from attacking them. Equally, it is unsurprising that a significant number of palatable species have evolved to mimic the patterns of toxic species in order to trick birds into avoiding eating.
For example, the unpalatability of the traditional Chinese, *Altinote dicaeus callianira*

(Geyer, 1837) (F: Nymphalidae) (**Figure 4a**). Birds can remember the colors and patterns of butterflies, and associate them with pleasurable or unpleasant experiences. If a bird pecks at a toxic butterfly, it finds the taste very unpleasant and is likely to suffer consequences including vomiting, nausea, and visual disturbance. Experiments with various insectivorous birds have shown that if they suffer this experience, they then avoid eating [26].

1.3.8 Diematic patterns

The diematic patterns are defensive markings that have the power to terrify or startle prospective predators. The usage of ocelli is the most prevalent type of diematic defense. These typically appear as a pair of false-eye marks that can scare off a predator. These ocelli are extremely noticeable and resemble the eyes of monkeys or raptors in many butterflies and moths. The eyed hawk-moth, *Smerinthus ocellatus* (Linnaeus, 1758) (Family: Sphingidae) (**Figure 2s**) is one example. The ocelli of the Neotropical bullseye moths, *Automeris liberia* (Cramer, 1780) (F: Saturniidae) (**Figure 4b**) are typically concealed beneath the forewings of dead leaves with cryptic patterns. When startled, they fall to the ground and begin to twitch rhythmically, bringing as much attention to the ocelli as possible. Its frightening appearance would be more than enough to stop a bird from attacking. **Figure 4c** shows the peacock butterfly, *Aglais io* (Linnaeus, 1758) (F: Nymphalidae), which is stunning to humans but terrifying to small birds. **Figure 4e** shows the adult snake-head markings on the apex of the enormous atlas moth, *Attacus atlas* (Linnaeus, 1758) (F: Saturniidae), while **Figure 4d** shows the adult snake-head markings on the apex of the silk moth, *Rothschildia* spp. Grote, 1896 (F: Saturniidae). These species' forewing apexes are lobed and have markings that resemble a snake's mouth and eyes. The illusion is strengthened by the wings' rhythmic movements, which highlight the snake-head markings [27, 28].

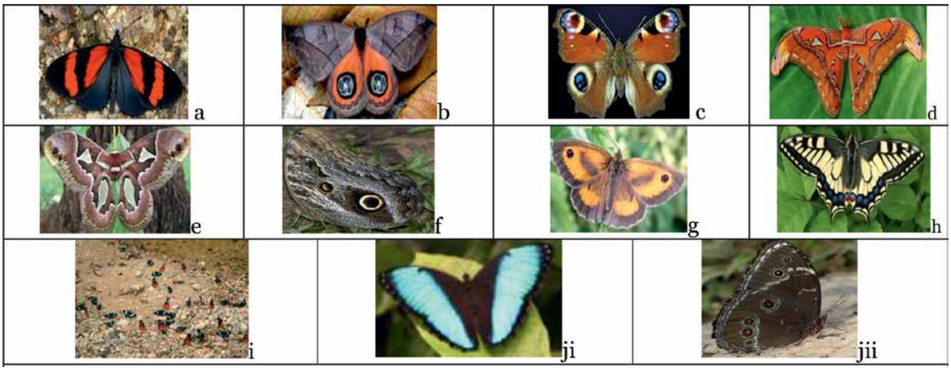

Figure 4.
Aposematic coloration in Lepidoptera: a: traditional Chinese, Altinote dicaeus callianira *(Geyer, 1837) (F: Nymphalidae); b: bullseye moths,* Automeris liberia *(Cramer, 1780) (F: Saturniidae); c: peacock butterfly,* Aglais io *(Linnaeus, 1758) (F: Nymphalidae); d: giant atlas moth,* Attacus atlas *(Linnaeus, 1758) (F: Saturniidae) e: silk moths,* Rothschildia spp. *Grote, 1896 (F: Saturniidae); f: Neotropical owl butterfly,* Caligo teucer *(Linnaeus, 1758) (F: Nymphalidae); g: gatekeeper,* Pyronia tithonus *(Linnaeus, 1771) (F: Nymphalidae) h: old world swallowtail,* Papilio machaon *Linnaeus, 1758 (F: Papilionidae); i: prola beauty or red flasher,* Panacea prola *(Doubleday, 1848) (F: Nymphalidae); j: helenor or common blue morpho,* Morpho helenor *(Cramer, 1775) (F: Nymphalidae) i: dorsal surface; ii: ventral surface [21].*

1.3.9 Decoys to distract birds

The terrifying effect of diematic marks frequently last a short while. A bird may renew its attack, once it has recovered from its first fright. The Neotropical owl butterfly, *Caligo teucer* (Linnaeus, 1758) (F: Nymphalidae) (**Figure 4f**) and the gatekeeper, *Pyronia tithonus* (Linnaeus, 1771) (F: Nymphalidae) (**Figure 4g**) are two examples of predators that the ocelli on the butterfly's wings divert and confuse in such situations. An attacking bird will always try to predict, where its victim will flee; thus, it will aim to attack just in front of the head. The bird is tricked by the phony head into shooting behind the butterfly instead. The butterfly then escapes by darting off in the opposite direction from where the bird anticipates it [29, 30].

1.3.10 Patterns to confuse

There are patterns on many butterflies that, at first glance, appear to have no purpose. What would be the purpose, for instance, of a visually appealing pattern that is simple to recall, like the chequered pattern on the old world swallowtail, *Papilio machaon* Linnaeus, 1758 (F: Papilionidae) (**Figure 4h**). When disturbed, it suddenly flicks its closed wings open in the same manner as the peacock and other ocelli-equipped species. It generally rests with its wings closed [31, 32].

1.3.11 Signaling danger to other butterflies

Although, it is well-known that warning coloration is intended to directly deter birds and other predators, the prola beauty or red flasher, *Panacea prola* (Doubleday, 1848) (F: Nymphalidae) (**Figure 4i**) from Peru serves an entirely opposite purpose. On riverbanks, a lot of males congregate to consume mineralized moisture. They expose their metallic blue dorsal surface while basking and feeding, which aids other passing males in identifying them and homing in on the feeding grounds. The butterflies must use a defense mechanism since birds may readily attack a gathering of butterflies on the ground. When there is even a slight disturbance, 1 or 2 butterflies become agitated and begin nervously fanning their wings, causing the other butterflies to see their bright red undersides [14, 33].

1.3.12 Flash coloration

Many butterflies have alternate display of a bright dorsal surface and somber underside. This is recognized as flash coloration. An example is the South American Telenor blue morpho or common blue morpho, *Morpho helenor* (Cramer, 1775) (F: Nymphalidae) (**Figure 4j**), which has a brilliant iridescent blue dorsal surface (**Figure 4ji**) and black with eyes on ventral surface (**Figure 4jii**) that makes it highly visible to predators as well as to potential mates. A colorful dorsal surface and a somber underside are common displays of many butterflies. This is recognized as flash coloration. For instance, the South American the helenor blue morpho or common blue morpho, *Morpho helenor* (Cramer, 1775) (F: Nymphalidae) (**Figure 4j**) is highly visible to predators as well as for potential mates due to its blue brilliant iridescent on the dorsal surface (**Figure 4ji**) and several eyes pattern on black ventral surface (**Figure 4jii**). When startled, a butterfly will land quickly and close its wings, consequently, that only the dark brown underside is visible. The secondary decoy-ocelli defense may enable the butterfly to flee, if a chasing bird spot it at rest after landing, since it will direct the bird's beak away from the body of the butterfly and toward the wing margins [34].

2. Conclusion

Lepidoptera have a wonderful defense mechanism called camouflage that enables them to efficiently hide their appearance and blend with their surroundings. This fascinating subject has been investigated by the zoologists for well over a century. It is distinguished from disguise by its ability to harmoniously merge with a natural background or substrate through color, pattern, and texture. Many butterflies and moths have developed colors and patterns that seamlessly blend with their surroundings, such as leaves, soil, rocks, and tree trunks. Disguise, on the other hand, refers to species that resemble other natural objects like leaves or flowers. Another fascinating facet of how Lepidoptera defend themselves is mimicry. Batesian mimicry involves edible species mimicking avoided ones to protect themselves from predators, while Mullerian mimicry involves multiple harmful or unpalatable Lepidoptera developing similar appearances for shared protection. In addition to the well-known tiger complex, the Lepidoptera kingdom contains several other mimicry rings, such as the glass-wing ring, which represents species with transparent wings, and the orange-ring, which is made up of a collection of species with brilliant orange wings. The diversity of mimicry strategies extends to wasp mimicry, transformational mimicry, aposematic coloration, diematic patterns, decoys to distract predators, patterns to confuse them, and even signaling danger to other butterflies through flash coloration. As scientists work to understand the intricate workings of these astonishing adaptive tactics, the world of camouflage and mimicry in Lepidoptera never ceases to astound both scientists and nature lovers.

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Chapter 3

Gynandromorphy Behavior of Lepidopterans

Soumya Shephalika Dash and Satya Narayan Satapathy

Abstract

Gynandromorphism is a rare phenomenon where an individual develops with a mosaic of both male and female traits. It is observed in various groups of organisms, including arthropods, birds, reptiles, amphibians, and mammals. Among arthropods, gynandromorphs have been frequently documented in both natural populations and laboratory settings. In insects, bilateral gynandromorphs are the most commonly observed, where the left and right halves of the body display different sexes. This phenomenon has been reported in 12 orders within the class Insecta. Within the order Lepidoptera (butterflies and moths), gynandromorphism has been documented in 18 families, with a higher number of cases observed in the families Papilionidae, Pieridae, and Saturniidae. Lepidopterans are known for their sexual dichromatism, primarily expressed through wing coloration. While gynandromorph specimens have been reported in various butterfly families such as Lycaenidae, Nymphalidae, Pieridae, Papilionidae, and Geometridae, there have also been documented cases in certain moth species, including Noctuidae species like *Agrotis segetum* and *Agrotis ipsilon*.

Keywords: Lepidoptera, gynandromorphy, chromosome, geometridae, papilionidae

1. Introduction

Gynandromorphism is a condition where a single individual develops with a combination of male and female characteristics [1]. A gynandromorph is a chimaera individual whose body has both masculine and feminine parts [2]. The term 'gynandromorph' is derived from the Greek words (gyne = woman; aner = man and morphe = form). It is extremely uncommon in arthropods, as compared to plants. To put in another way, arthropods are sexually dimorphic, which means that each individual develops into either an entirely male or an entirely female but does not become an intermediate. Yet several gynandromorphs, particularly in arthropods, have been reported both in natural and laboratory populations. These morphologically abnormal individuals who possess mixed male and female characteristics might result from developmental defects which are rare in natural environments. Gynandromorphs have been estimated to occur in insect populations at a frequency of 0.01 to 0.05% of their natural occurrence [3]. Along with arthropods, this peculiar phenomenon has been described in birds [4], reptiles [5], amphibians [6], fish [7] and mammals [8]. Gynandromorphs are often exhibited in two ways: bilaterally and non-bilaterally (sexual mosaics), depending on how male and female characteristics are distributed throughout the body. Additionally, they can be categorized as anteroposterior (where the anterior and posterior sides are of different sexes), bilateral (where the right and left sides are of different sexes), transversal (where the distribution is asymmetric), or mosaic (where the features are distributed randomly over the body). Embryogenesis defects at early phase leads to the formation of insect's body part that are marked with characters of different sex. The aberrant division of nuclear substance during the division of the fertilized egg is the cause of gynandromorphism. If this occurs at the very first division, a bilateral gynandromorphism develops. The gynandromorph will be a mosaic if such an abnormality happens in the later phases of the egg division. In insects, the bilateral form most commonly occurs when the left and right portions are of different sexes. In contrast, mosaics have both sexual features mixed together and unevenly dispersed throughout the body, giving it a patchy appearance [9].

Numerous studies have documented the existence of arthropods with phenotypically male and female parts. These individuals exhibit a various of male and female part distribution patterns. For instance, the male and female reproductive organs may be distinctly bilateral, unevenly distributed, or mixed. Although this classification is frequently used, it does not take into account the genetic and developmental processes. Twelve orders of the class Insecta have bilateral gynandromorphs. Gynandromorphism is a well-known phenomenon among Lepidoptera, but it can occur more or less frequently depending on the group. In Lepidoptera, presence of gynandromorphism is at low rate (0.000125%), that is, one in 8000 reared insects. Lepidopteran gynandromorphs have been found in species that exhibit sexual dimorphism, indicating different morphological characteristics of male and female, such as wing pattern and antennae. Because of this, two halves of the adult insect exhibit distinct sexual phenotypic characteristics. There have been reports of gonandromorphism from 18families in this order. Among them, a lot of specimens have been reported for butterfly species (e.g., in Geometridae, Lycaenidae, Nymphalidae, Pieridae, Papilionidae) [3, 10–15] and also for moths (e.g., Noctuidae) [16, 17]. However, the Papilionidae, Pieridae, and Saturniidae families account for the majority of cases [9].

2. Hypothesis related to gynandromorphism

a. Boveri's hypothesis. A postponement in the fusion of the spermatozoon with the egg, causing the egg to initiate cell division before the sperm nucleus reaches it, resulting in only half of the egg being fertilized.

b. Only one polar body is extruded; In Hymenoptera, a second maturation division occurs after which two ootids are formed. Of these two ootids, one undergoes fertilization and develops into female parts, while the other ootid remains unfertilized and develops into male parts.

c. Morgan's first hypothesis. In certain cases, multiple spermatozoa enter the egg, resulting in a unique reproductive process. One of the spermatozoa successfully fertilizes the egg, giving rise to the development of female parts. Meanwhile, the development of male parts occurs through the utilization of one or more of the remaining spermatozoa. This mechanism allows for the formation of both female and male structures.

d. Morgan's second hypothesis. At the first division of the fertilized egg or during subsequent cell divisions, an X-chromosome is eliminated. This process results in the loss or exclusion of one of the X-chromosomes from the developing cells.

e. Through a process called non-disjunction, an individual with three X-chromosomes (XXX) can be produced. When the fertilized egg divides for the first time or after subsequent cell divisions, one of the X-chromosomes separates and moves to a cell, while the other two X-chromosomes pass to the other cell. In Lepidoptera, the cell that contains two X-chromosomes would give rise to male parts, while the cell with only one X-chromosome would develop into female parts.

f. In certain cases, such as in Abraxas, a binucleate ovum can occur where each different nucleus is fertilized by a different spermatozoon. Both nuclei in this binucleate structure may originate from the egg itself. However, in species like *Bombyx mori* (silkworm), one nucleus may be an egg nucleus while the other is a polar nucleus. The polar nucleus is typically involved in the process of polar body formation during oogenesis.

g. The pupa experiences mechanical shock as it sheds its larval skin.

In Lepidoptera, it is believed that most gynandromorphs (organisms with both male and female characteristics) likely originate from binucleate eggs of different types. The elimination of an X-chromosome has been demonstrated to cause gynandromorphism in Abraxas, but it can only be confirmed when a sex-linked trait is involved, and very few such traits are known in Lepidoptera. There is one known example of a mosaic pattern developing as suggested in the initial hypothesis by Hlorgan, indicating that some gynandromorphs may be produced in this manner. Somatic non-disjunction is considered the most probable explanation for Lepidopterous gynandromorphs, where only a small portion of the organism is male. In these cases, the individuals would begin as females (XY), and the male parts would possess an additional X chromosome (XXY). However, further confirmation is needed regarding the production of gynandromorphs through shock or other external factors (**Figure 1**).

3. Occurrence of gynandromorph

In arthropods, the existence of individuals with phenotypically male and female parts has frequently been documented. They have been discovered in populations of all orders of non-insects as well as in insects both in natural environments and in laboratory populations. The term "gynandrous" or "gynandromorphous" was used to describe them long before any dissections were performed. Rudolphi performed the first dissection on a half-gynandromorphous *Gastropaha quercifolia*, the lappet (Lepidoptera: Lasiocampidae), in 1825 [18].

Conventional karyotyping is commonly practised for determining the sex of species where molecular analyses are not feasible. Simply observing the sex chromatin

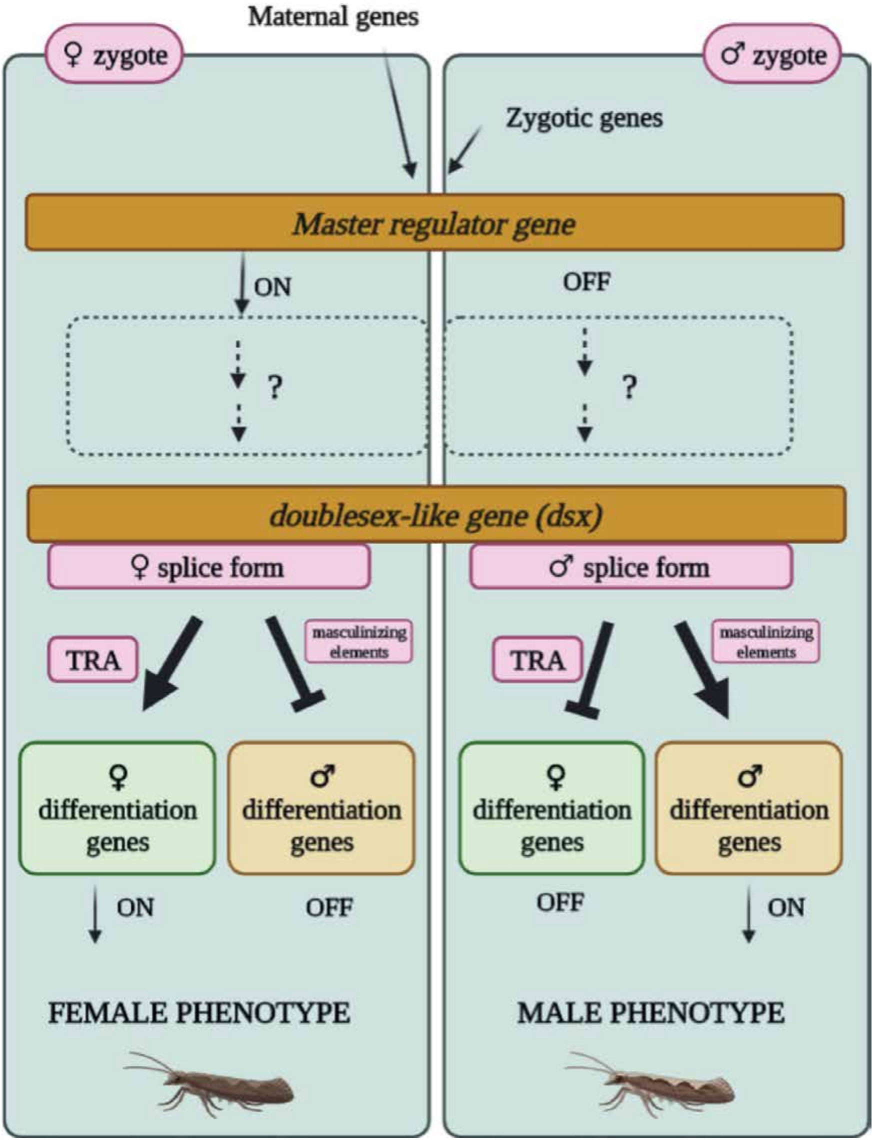

Figure 1.
Sex determination in lepidopteran insects. (This model operates under the assumption that the functioning of a key/master regulatory gene, analogous to sex-lethal in insects, is influenced by the combined actions of maternal genes and zygotic genes. The regulatory gene's expression plays a pivotal role in either activating or inhibiting the expression of subsequent genes (referred to as downstream genes). Progressing through the hierarchical cascade of gene expression, a highly conserved gene similar to double sex undergoes alternative RNA splicing, resulting in the production of either the male-specific DSX M protein or the female-specific DSX F protein. These sex-specific DSX proteins then go on to activate or suppress a set of genes responsible for sex-specific differentiation, ultimately leading to the development of either the male phenotype or the female phenotype.

in lepidopteran insects with a ZW-ZZ sex chromosome constitution enables us to distinguish between the male (ZZ) and female (ZW) genotypes. The sex chromatin refers to a tightly compacted W chromosome that can be observed within interphase nuclei [19]. For insects where molecular data is accessible, distinguishing between male and female genotypes can be achieved through diagnostic polymerase chain reaction (PCR) amplifications using Y-specific or W-specific molecular markers. This technique enables the identification of genetic markers unique to the Y chromosome in males or the W chromosome in females. Furthermore, analyzing the transcriptional activity of genes that are expressed in a sex-specific manner can serve as an additional method to confirm the male and female phenotypes within an individual.

4. Pattern of gynandromorphism

Gynandromorphs, which consist of individuals with a combination of male and female anatomical features, can occur naturally or be induced experimentally in various animal species. These individuals typically exhibit a mosaic appearance, where male and female tissues coexist, often following a symmetrical pattern. Bilateral gynandromorphs possess male characteristics on one side of the body and female characteristics on the other side. Transverse gynandromorphs, also known as polar gynandromorphs, exhibit a clear division between male and female body parts along a transverse plane perpendicular to the main body axis. The third type, oblique gynandromorphs, display a diagonal boundary between the different-sex body parts, crossing the sagittal plane at an angle [20]. Gynandromorphism has been observed in various species, primarily insects, chelicerates (spiders and mites), crustaceans, and birds. In contrast to gynandromorphism, which can be classified based on symmetry, the patterns of phenotypic expression in intersexual states are more specific to each taxonomic group.

Patterns of intersexuality encompass the characterization of sexually dimorphic anatomical structures and body organs, such as genitalia and gonads, that are affected by the anomaly. In the case of Armadillidium vulgare, a type of isopod, Legrand, Juchault [21] have identified four primary intersex types: (1) functional males displaying female genital openings; (2) individuals possessing a functional ovary accompanied by a vestigial testicular vesicle and underdeveloped vas deferens; (3) female individuals closely resembling the previous category, distinguishable primarily through physiological differences; and (4) males with testes that possess an oviduct.

5. Gynandromorphism in lepidopterans

Gynandromorphism is exceedingly uncommon in the natural world. Due to its rarity, the collection and description of gynandromorph specimens have been infrequent (0.01 to 0.05%).

A large number of gynandromorphs have been reported in Lepidoptera from at least 10 different species of Papilionidae, eight species of Lycaenidae, four Hesperiidae, ten Pieridae, four Nymphalidae, and several families of moths including Saturniidae, Pyralidae, Geometridae, and Lymantriidae (**Table 1**) [22–26].

6. Family-Geometridae

In a study by Seven, Özdemir [3], a gynandromorphic specimen of *Gnopharmia cholcidaria* (Lederer, 1870) was documented, displaying lateral deviations. The left side of the specimen exhibited female characteristics, while the right side displayed male traits. Notably, the gynandromorphic individual of *G. cholcidaria* demonstrated distinct differences between the two sides.

Sl. no.	Family	Examples
1	Adelidae	*Lemyra imparilis*, *Nemophora rubrofascia*, *Spilosoma mendica*
2	Bombycidae	*Bombyx mori*
3	Crambidae	*Hedylepta accepta*, *Ostrinia furnacalis*, *O. scapulalis*
4	Geometridae	*Bupalus piniarius*, *Phaeoura mexicanaria*, *Thyrinteina arnobia*, *Paleacrita vernata*
5	Lasiocampidae	*Gastropaha quercifolia*, *Malacosoma neutria*
6	Lycaenidae	*Lycaena argus*, *Lycaeides argyrognomon*, *Neozephyrus taxila*, *Strymon bazochii*
7	Lymantriidae	*Lymantria dispar*
8	Noctuidae	*Agrotis ipsilon*, *A. segetum*, *Helicoverpa armigera*, *Spodoptera frugiperda*
9	Nymphalidae	*Argynnis paphia*, *Epinephele tithonus*, *Hyponephele narica*, *Ladoga camilla*
10	Papilionidae	*Ornithoptera Croesus*, *O. priumus*, *O. victoriae*, *Papilio bianor*, *P. glaucus*, *P. memnon*, *P. polyxenes*, *Troides rhadamantusrhadamantus*
11	Pieridae	*Colias erate poliographus*, *Eurema mandarina*, *Gonepteryx cleopatra*, *Gonopteryx rhamni*, *Pieris melete*, *P. napi*
12	Pyralidae	*Galleria mellonella*
13	Saturniidae	*Aglia japonica*, *Antheraea mylitta*, *A. yamamai*, *Arsenura armida*, *Automeris io*, *Hyalophora cecropia*, *Periga circumstans*
14	Satyridae	*Maniola jurtina*
15	Sphingidae	*Smerinthus ocellata*, *S. populi*
16	Tortricidae	*Acleris celiana*
17	Tineidae	*Harmaclona tephrantha*
18	Yponomeutidae	*Yponomeuta cagnagellus*

Table 1.
Examples of Gynandromorph seen in Lepidoptera.

On one side, the antenna was bipectinate, the wings were smaller in size, and the frenulum consisted of a single hair. Conversely, on the other side, the antenna appeared filiform, the wings were larger, and the frenulum exhibited a hair bundle shape. This dichotomy in antenna structure, wing size, and frenulum characteristics clearly distinguished the male and female traits within the gynandromorphic specimen of *G. cholcidaria*. Another gynandromorphic individual of *Idaea deversaria* (Herrich-Schäffer, 1847) exhibited frontal deviations. In this case, both sides of the specimen had filiform antennae, similar wing measurements, and a frenulum with a hair bundle shape resembling the female characteristics. Additionally, the hind legs lacked hair and shared the same shape on both sides. While the male genitalia structures dominated in *G. cholcidaria*, the characteristics related to male and female genitalia were found to be equal in *I. deversaria*. This indicates that the expression of genitalia traits in the gynandromorphic specimens varied between the two species.

7. Family-Lycaenidae

Jahner et al. [13] studied comparing the genitalia and wing patterns of gynandromorphic Melissa blue butterflies (*Lycaeides melissa*) and one gynandromorphic

Anna blue butterfly (*L. anna*) with the morphology of normal individuals, both from wild-caught specimens and those reared from wild populations. The researchers aimed to investigate the underlying mechanisms of gynandromorphism in Lycaeides species. It is commonly expected that Lycaeides bilateral gynandromorphs arise from double fertilization of a binucleate egg, which is considered the most prevalent mechanism of gynandromorphism [18, 27]. However, in certain cases, mosaicism can also occur in Lepidoptera due to the loss of the W chromosome during a nondisjunction event that takes place in later stages of development.

In Lepidoptera, the W chromosomes generally carry limited genetic information, and sex determination is primarily based on the number of Z chromosomes present in a cell. Males possess two copies of the Z chromosome, while females have only one [28]. This information suggests that the sex of an individual is predominantly determined by the number of Z chromosomes rather than the presence of W chromosomes.

8. Family-Saturniidae

In 2010, Rajkhowa et al. [29] reported a rare occurrence of bilateral gynandromorphy in the muga silkworm, *Antheraea assamensis* Helfer, discovered in Lahdoigarh, Assam, India. This unique gynandromorph specimen exhibited male characteristics on the left wings and female characteristics on the right wings. Notably, the wings on one side of the body were larger and darker compared to the other side. The measurements revealed a wing expanse of 75 mm on the left side (male) and 80 mm on the right side (female).

Distinct differences were observed in the antennae of the gynandromorph. The male antenna displayed a dark brown color with reddish-pink bases, while the female antenna appeared paler. The abdomen of the specimen was chestnut brown in color. The wing lines on the left side (male) were somewhat whitish, incurved, and outlined by dark brown, whereas on the right side (female), the wing lines were dark brown, incurved, and outlined by white. The left wings of the gynandromorph were dark brown, resembling the male, while the right wings were light pale brown, resembling the female.

In *A. assamensis* Helfer, both male and female individuals possess antennae that are quadripectinate, meaning they have four branches. Interestingly, the gynandromorph specimen exhibited pectens of different sizes, which are characteristic features of both males and females. This complete bilateral gynandromorphy is evident from the morphological variations observed.

9. Family-Noctuidae

Sagar et al. [30] conducted a study on sexual dimorphism in the fall armyworm, *Spodoptera frugiperda*, focusing on the distinguishing features between males and females. They found that male forewings exhibited a brown ground color, while females had a darker shade of brown. Additionally, the male forewing displayed a prominent longitudinal black dash at the base of the Cu vein, an indistinct reniform spot with a white v-shaped mark at the apex, a small patch of white scales at veins M3 and CuA1, and a light brown patagium (part of the thorax) with a transverse grayish-black band. In contrast, the female forewing had a less conspicuous black dash, an absence of the reniform spot except for a few white scales, no white patch at veins M3 and CuA1, and a patagium without any distinct grayish-black band.

Interestingly, the gynandromorph specimen exhibited a notable variation where the left half of the wings resembled those of a male, while the right half resembled those of a female. Similarly, the patagium on the left side resembled that of a male (prominent), while on the right side, it resembled that of a female (inconspicuous). This indicated a bilateral type of gynandromorphism. In terms of wing coupling, *S. frugiperda* showed sexual dimorphism in the number of frenulum, with males having a single frenulum and females having three. The gynandromorphic moth had a left half with a single frenulum and a right half with three frenula.

Furthermore, an examination of the genitalia revealed asymmetry in the genital structures of the gynandromorph. One half exhibited well-developed male genitalia structures, including a distinct uncus, valvae, aedeagus, and ampulla. The right valva was also partially developed with a distinct ampulla. On the other half, the female genitalia showed well-developed anterior apophysis, ovipositor lobes, ductus bursae, ventral plate of ostium bursae and appendix bursae, while the corpus bursae was not distinct. Based on the genitalia assessment, the gynandromorph specimen appeared to have a male-to-female proportion of approximately 60:40.

10. Family-Papilionidae

Gynandromorphs in Papilio were discovered to most frequently arise via interspecific (or intersubspecific) crosses [22]. Both polymorphic yellow and black variants can be seen in *Papilio glaucus* and *P. alexiares* Hopffer [31]. The polymorphic dark and yellow morph females are only seen in these two *Papilio* species, and they prominently display the color contrast in mosaicism and gyndromorphism. Scriber et al. [32] described a *P. glaucus* female with one golden half and one mainly dark half.

One instance of interspecific bilateral gynandromorphs was produced by mating a female *Papilio glaucus* with a male *P. canadensis* [33]. In the resulting gynandromorph, this interspecific mating brought together genes that had previously been segregated into the two gender-differentiated halves. The heterogametic female [XY (=ZW)] possessed both the X-linked suppressor (s+) gene and the Y-linked black (b+) gene of dark morph females, which resulted in the yellow (suppressed) tiger pattern for the female half. Contrast this with the interspecific (*P. glaucus*, *P. alexiares*) gynandromorph, where the female half was black, and which had the dark melanic (b+) gene and no suppressor (s-, an enablement).

The numerous enzyme activities of the melanin (black) and papiliochrome (yellow) specific pathways appeared to be coordinated with the biochemical pathways, which were temporally and spatially regulated by enzymes, including dopa carboxylase. The single major Y-linked factor on the female side of the individual coordinated all of these enzyme regulations, which had to be carried out concurrently in the same gynandromorphs [34]; however, these genes were either not expressed or absent on the male side of the individual. The activity of other sex-linked traits such as obligate diapause regulators would explain the direct development (non-diapause) of both halves of the *P. glaucus*/*P. alexiares* gynandromorph and the 1 year delay (diapause) of the *P. glaucus*/*P. canadensis* gynandromorph (with both the hemizygous female and the heterozygous male having the X-linked obligate diapause gene, od+). The distribution of sex-linked diagnostic allozymes and mitochondrial DNA variation in the two halves of such gynandromorphs may be studied in the future, but other sex-linked features, such as oviposition preferences, could not be examined [31].

11. Benefits of gynandromorphism

- Studying gynandromorphs can provide valuable insights into the molecular and genetic mechanisms underlying sexual development.

- Studying the sexual dimorphism can help researchers to better understand the factors that contribute to the evolutionary processes shaping the differences.

- Gynandromorphs can be useful for comparative studies between sexes. By analyzing the morphology, behavior, and physiology of gynandromorphs alongside typical males and females, scientists can identify and explore the similarities and differences in these characteristics. This can provide insights into the roles of specific genes or hormones in shaping sex-specific traits.

- Gynandromorphs offer a window into the intricate process of embryonic development. They provide visual evidence of how cells differentiate and interact during early development, offering valuable insights into the mechanisms involved in tissue patterning, cell signaling, and cell fate determination.

- Gynandromorphs, being rare individuals, can have a special conservation value. These unique specimens can be of interest to collectors, museums, and scientific institutions, which can help raise awareness about the diversity and conservation needs of lepidopteran insects and their ecosystems.

- Gynandromorphs offer unique opportunities to study the genetic basis of sexual development and sexual dimorphism.

- Public engagement and education: Gynandromorphs can generate interest and curiosity about the natural world and provide opportunities for educational outreach.

12. Conclusion

The study of gynandromorphy in lepidopterans provides a fascinating glimpse into the complex world of sexual development and phenotypic variation. By examining these unique individuals with a mosaic of male and female traits, researchers have gained valuable insights into sex determination mechanisms and the modular nature of lepidopteran morphology. The occurrence of gynandromorphic mutations also sheds light on the evolvability and adaptability of lepidopteran species. Overall, this book chapter highlights the importance of gynandromorphy as a fascinating behavioral phenomenon that deepens our understanding of the intricate processes underlying the diversity and complexity of lepidopteran biology.

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Chapter 4

Non-Coding RNAs in Lepidoptera

Dandan Li, Yunchao Kan and Zhongwei Li

Abstract

In the last few years, the amount of genomic sequence data has grown exponentially. A large number of non-coding RNAs (ncRNAs) have been identified from bacteria to humans. ncRNAs are various and multi-faced; they can regulate gene expression through chromosomal, transcriptional, post-transcriptional, and translational levels and then participate in the whole process of development in different organisms. ncRNAs have been identified in the 1980s in Lepidoptera; they can play roles in growth, metamorphosis, metabolism, sex determination, reproduction, and immune response of insects. Now, the use of ncRNAs in pest control of Lepidoptera is also in process. This chapter will review the recent advance of ncRNAs in Lepidoptera and prospect the future studies of ncRNAs in insects.

Keywords: non-coding RNA, Lepidoptera, metamorphosis, immune response, reproduction

1. Introduction

Non-coding RNAs (ncRNAs) are a large class of RNAs with no protein products, including transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), PIWI-interacting RNA (piRNA), endogenous small interfering RNA (siRNA), circular RNA (circRNA), long non-coding RNA (lncRNA), protein functional effector small ncRNA (pfeRNA) and other ncRNAs with unknown functions [1, 2]. ncRNAs have been identified in bacteria and humans and are multi-faced. They can regulate gene expression through chromosomal, transcriptional, post-transcriptional, and translational levels and then participate in the whole process of development. A large number of ncRNAs has been identified since the 1980s in Lepidoptera. More and more ncRNAs have been found to play roles in the growth, metabolism, sex determination, reproduction, and immune response of insects. Now, the use of ncRNAs in pest control of Lepidoptera is also in process. This chapter will review the recent advance of ncRNAs in Lepidopteran, and the prospect for future studies of ncRNAs in insects.

2. A brief history of non-coding RNAs

In 1958, the "Central Dogma" was first proposed by Francis Crick and published in Nature in 1970, which described that the genetic information was generally transferred from DNA to RNA and then from RNA to protein, named transcription and translation, respectively [3]. For a long time, the main research was focused on messenger RNA (mRNA), which worked as the "regular army" in the RNA Legion. But in the RNA corps, there is another "non-regular army" that function as "Jack of all trades", named non-coding RNAs (ncRNAs).

In the 1950s, the ribosome RNA (rRNA) and transport RNA (tRNA) were first discovered as ncRNAs, followed by small nucleus RNA (snRNA) (1980s), small nucleolar RNA (snoRNA) (1990s), microRNA (miRNA) (1990s), long non-coding RNA (lncRNA) (1990s), circular RNA (circRNA) (1990s), and piRNA (2000s). With the completion of the Human Genome Project (HGP) in 2003 [4], the project Encyclopedia of DNA Elements (ENCODE) was launched to investigate all the functional elements in the human genome [5]. From then on, a new world of RNA was emerging, a variety of ncRNAs were identified through bacterial, viral, plant, insect, mammalian, *etc*. At the same time, scientists were surprised to find that less than 2% of genome sequence was translated into proteins in humans, and the remaining 98% were considered "garbage" and "noise". But now we know that at least 80% of the remaining "junk" sequences of the human genome are functional and 93% of the human genome has the ability to transcribe into ncRNAs [6, 7].

3. Differential class of ncRNAs in Lepidotera

3.1 Small nuclear RNA (snRNA)

Small nuclear RNA (snRNA) is a conserved class of ncRNAs in the nucleus, with a length of approximately 100–215 nucleotides in mammals. Generally, snRNAs contain seven categories due to their abundant U content, numbered U1–U7. U3 snRNA is in the nucleolus and the other six categories are present in the non-nucleolar region. Except for U6, which is transcribed by RNA polymerase III, the other snRNAs are transcribed by RNA polymerase II. snRNAs always combine with protein to form the small nuclear heterogeneous ribonucleoprotein particle particle (SnRNP), to play a conserved role in the alternative splicing of mRNAs [8–11]. High-throughput sequencing studies indicate that alternative precursor messenger RNA (pre-mRNA) splicing occurs in >95 to 100% of human genes and in nearly 63% of mouse genes [12, 13]. Five snRNAs U1, U2, U4/U6, and U5 recruit the proteins, forming snRNP complexes to recognize introns to form a catalytically active spliceosome and remove the intron from a pre-mRNA containing two exons [14, 15]. Alternative splicing plays roles in the sex determination of fruit flies and in learning, memory, and neurotransmission of mammalian [16]. Mutations occur in splice sites, RNA regulatory silencers or enhancers, or genes that encode splicing factors resulting in disease. For example, the first example of human disease mutations affecting splicing were the β-globin thalassemia mutations and mutations in the SMN-2 gene, which give rise to spinal muscular atrophy [17–20]. The splicing factor hnRNPA1 plays a role in the pre-mRNA splicing of SMN-2 and pyruvate kinase in cancer [21–24].

The function of snRNAs is conserved and fundamental across different organisms. Recent research in silkworm *Bombyx mori* showed that depletion of U1 snRNP exhibited abnormal cellular phenotypes with enlarged cell nucleus, scant cytoplasm, and enlarged nuclei. Genes involved in metabolic pathways, biosynthesis of secondary metabolites, and steroid hormone biosynthesis were significantly affected, which led to either delayed or stationary first instar larval development [25].

3.2 Small nucleolar RNAs (snoRNAs)

Small nucleolar RNAs (snoRNAs) are small RNAs found in the nucleolus with lengths of 60–300 nucleotides. snoRNAs are responsible for posttranscriptional modification of rRNAs [26]. In previous research, snoRNAs were categorized as C/D box snoRNAs and H/ACA box snoRNAs, regulating the 2′-O-ribose methylation and pseudouridylation of rRNAs, respectively [26]. However, recent studies showed a large number of snoRNAs without specific targets or determined cell functions, named orphan snoRNAs [27]. Studies showed that besides rRNA modification, snoRNA had other novel functions: (1) alternative splicing, RNA editing, or 3′-end processing of pre-mRNA [28–32]. In humans, SNORD27 not only guides the 2′-O methylation of A27 in 18S rRNA but also regulates the alternative splicing of the transcription factor E2F7 pre-mRNA [31]. (2) Serving as the precursors of microRNAs [33] or end of long-non-coding RNAs [34, 35]. High-throughput sequencing has revealed that more than half of all snoRNAs were processed to generate smaller fragments [36], named snoRNA-derived RNAs (sdRNAs). sdRNAs can be divided into several categories based upon their origin and length, H/ACA box snoRNAs generated sdRNAs mainly derived from the 3′end with a length of 20–24 nt, the C/D box snoRNAs generated sdRNAs derived from the 5′end and produce two fragments, one greater than 26 nt and another 17–19 nt in length [37, 38], sdRNAs are considered as a novel source of miRNAs.

Dysregulation of snoRNA causes disease and cancer progression [39]. In humans, snoRNA h5sn2 is downregulated in meningiomas compared with normal brain tissue, and 5S snoRNA and SNORA18L5 were linked to the function of the p53 tumor suppressor [40, 41]. Some snoRNAs were ascribed to cancer-associated signaling pathways, such as PI3K/AKT, which plays a pivotal role in cell differentiation, proliferation, and survival [42]. As more and more cancer-related snoRNAs were identified, they were used as potential candidates for cancer biomarkers [43]. Such as in angioimmunoblastic T-cell lymphoma, the upregulation of HBI-239 is used as a prognostic marker [44]. In colorectal cancer, the overexpression of SNORA21 is related to poor patient survival, which can be used as a prognostic marker [45].

Furthermore, as the main regulator of RNAs in nucleoli, small nucleolar RNAs can also respond to cell stress. Michel et al. found that in mice, box C/D snoRNAs origin from the introns of the ribosomal protein L13a (Rpl13a) are key mediators of cell death under oxidative stress [46]. Cytoplasmic accumulation of box C/D snoRNAs can be induced by oxidative stress doxorubicin [47]. Li et al. found that a C/D box snoRNA Bm-15 can target the 2′-O methylation modification at C434 of 18S rRNA in silkworm *B. mori* [48]. Bm-15 is located in both the nucleus and cytoplasm of silkworm cells [49] and can translocate dynamically from the nucleolus to the cytoplasm under the abiotic stress of nutritional deficiency or UV radiation, which might slow down the maturation of rRNAs and synthesis of ribosomes to enhance stress resistance of cells [50].

snRNAs and snoRNAs are known to be functionally and evolutionarily conserved elements of transcript processing machinery. But recently, with abundance measuring of snRNAs and snoRNAs in the frontal cortex of humans, chimpanzees, rhesus monkeys, and mice, 44% of the 185 measured snoRNA and 40% of the 134 snoRNA families showed significant expression divergence among different species. A 10-fold elevated expression of U1 snRNA and a 1000-fold drop in expression of SNORA29 were shown in humans compared to the other species, indicating that snoRNA abundance changes specific to the human lineage and suggests a possible mechanism underlying these changes [51].

Identification of snoRNAs was accelerated by high-throughput sequencing in Lepidoptera. With library construction and sequencing, 141 snoRNAs were identified in silkworm *B. mori*, and many snoRNAs were orphan snoRNAs [48]. Knocking down the homolog of C/D box snoRNA Bm-15 in *Spodoptera frugiperda* can induce the apoptosis of Sf9 cells [52]. Bm-152 might participate in the development of silkworms through the ecdysone and juvenile hormone signaling pathways [53]. Two silk gland-enriched ncRNAs Bm-102 and Bm-159 can be found in the histone modification complex and might play roles through epigenetic modifications in silkworms [54].

3.3 MicroRNA

In 1993, Victor Ambros and his colleagues, Rosalind Lee and Rhonda Feinbaum, found that *lin-4* does not code for a protein to control the timing of *C. elegans* larval development, but produces small RNA lin-4 [55], which had antisense complementarity to multiple sites in the 3′ UTR of the *lin-14* gene [55, 56], which mediate the repression of *lin-14* by the *lin-4* gene product [57]. With more and more discoveries of miRNAs, the functional roles of them were revealed. miRNA plays regulatory roles by targeting mRNAs for cleavage or translational repression. More than 50% of protein-coding genes in animals are regulated by miRNAs [58].

The earliest report of insect miRNAs was in 2001, Lagos Quintana et al. first found 22 miRNAs in the *Drosophila melanogaster* [59]. From then on, a large number of miRNAs were identified from bacteria, viruses, and plants to Metazoa. Now, 3857 mature miRNAs were identified through 31 species of Hexapoda according to the miRBase database (**Table 1**), includes 123 mature miRNAs in *Acyrthosiphon pisum*, 155 in *Aedes aegypti*, 130 in *Anopheles gambiae*, 254 in *Apis mellifera*, 80 in *Bactrocera dorsalis*, 2 in *Biston betularia*, 487 in *Bombyx mori*, 74 in *Culex quinquefasciatus*, 102 in *Dinoponera quadriceps*, 1470 in Drosophila, 92 in *Heliconius melpomene*, 7 in *Locusta migratoria*, 98 in *Manduca sexta*, 32 in *Nasonia giraulti*, 28 in *Nasonia longicornis*, 53 in *Nasonia vitripennis*, 133 in *Plutella xylostella*, 73 in *Polistes Canadensis*, 122 in *Spodoptera frugiperda*, and 342 in *Tribolium castaneum* [60–65].

In Lepidoptera, research on miRNAs focused more on *B. mori*, *M. sexta*, *P. xylostella* and *S. frugiperda*. miRNAs contribute to the modulation of a wide range of biological processes of insects, such as development [66, 67], metamorphosis [68], metabolism and longevity [69], reproduction [70], sexual dimorphism [71], cast determination [72], memory formation [73], behavior [74], insecticide resistance [75], endosymbiosis [76], and host-pathogen interactions and immunity [77, 78].

In silkworm *B. mori*, microRNA-14 regulates larval development time; the hormone receptor E75 (*E75*) and the ecdysone receptor isoform B (*ECR-B*) were the putative target genes of miR-14 [79]. let-7 is required for the developmental transition of *B. mori* through coordinating the biosynthesis of ecdysone and JH. The let-7 knockout larvae were developmentally arrested in the prepupal stage and became pupal-adult intermediates after apolysis [80]. Genetic disruption of miR-2738 increased the levels of BmPSI and BmMasc transcripts and might be a minor regulator of sex-determination genes in the silkworm [81]. bmo-miR-2739 and the miR-167 coordinately regulate the expression of the vitellogenin receptor in *B. mori* oogenesis, disruption of bmo-miR-2739 or miR-167 resulted in increased amounts of BmVgR protein in the ovaries and high level of mRNA expression of BmVgR in the fat body [82]. In *H. armigera*, microRNA-277 regulates dopa decarboxylase to control larval-pupal and pupal-adult metamorphosis [83].

Species	Number of rniRNA	Species	Number of miRNA
Acyrthosiphon pisutu	123	*Drosophila sechellia*	103
Aedes aegypti	155	*Drosophila simulans*	148
Anopheles gambiae	130	*Drosophila virilis*	180
Apis mellifera	254	*Drosophila willistoni*	77
Bactrocera dorsalis	80	*Drosophila yakuba*	89
Biston betularia	2	*Heliconius melpomene*	92
Bombyx mori	487	*Locusta migratoria*	7
Culex quinquefasciatus	74	*Manduca sexta*	98
Dinoponera quadriceps	102	*Nasonia giraulti*	28
Drosophila ananassae	76	*Nasonia longicornis*	53
Drosophila erecta	101	*Nasonia vitripennis*	32
Drosophila grimshawi	82	*Plutella xylostella*	133
Drosophila melanogaster	258	*Polistes Canadensis*	73
Drosophila mojavensis	71	*Spodoptera frugiperda*	122
Drosophila persimilis	75	*Tribolium castaneum*	342
Drosophila pseudoobscura	210	Total	3857

Table 1.
Mature miRNAs in insect according to miRbase 22.1.

Recently, microRNAs were found to play key roles in the host-pathogen interaction of insects. Cellular miRNAs could affect virus infection by regulating the expression of virus genes. A cellular lncRNA Lnc_209997 inhibits the proliferation of *Bombyx mori* nuclear polyhydrosis virus (BmNPV), but miR-275-5p can facilitate the replication of BmNPV, the expression of miR-275-5p can be induced by BmNPV through inhibiting the expression of Lnc_209997 [84]. Overexpression of bmo-miR-2819 could suppress BmNPV replication by down-regulating the expression of BmNPV ie-1 gene [85]. BmCPV-miR-1 could enhance viral replication by suppressing the expression of its target gene, the inhibitor of nuclear factor kappa-B kinase subunit beta of *B. mori* (BmIKKβ), the key gene of the NF-κB signaling pathway [86].

3.4 Long non-coding RNAs (lncRNAs) in Lepidoptera

Long non-coding RNAs (lncRNAs) were discovered in the early 1990s. Brannan and Brockdorff found that two lncRNAs, H19 and Xist, can be involved in the epigenetic regulation of mammals [87, 88]. Then, with genomic screening of mouse [89, 90] and other organisms, a large number of lncRNAs were identified [91].

lncRNAs are RNAs longer than 200 nucleotides that do not code for proteins [92, 93]. Based on Kung and Lee described [94], lncRNAs can be divided into three categories: (1) Long intervening non-coding RNA (lincRNA), which can be transcribed independently, such as H19 [87], Xist [88], HORTAIR [95], MALAT1 [96], *etc.* (2) Intronic lncRNAs are transcribed from the introns of protein-coding genes. Such as COLDAIR, which is transcribed from the intron of FLC [97]. (3) Sense-antisense pair, lncRNAs are transcribed from the antisense strand of protein-coding genes, including completely non-overlapping dispersed antisense transcripts; completely overlapping inverse semantic transcripts, and partial overlapping antisense transcripts.

lncRNAs can regulate gene expression in multiple levels [98, 99], such as epigenetic regulation of chromatin [100, 101], transcriptional [102–105], post-transcriptional [106, 107], and translational regulation [108], as well as having effects on protein transportation or location [109, 110]. Moreover, some lncRNAs function as a decoy/sponge or precursor of miRNAs [107, 111–113]. Such as H19, the oncofetal lncRNA is the precursor RNA of miR-675 [114, 115]. The association of H19 in tumorigenesis and invasion is attributable to the regulation of miR-675 [116, 117].

In Lepidoptera, lncRNAs were identified more in *B. mori*, *H. armigera* and *P. xylostella*. In *P. xylostella*, 3844 long intergenic ncRNAs (lincRNA) were identified, 280 and 350 lincRNAs are differentially expressed in Chlorpyrifos and Fipronil-resistant larvae [118]. 1309 lncRNAs were identified in one susceptible and two chlorantraniliprole-resistant *P. xylostella* strains, of which 877 were intergenic lncRNAs, 190 were intronic lncRNAs, 76 were anti-sense lncRNAs and 166 were sense-overlapping lncRNAs [119]. A total of 8096 lncRNAs were identified and classified into three groups. Expression profiling identified 114 differentially expressed lncRNAs during the development and found that the majority were temporally specific. Many lncRNAs are microRNA precursors or competing endogenous RNAs involved in micro-RNA regulatory pathways [120]. A total of 3,463 *H. armigera*, 6,245 *P. xylostella* differentially expressed lncRNAs were identified; the differentially expressed lncRNAs were mainly enriched in the metabolic, digestive, and synthetic signaling pathways [121].

In *B. mori*, 11,810 lncRNAs were identified from different tissues and developmental stages, including 474 intronic lncRNAs (ilncRNAs), 6250 intergenic lncRNAs (lincRNAs), and 5086 natural antisense lncRNAs (lncNATs) [122]. Using RNA sequencing technology data, 599 lncRNAs were identified in the silk gland of domestic and wild silkworms [123]. The functional roles of lncRNAs in silkworm were revealed gradually. lncRNA Bmdsx-AS1, origin from the antisense strand of sex-determining gene Bmdsx of silkworm, binding to the splicing factor hnRNPH, then interacted with BmPSI, one of the upstream regulating factors of Bmdsx. The splicing pattern of Bmdsx pre-mRNA was altered in male silkworms after Bmdsx-AS1 knockdown, but overexpression of Bmdsx-AS1 induced the male-specific splicing form of Bmdsx in the females [124]. A nucleus-enriched lncRNA lncR26319 regulates *Endophilin A* through competitively binding to miR-2834 and regulates oogenesis of silkworms [125].

A recent study showed that lncRNAs also play roles in pathogen-insect interaction. A total of 1845 candidate lncRNAs were identified in the virus-infected and non-infected midgut of silkworm larvae, 41 lncRNAs were differentially expressed, the apoptosis, autophagy, and antiviral response genes, such as ATG3, PDCD6, IBP2, and MFB1, could be targeted lncRNAs with differential expression [126]. LincRNA_XR209691.3 could promote BmNPV replication by interacting with BmHSP70 [127]. Overexpression of LINC5438 promoted the proliferation of BmNPV, while interference with LINC5438 inhibited its proliferation, LINC5438 can regulate the expression of Bmiap, BmDronc, BmICE, and its predicted target gene BmAIF [128].

3.5 PIWI-interacting RNA (piRNA)

piRNAs are generally 24–32 nucleotides in length and bind specifically to the PIWI subfamily of Argonaute proteins. So far, the large-scale sequencing of the sRNAs from different organisms has discovered a variety of piRNA sequences [129–133]. Mature piRNAs have a strong preference (≥60%) for the 5′ uridine residue, with adenosine signature at position 10, and 2′-O-methylation at 3′ end, assembling as clusters in the genome [134]. Over 80% of piRNAs have unique genomic locations, from which nearly 75% are mapped to transposon loci [135], the others are mapped to multiple genomic locations. piRNAs exist in both germline and somatic cells, including neurons. The most widely-recognized and well-characterized function of piRNAs is to suppress the activities of transposable elements in the germline development, where piRNAs are highly abundant, and in somatic cells like neurons, piRNAs are modestly abundant [136–145]. For the low conservation of individual piRNAs, the deductions of their functions are challenging. Piwi-piRNAs have important functional roles in suppressing transposon [142], preserving genomic integrity [144, 146], regulating translation [147], regulating target mRNAs [140], modulating mRNA stability [147] through epigenetic modifications including DNA methylation, and histone modifications [148, 149].

In silkworm, a systematic discovery of transposable-element (TE)-associated small RNAs in the genome were carried out, 182, 788, and 4990 TE-associated small RNAs in the miRNA, siRNA, and piRNA species were identified, respectively [150]. In female embryos, the mRNA of the male-determining gene Masculinizer (Masc) can be cleaved by the fem piRNA-Siwi complex, disruption of Masc directed to the female-determining pathway. But in male embryos, Masc activates the male-determining pathway for the absence of Fem piRNA [151]. In *P. xylostella*, the W chromosome-derived piRNAs complementary to Masc mRNA have also been identified, indicating the convergent evolution of piRNA-dependent sex determination in Lepidoptera [152]. But this is not the case in the Asian corn borer, *Ostrinia furnacalis* (Pyraloidea) [153].

Recently, piRNA pathway has also been shown to be involved in the host-pathogen interaction of insects. In mosquitoes, besides germline transposon control, the piRNA pathway also plays a very important role in antiviral immunity [154]. Piwi-like-1 and Piwi-like-2-3 could inhibit AcMNPV replication in *B. mori*, while Piwi-like-4-5 could promote virus replication in Sf9 cells [155].

3.6 Circular RNA (circRNA)

circRNAs are covalently closed single-stranded RNAs (ssRNAs) that have recently re-emerged as a widespread class of RNA species. Circular RNA was first identified in plant viroids [156, 157], followed by the discovery of eukaryotic circular transcripts via electron microscopic evidence showing a circular morphology with unknown function [158]. Circular RNAs extensively exist in eukaryotes from yeast [159–161], worms [162], insects [163, 164], plants [165, 166], and mice to humans [161, 167]. Eukaryotic circular RNAs are usually classified into three groups according to their biogenesis pathways [168]: the exonic circRNAs are produced from pre-mRNA back-splicing, a downstream 5′ splice site joined to an upstream 3′ splice site, resulting in an RNA molecule in a circular format, which is the major biogenesis pathway of circRNAs, a, named. Another type of circRNA is the circular intronic RNA (ciRNA) generated from intronic lariats that failed to be debranched after splicing, this type of circular RNAs ligated with a 20–50 phosphodiester bond [169, 170]. Furthermore, circRNAs with inside unspliced introns were also found and named the exon-intron circRNAs (EIciRNAs) [171].

Circular RNAs play roles in gene expression by modulating transcription in the nucleus and regulating translation in the cytoplasm. In the nucleus, circRNAs play a diverse biological role including chromatin looping, transcription regulation, and alternative splicing [170–174]. Circular RNAs can form three-stranded structures harboring a DNA:RNA hybrid named R-loops with their producing locus to impact transcription [171]. ci-ankrd52 originated from the second intron of the *ANKRD52* locus. Degradation of ci-ankrd52 by RNase H1 resolves the transcribing R-loop to enhance transcriptional elongation [175]. Conn et al. discovered circSEP3 in the cell nucleus of *Arabidopsis thaliana* regulated the splicing of SEPALLATA3, a homeotic MADS-box transcription factor important for floral homeotic phenotypes [173]. circRNAs play various biological roles in cytoplasm, they can act as decoys for miRNA, serve as protein scaffolds, or sequestering proteins. Several abundant circRNAs act as miRNA sponges, or competing endogenous RNAs (ceRNAs), that subsequently suppress their bio-accessibility and thereby targeted mRNAs [176, 177]. circRNA supercont3.352:252102|253283 acted as sponge of cpp-miR-1671 and decreased the expression of cpp-miR-1671 target gene *CYP4G15,* then participated in deltamethrin resistance of *Culex pipiens pallens* (L.) [178]. In *Aedes albopictus*, aal-circRNA-407 acts as a sponge of aal-miR-9a-5p to promote the expression of its target gene *Foxl*, then regulate the ovarian development of mosquito, knockdown of circRNA-407 resulted in a decreasing number of developing follicles and a reduction in follicle size after a blood meal [179].

CircRNAs play roles in development [180], reproduction [181, 182], metamorphosis [183, 184], life-span [163], insecticide resistance, aging [185], and host-pathogen interactions and immunity [186, 187]. The biological role of circRNAs in host-pathogen interactions has been broadly investigated. In Lepidoptera, the expression levels of circRNAs in the silkworm midgut are altered after BmNPV infection, these altered circRNAs can modulate various immune pathways, such as the Notch, ABC transporters, and the endocytosis pathway, indicating circRNA might be an anti-viral factor [188]. Another vcircRNA_000048 encoded by BmCPV can translate a small peptide vsp21 with 21 amino acid residues–, which attenuates the viral replication [189].

CircRNAs have recently been reported to be implicated in the regulation of anti-bacteria [190], anti-fungal [191], and anti-parasite immunity [192]. circRNAs modulate various immune pathways, including lysosomes, phagosomes, endocytosis, ubiquitin-mediated proteolysis, the metabolism of xenobiotics by cytochrome P450, and insect hormone biosynthesis [193], as well as cellular renewal and structure and carbohydrate and energy metabolism [194]. Pathogen-encoded circRNAs hijack the host system for proliferation [195], and the host immune system also has the ability to hijack circRNAs encoded by pathogens to inhibit their infection [196, 197].

4. Conclusion

Over the last decade, we have gained a deeper understanding of the biological role of ncRNAs in the development, sex determination, oogenesis, spermatogenesis, and pathogen-interaction in insects. Now a growing body of evidence demonstrating that ncRNAs are involved in immune regulation in insects, especially miRNAs, piRNAs, circRNAs, and lncRNAs, which provide new insight into the immuno-interaction of host and pathogen. Recently, new techniques are likely to improve our understanding of the biogenesis and biological roles of ncRNAs. As more and more functional roles of ncRNAs are revealed, ncRNAs will be used as the potential target of pest control for Lepidoptera in the near future.

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Conflict of interest

The authors declare no conflict of interest.

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Section 2

Moths: Bioecology and Genetics

Chapter 5

Hawkmoths (Lepidoptera, Sphingidae) Flight Potential Trajectories from Wind Systems in Atlantic Rainforest in Southeast Brazil

Marcio D'Arrochella, André Zaú and Jorge Oliveira

Abstract

In tropical regions, pollination is primarily carried out by animals, hawkmoths (Lepidoptera, Sphingidae), being perceived as one of the most important groups. Lepidoptera, in general, comprises approximately 146.000 species of hawkmoths, 87% of them being nocturnal. In these regions, there is a total of 7.100 species, approximately 3.100 of which are found in Brazil. Sphingidae family is one of the most representative families when it comes to pollination, being more abundant in low-altitude environments. With assistance of the wind, they migrate long distances from coastal woodlands to mountainous areas during the hot rainy season. Their abundance is even greater during cloudy nights, and during new moon phases, as they are always in search of illumination. In Brazil, most of the studies focus on the morphology of individuals for taxonomic research, being few publications on their dispersal available. Aiming mapping flight paths for feeding and reproduction, the occurrence of pollination is estimated, enabling the connectivity of forest fragments and ensuring gene exchange. To visualize this study, atmospheric models of breeze circulation such as Brazilian Regional Atmospheric Modeling System (BRAMS), GRADs, and the Three-Dimensional Kinematic Trajectory (TC3D) were employed in the Atlantic Forest of the state of Rio de Janeiro, where observations and descriptions of over 80 species are accumulated, allowing the identification of spatial patterns through the use of Geographic Information Systems. The north/south orientation proved to be dominant, potentially connecting fragments of forests with varying sizes, shapes, and conservation states, extending from coastal areas to the mountainous regions within the southeastern part of Brazil.

Keywords: potential trajectories, pollination, atmospheric models, connectivity, Atlantic rainforest

1. Introduction

The Atlantic Rainforest is the second largest tropical rainforest of the American Continent and originally was extended along the Brazilian coast up to east Paraguay and the northeastern Argentina. Currently, the forest is recognized as one of the 25 global biodiversity *hotspots*[1] due to its over 8.000 endangered endemic species, even though being reduced to less than 7% of its original area [1]. Other authors [2, 3] indicate that there is approximately 15% coverage of the Atlantic Forest in Brazil, including fragments in early succession stages and reforested areas, which actually do not in fact qualify as remaining forest.

The fragmentation of the Atlantic Forest has detrimental effects on biodiversity. The reduction of forested areas, whether for urban development or agricultural practices, has drastically diminished and transformed *habitats*, potentially leading to extinction of species, the blocking of migration routes and hindering genetic exchange [4]. Genetic exchange guarantees the maintenance of the gene *pool*[2], on an evolutionary scale, which enables certain species to have more resistance to pests or changes in the physical environment [5].

Fragmentation occurs when an ecosystem is subdivided by human actions or even natural disturbances, such as fire, resulting in a landscape where only a few remnants of the original vegetation cover remain inserted in a matrix with completely different characteristics [6].

Depending on spatial orientation, the shape, and size, forest fragments can become less isolated, theoretically then, reducing the possibility of population decline due to mortality. For this reason, some authors [7, 8] emphasize the importance of the dispersal syndromes as the basis for connectivity, which is not necessarily, is what is used as basis to establish an ecological corridor [9–11]. In similar fashion, priority should be given to the creation of large-scale conservation units, since small fragments, even when arranged in corridors, are subject to both area and edge effects, which may hinder dispersal, migrations, and barrier crossing.

In this regard, the Guapiaçu-Macacu watershed, located in the county of Cachoeiras de Macacu (RJ), shows as a fertile ground for research on connectivity due to its spatial configuration of Atlantic Forest fragments with different shapes, sizes, and degrees of isolation in a pasture matrix. Furthermore, this biome is recognized as one of the 18 centers of lepidoptera dispersion in the neotropical region [12] and yet still exhibits an annual extinction rate of 1.8% within Lepidoptera order [13]. This fragments form a corridor that integrates themselves in private areas and diverse conservation units. Understanding how gene exchange occurs in these fragments can both make their management more efficient, as it can allow the possibility to identify problems and potentialities that assist in the creation and management of other conservation units. In a similar fashion, understanding the orientation of dispersers migratory paths[3] can allow us to improve the planning and execution of ecological restoration projects for the establishment of ecological corridors.

To evaluate the dispersal syndrome, it is necessary to understand the seed and pollen dispersers of the area. When it comes to a matrix dominated by rural areas with patches of Atlantic Forest nonetheless, it is understood that terrestrial dispersers are

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[1] The term refers to prime concern areas for biodiversity conservation worldwide [14].

[2] The term can be understood as genetic elasticity.

[3] Pollen and seeds.

not as efficient on promoting connectivity and genetic exchange. Therefore, for long-distance dispersal, seed and pollen require aerial transportation, which can be eased by the wind or a flying agent [15].

2. Study area

Looking over the map of use and land cover of the watershed (**Figure 1**), it is possible to have a preliminary overview of the landscape conjuncture and structure, which appears as a pasture matrix with forested water dividers. In the center, numerous forest fragments of different sizes are arranged **s**patially forming a corridor in the SW-NE direction. Bordering this fragment, we have the presence of some agricultural areas. It is noticeable that urban occupation in the center of the watershed is incipient, with a greater concentration to the south and northeast. The area covers approximately 573, 54 Km^2 and is a part of the Guanabara hydrographic watershed, located at the Atlantic slope of the Serra do Mar (locally known as Serra dos Órgaos) with rivers draining into the Guanabara bay [16].

The vegetation in the area is classified as dense ombrophilous forest of submontane formation (ranging from 50 to 500 m in altitude), on slopes and lowland forests on the hills (ranging from 5 to 50 m in altitude). The area displays high structural and floristic diversity; however, most of the fragments are found stripped of their original diversity [17].

The study area is under the influence of the South Atlantic Subtropical Anticyclone (ASAS) which is a system of great scale, semi-stationary, in action all over the year, but presenting variations in its position, depending on the season of the year, and the passage of cold fronts in the area.

In the summer, the strong surface heating makes it less intense over the continent and more intense over the sea. At winter, when the continent is colder than the sea, the ASAS advances on the continent, exerting a strong influence over the study area [18].

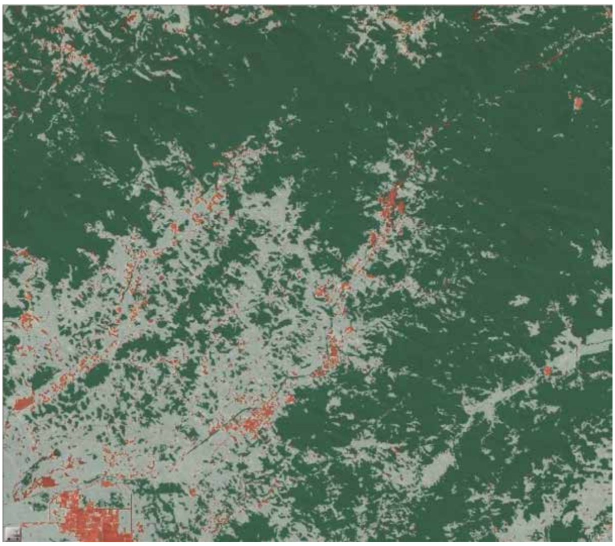

Figure 1.
Processed image from ***sentinel 2ª satellite*** *of the land cover in the Guapiaçu watershed.*

At mesoscale, the study area is under the influence of maritime, land, valley, and mountain breezes. The maritime and continental breezes occur due to the differential heating between the ocean and the continent. When the wind blows from the ocean to the continent, it is named “maritime breeze,” and when it blows from the continent to the ocean we address it as “land breeze.” The mountain breeze occurs when cold mountain air descends down the slope during the night. The valley breeze occurs during the day when warm air rises up the slope. As the study area is located in a valley and mountainous region, it is under the influence of all four breezes in different sectors.

The breezes occur within the Planetary Boundary Layer, situated between the first 2 Km of the troposphere, where the airflow (wind) is strongly influenced by the surface roughness (relief, vegetation, buildings, etc.). The friction with these obstacles is significant, thereby reducing the wind speed [19].

3. The hawkmoths and the Atlantic rainforest

Lepidopterans are approximately 146.000 species of butterflies and hawkmoths, 87% being nocturnal and 13% diurnal [20]. In the neotropical regions, they add 7.100 and 7.900, respectively, in Brazil occurring between 3.100 and 3.200 species [21].

It is viable to characterize lepidopterans as holometabolic organisms, as they undergo a complete metamorphosis in four stages of their life cycle, as follows (**Figure 2**): the first stage is the egg; the second is the larval phase (popularly known as caterpillar), feeding as herbivores and growing; in a relatively shorter third phase, they transform into a pupa, enclosing themselves in a cocoon, where physical changes such as the development of wings occur; finally, the fourth stage is the winged one in the form of butterflies or hawkmoths, in which they will reproduce and eventually die [22, 23]. The fourth stage lasts on average 2 months, in which the lepidopterans can fly long distances to feed on nectar and potentially contribute to pollination [23, 24].

Hawkmoths are the least studied lepidopterans in tropical areas, and the Sphingidae is one of the most representatives when it comes to pollination. These

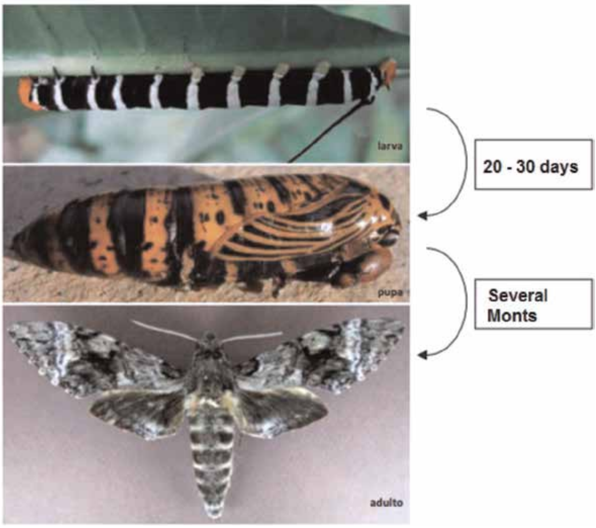

Figure 2.
The last three life stages of a Lepidoptera. Adapted from [22].

insects are commonly found in low-altitude environments and comprise over 50 species [25]. In a study conducted in a High Mountain Forest area[4] at southeastern region, it was observed that hawkmoths visited more than 80 plant species. This situation is ideal for the chosen study area. The same authors state that hawkmoths migrate from restingas to mountainous areas during the hot and rainy season.

Hawkmoths, for the most part, have nocturnal flight habits, which are closely related to temperature and precipitation[5] increase, also under the influence of lunar phases [26]. At Serra do Mar in Paraná, it was perceived an increased flight activity under cloudy skies, followed by drizzle or fog, with high temperatures and moderate winds [27]. This behavior is believed to be driven by hawkmoths that need to seek light, which leads them to migrate and allows pollen dispersal. Warmer areas tend to have highest abundance of hawkmoths, although this does not necessarily reflect on the number of species [25]. Flowers pollinated by hawk moths typically exhibit nocturnal anthesis, pale coloration, nectar rich in sucrose, and sweet floral scent [22].

Recent studies investigating pollen residues and hawkmoths collected in São Paulo (Picinguaba) recorded pollination interactions between sphingid lepidoptera and the families *Apocynaceae*, *Asteraceae*, *Convolvulaceae*, *Malvaceae*, *Fabaceae*, *Scrophulariaceae*, and *Rubiaceae* which were already known to occur, and for the first time occurrence, *Bromeliaceae*, *Arecaceae*, *Begoniaceae*, *Celastraceae*, *Combretaceae*, *Cyperaceae*, *Erythroxylaceae*, *Malvaceae*, *Melastomataceae*, *Piperaceae*, *Poaceae*, *Polygonaceae*, *Sapotaceae*, *Scrophulariaceae*, *Solanaceae*, *Ulmaceae,* and *Verbenaceae* [26]. Authors emphasize that in terms of abundance, the most prevalent families were *Rubiaceae* (15,6%), *Bromeliaceae* (7,8%), and *Fabaceae* (6,2%).

The studies at the state park of Serra dos Órgãos, in the Picinguaba and Santa Virgínia nuclei, located in northern coast of São Paulo state, provide important contributions to the identification of families pollinated by sphingid hawkmoths. However, there is progress, as it also provides species information [25]. The identified species by the author are *Apocinaceae*, *Caprofoliaceae*, *Chrysobalaneceae*, *Dabaceae*, *Malvaceae*, *Orquidaceae*, *Rubiaceae*, *Solanaceae*, and *Zingiberaceae*.

The most representative families in terms of species number in this study were *Orchidaceae*, *Rubiaceae*, and *Solanaceae*. This is a pattern, also found by [26] in the same study area. The relationship between hawkmoth flight and increased temperature and precipitation was also evidenced by studies in the zone of Mata Mineira [28]. In their collections, the maximum number of individuals were observed in the months of January and February, while the minimum were observed in August. Regarding the flight of sphingid hawkmoths, it is known that:

> These Hawkmoths are excellent flyers and have a very rapid wing beat; some fly during the day, but mostly, moths are active only at night. A large number of them feed in a manner very similar to hummingbirds, hovering in front of the flower and extending their proboscis into it [29].

The majority of hawkmoths that inhabit the Atlantic Forest, particularly in the area of Cachoeiras de Macacu and Serra dos Órgãos, show a higher number of individuals during nocturnal and crepuscular flights, in autumn months and under light rain or

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[4] Forests with altitude greater than 1600 m [25].

[5] The low luminosity of the sky on rainy days causes more hawkmoths to fly in search of luminosity [23].

high cloudiness. Regarding the moon phases, the same authors describe the new moon as the ideal situation, as the absence of light prompts hawkmoths to search for this resource [30].

4. Methodology

The chosen control point for obtaining the flight trajectories of the sphingid hawkmoths was the Guapiaçu Ecological Reserve (REGUA). This choice was based the fact that a significant number of species described in the reference catalog [31] were found in REGUA. Furthermore, one of the authors works there and regularly observes and describes the behavior and morphology of hawkmoths.

REGUA (**Figure 3**) covers and manages approximately 7.1000 hectares and was established and registered in 2001 as a nonprofit private association [31]. The reserve is sustained through tourism, mainly birdwatching, homing to over 500 bird species. It also boasts a great diversity of dragonflies and moths, with occasional visits from jaguarundi, capybaras, and even a jaguar (locally known as "onça pintada") [30].

From the analysis of the "Sphingidae Guide of Serra dos Órgãos" [31], it was possible to identify 110 species present in the study area and their flight months throughout the year (**Table 1**). It is worth to say though that some species may fly in different months around the year.

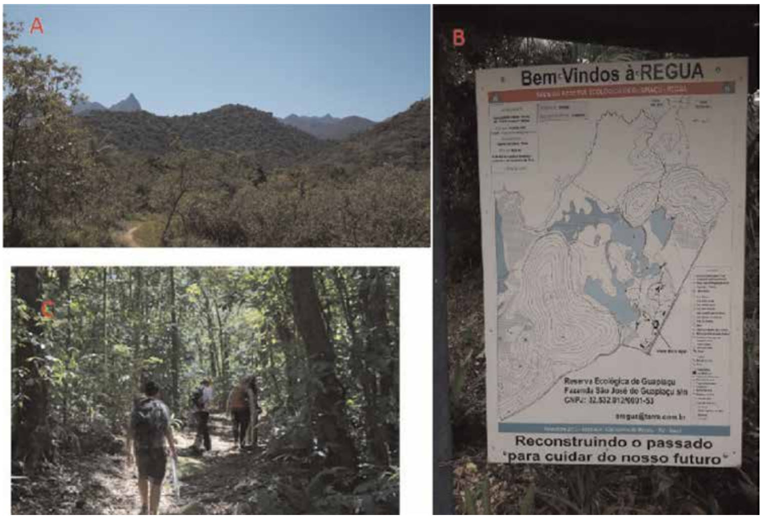

Figure 3.
Control point at the Guapiaçu ecological reserve. A—General view, B—Field visits and C—Map of the reserve. Personal archive.

Month	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
N° of species	26	14	40	27	12	13	23	25	28	26	24	29
Rain precipitation*	210	150	158	117	81	47	52	56	90	106	181	213

**Data in mm, taken from [32] for the period of 1968–2009.*

Table 1.
Number of species found flying in Cachoeiras de Macacu (RJ) by the month of the year.

Using Pearson's correlation test, we obtain the index of 0.3037, indicating a weak correlation between the number of species and precipitation. It is noticeable nevertheless that the typically drier months, such as May and June, coincide with lower species numbers. On the other hand, the month with the highest occurrence was March, which denotes the transition from summer to autumn, confirming the previously mentioned findings [26].

The most efficient criterion for selecting specific species to be analyzed is abundance, which reflects the most representative ones in the area of study [31]. However, during the construction of the hawkmoth [31], there was not exactly the concern of generating abundant data, given the objective was solely to identify and describe the species.

As an alternative for selecting the most representative species in the area, we chose to rely on the expertise of Dr. Jorge Bizarro,[6] a specialist zoologist with over 20 years of study on the subject. In the interview, 13 species were indicated as the most representative (**Table 2**), which are included in the catalog.

From this table, it is possible to understand which species fly in which seasons of the year and thus infer about pollination by month and season. It is also possible to understand which species are more tolerant, such as *Adhemários daphne daphne*, that was observed every month of the year, and the less tolerant species, such as *Nyceryx coffaeae*, observed in only 5 months. The month of March was also the month in which the most representative species of the area were seen, with exception of *Xylofanes porcus continentalis*.

Recognizing the geographic distribution of these species allows us to speculate that their local behavior can be extrapolated to other areas with similar topography,

Species	J	F	M	A	M	J	J	A	S	O	N	D
Adhemarius daphne daphne	X	X	X	X	X	X	X	X	X	X	X	X
Manduca diffussa petuniae	X		X	X			X			X	X	X
Manduca hannibal almicar	X		X					X	X		X	
Enyo lugubris lugubris	X		X	X		X			X			
Enyo ocypete			X	X			X	X	X			
Nyceryx coffaeae			X					X	X			
Nyceryx riscus			X	X			X	X	X			
Pseudosphinx tétrio		X	X	X		X	X	X	X	X	X	X
Erinnyis alope alope	X	X	X		X	X	X	X	X	X	X	X
Erinnyis ello ello	x	x	X	x			x	x		x	x	X
Xylophanes chiron nechus	x		X	x	X		x	x	x	x	x	X
Xylophanes porcus continentalis	x			x	X			x	x		x	X
Xilophanes tersa tersa	x	x	X	x	X		x	x	x		x	x

Source: Adapted from [31].

Table 2.
Hawkmoth species most observed in REGUA and their months of appearance.

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[6] Jorge Bizarro is a PhD in Zoology, co-author of the hawkmoth catalog, and REGUA director (Ecologic Reservation of Guapiaçu), used as a reference on a great number of scientific papers.

climate, vegetation cover, and land use. To facilitate studies, the use of atmospheric modeling was chosen, as it presents a more feasible alternative in the absence of local baseline data, allowing approximations to be generated regarding the atmospheric behavior of these areas.

Models are simplified approximations of reality, resulting from how we perceive it, subjective representations of our interpretation as a result of rationality. Above all, it is a methodological approach, constructing "a simplified and intelligible framework of the world" [33].

Models are used according to specific objectives, which are expressed in their functioning and structure, serving as parameters for choosing which one to use. Therefore, for a research project that relies on atmospheric dynamics, it is important to remember the dependence between the atmosphere and the characteristics of the Earth's surface due to the inherent exchange of heat, moisture, and linear momentum, with exchanges occurring between the surface and lower layers of the atmosphere. In this way, it is possible to define local forcing and local determinants for the behavior of currents, winds, and breezes [34]. This can be expressed in models.

Currently, Numerical Weather Prediction (MNT) allows us to forecast events with a 15-day lead time, and 5-day accuracy, which is possible thanks to the computers at the Centre for Weather Forecasting and Climate Studies (CPTEC) of the National Institute for Space Research (INPE) and other institutions that combine regional- and global-scale data [34]. For climate models to yield reliable results, it is necessary to dispose of a solid database as long as a thorough understanding of the variables influencing the process, as well as a long period of data collection.

CPTEC employs various models such as the Global Circulation Model of the Atmosphere (GCM) for global weather prediction and ETA for regional prediction. The Regional Atmospheric Modeling System (RAMS) and the Brazilian Regional

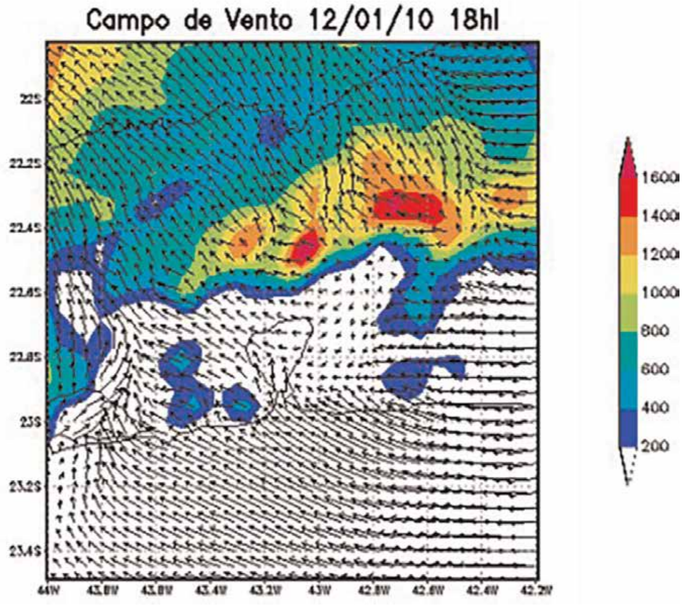

Figure 4.
Example of wind and temperature field generation in model BRAMS model with graphical interface of the TC3D model. BRAMS does not work under a graphical interface. It is a mathematical model, being necessary then the use of the program "GRADS" to further visualization of the generated atmospheric data. Source: [34].

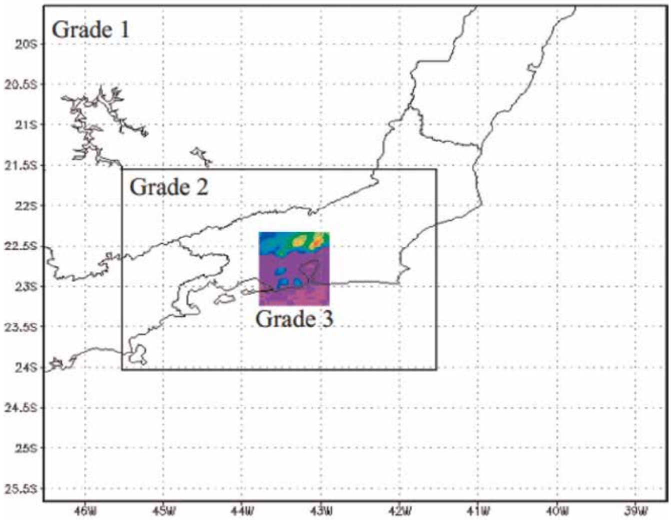

Figure 5.
Examples of grids that can be analyzed with the GRADs model. BRAMS does not operate under a graphical interface. BRAMS is a mathematical model, requiring the use of the GRADS program for the visualization of generated atmospheric data. Source: [35].

Atmospheric Modeling System (BRAMS) are used for studying spatially limited areas with high spatial resolution. BRAMS allows the interpolation of data from nearby meteorological stations to create wind field analysis (**Figure 4**), while GRADS provides analysis at three different scales as grids (**Figure 5**) [36].

One of the major advantages of numerical modeling of the atmosphere is the possibility of obtaining spatial and temporal information on a much larger scale than available in conventional synoptic networks. However though, it is important to validate the models for tropical atmospheric conditions, as most of them were developed for mid-latitude atmospheres on a global scale [35].

BRAMS, along with the GRADS (**Figure 5**) and Three-Dimensional Kinematic Trajectory (TC3D) programs, offer an advantage in this regard, as they have been adapted for validation in the tropical atmosphere. Using these models, it is possible to analyze potential connectivities and dispersal trajectories of hawkmoths at three different scales, with the third grid covering the entire watershed, the second grid connecting it to the upstream mountainous region, and the first grid covering the entire Serra do Mar.

BRAMS is a regional-scale numerical model, adapted from RAMS, developed by the University of Colorado, which allows us to predict variables such as temperature, wind, humidity, and precipitation. It solves the equations of atmospheric dynamics and includes numerous submodels that relate soil-vegetation-atmosphere system, turbulent flux exchanges, radiative transfers, cloud microphysics, and more [37].

The BRAMS was adapted to represent the state of the tropical atmosphere [35]. The equations used include the equations of motion, thermodynamics, continuity for the mixing ratio, and mass continuity. With parameterizations, it is possible to generate information on solar radiation, humidity processes (clouds, liquid, and ice precipitation), sensible and latent heat, soil layers, water surfaces, vegetation, among others [38].

As the nights of new moon are favorable for the increased occurrence of sphingid hawkmoths, all new moon nights [39] from the years 2015, 2016, 2017, and 2018 were investigated, each month, based on observations obtained from the website of the

Department of Astronomy at the University of São Paulo (USP), in order to run the BRAMS model for each of these nights, simulating their atmospheric conditions. This way, it was possible to understand under which atmospheric conditions the moths could have flown.

With this, it is possible to simulate the atmospheric conditions for each month, for the species of hawkmoths found during this month. Each new moon event presents the ideal conditions for hawkmoth flight, allowing the calculation of the flight trajectory for each species, since they could theoretically take advantage of the winds to minimize energy expenditure. Similarly, the data can be divided by season, to understand certain wind patterns and flight behavior. From the flow field (generated by the TC3D model), trajectories can be calculated forward (departure from a specific point) and backward (arrival at specific location). At this research, the team chose to use a departure point, the REGUA, which is believed to be the origin of dispersion of the described and observed hawkmoths.

To determine the possible flight trajectories from the wind field (**Figure 6**), The Three-Dimensional Kinematic Trajectory (TC3D) model was adopted. This is a nonconvective three-dimensional model applied over the surface, which allows estimation of directions and altitudes of particles suspended in the atmosphere based on wind circulation data[7].

The model uses a color scale that makes possible to estimate the altitudes reached by suspended particles. The author emphasizes that the application of this model

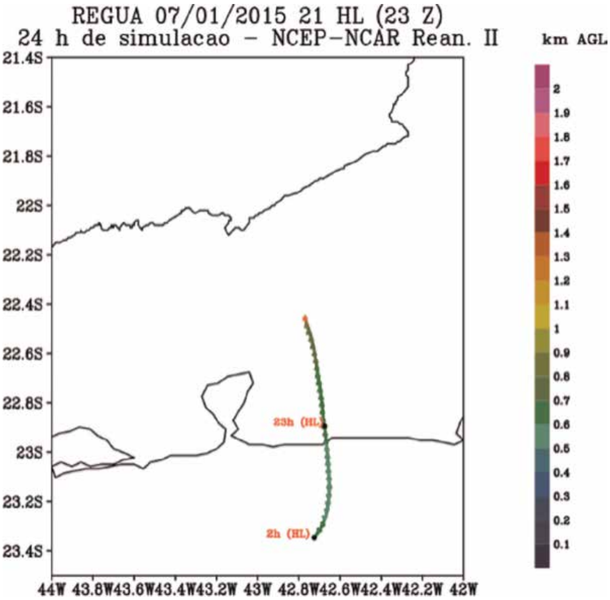

Figure 6.
Example from application of the TC3D model.

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[7] It is worth mentioning that TC3D model does not display detailed land cover and topography in its graphical interface. The program only uses political boundaries on a flat white surface. The vectors, however, represented by the particle in ascent trajectories are colored to express their altitude, and points with time stamps, providing information on the speed of dispersion.

enables the estimation of both outgoing and incoming trajectories, defining their vertical and horizontal paths within the atmospheric mixing layer [40].

Having REGUA as a reference point, it is possible to present flight trajectories of moths for each new moon night throughout the year, indicating which species would potentially be involved in pollination and the distances they could reach.

After conducting all the simulations for the fur dates in each season of the year, over 4 years, the trajectories were georeferenced using the ArcGIS software, creating a layer overlaying the satellite image of the area at the GRADS grid level 2 scale. All trajectories were vectorized to create a single synthesis map containing the 16 simulations, providing an overview of the spatial and biogeographic arrangement of the pollination potential and connectivity of the Southeast Brazilian Atlantic Forest.

5. Results

The preprocessing, processing, and postprocessing steps were performed using the BRAMS model, and the calculation of the trajectories was done using the Three-Dimensional Kinematic Trajectory (TC3D). Using the BRAMS model, it was simulated the wind field, while the TC3D model calculated the potential trajectories of a particle dispersed by the wind from a specific point, on a predefined date and time.

For this purpose, four nights of the new moon were chosen throughout the year, with intervals of 2 to 3 months, once each season. The models generated results for each season from 2015 to 2018. For each year starting from 2015, an additional day was added forward, as new moon events occur within a weekly period.

The interpretation of trajectories is based on state delimitation on a map without the geographical characteristics of the area. Thus, it is possible to identify only the direction, speed, and altitude at which an inert particle moves in the atmosphere. This way, the hypothesis was made that hawkmoths can take advantage of the sea and land breezes systems and potentially can follow the trajectories, given that they have full flight autonomy.

Breezes are atmospheric phenomena resulting from the differential heating between the land and the ocean. They are mesoescale phenomena whose reach depends on the topography. They occur in the planetary boundary layer which is in the first few kilometers above the surface.

It is important to note that even though there are no geographical elements in the graphic representation of GRADS, the evaluation is not arbitrary but requires some level of abstraction and knowledge of the landscape characteristics of the area and the political division of municipalities. This allows to make assumptions about the landscapes where a particle disperses, as well as its final destination during the chosen 24-hour period.

5.1 Summer trajectories

The trajectories were simulated starting at 8 PM, considering daylight saving (**Figure** 7) during the twilight period, for 4 years starting in 2015[8]. The species

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[8] The research considers that each event simulated by the model represents a flight path for feeding and reproduction.

observed in January and following these trajectories are *Adhemarius daphne daphne*, *Manduca diffussa petuniae*, *Manduca hannibal almicar*, *Enyo lugubris lugubris*, *Erinnyis alope alope*, *Erinnyis ello ello*, *Xylophanes chiron nechus*, *Xylophanes porcus continentalis*, and *Xylophanes tersa tersa*. That means that potentially 10 out of the 13 most representative species could follow these trajectories.

Regarding the predominant direction of the sea breeze system, it is evident that during summer, the behavior did not change drastically over the four simulated years. There was a predominance of the land breeze, blowing toward the south and southwest.

5.2 Autumn trajectories

The new moon experiment in March, as a sampling for autumn, presents the species that would follow such trajectory, including among them: *Adhemarius daphne daphne*, *Manduca diffussa petuniae*, *Manduca hannibal almica*, *Enyo lugubris lugubris*, *Enyo ocypete*, *Nyceryx coffaeae*, *Nyceryx riscus*, *Pseudosphinx tétrio*, *Erinnyis alope alope*, *Erinnyis ello ello*, *Xylophanes chiron nechus,* and *Xylophanes tersa tersa*. That shows that potentially 12 out of the 13 most representative species can fulfill these trajectories.

It is noticeable that there is no standard behavior (**Figure 8**). In the years 2015 and 2017, there was a predominance of the sea breeze in the north direction, with changes in direction after crossing the limits of Serra dos Órgãos. In 2016 and 2018, the initial direction of the land breeze was not the same but later followed the southwest pattern.

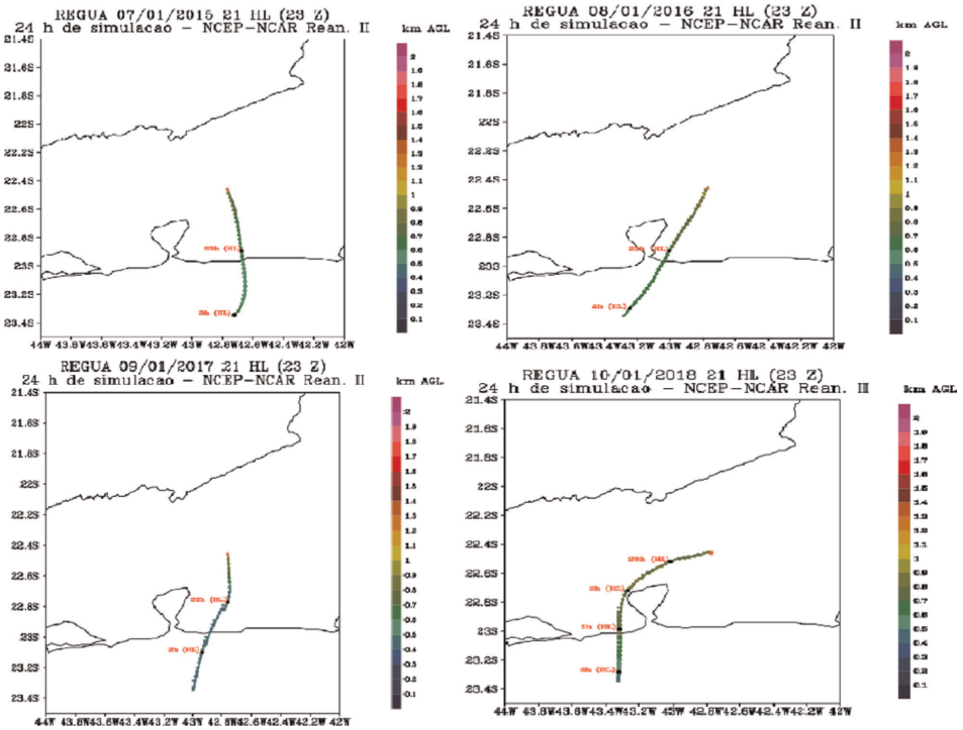

Figure 7.
Potential summer trajectories.

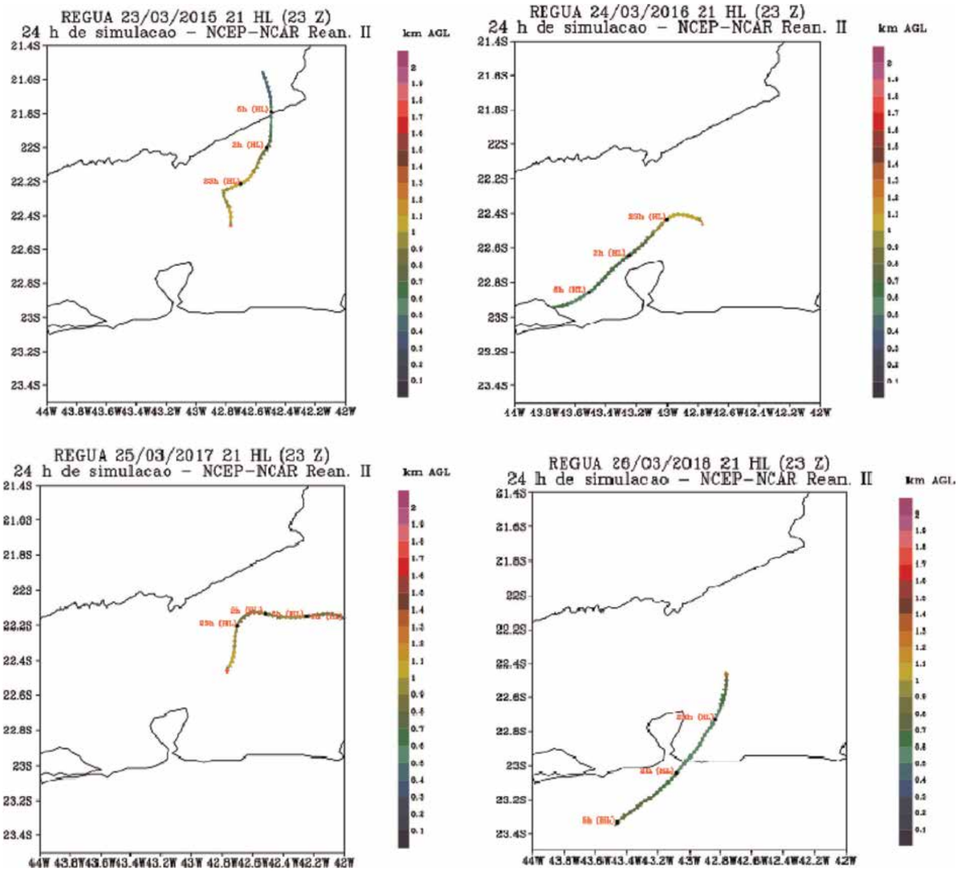

Figure 8.
Potential autumn trajectories.

5.3 Winter trajectories

Regarding the species that fly in June, as a sampling for the winter period, we have then: *Adhemarius daphne daphne*, *Enyo* lugubris *lugubris*, *Pseudosphinx tétrio*, and *Erinnyis alope alope*. This demonstrates that out of the 13 most significant hawkmoth species in the area, only 4 of them would potentially follow that trajectory. The winter season breezes (**Figure 9**) behaved similarly throughout the years 2015, 2016, and 2017, originating from the sea with an initial direction tending toward the northwest. Among these 3 years, 2016 showed a tendency toward the northeast at the end. The year 2018 presented a very different result, with the occurrence of the land breeze, following a westward direction, with a tendency toward the west and later south.

5.4 Spring trajectories

September represents the beginning of spring in the Southern Hemisphere and includes the species here mentioned that would potentially follow this trajectory: *Adhemarius daphne daphne*, *Manduca hannibal almicar*, *Enyo lugubris lugubris*, *Enyo*

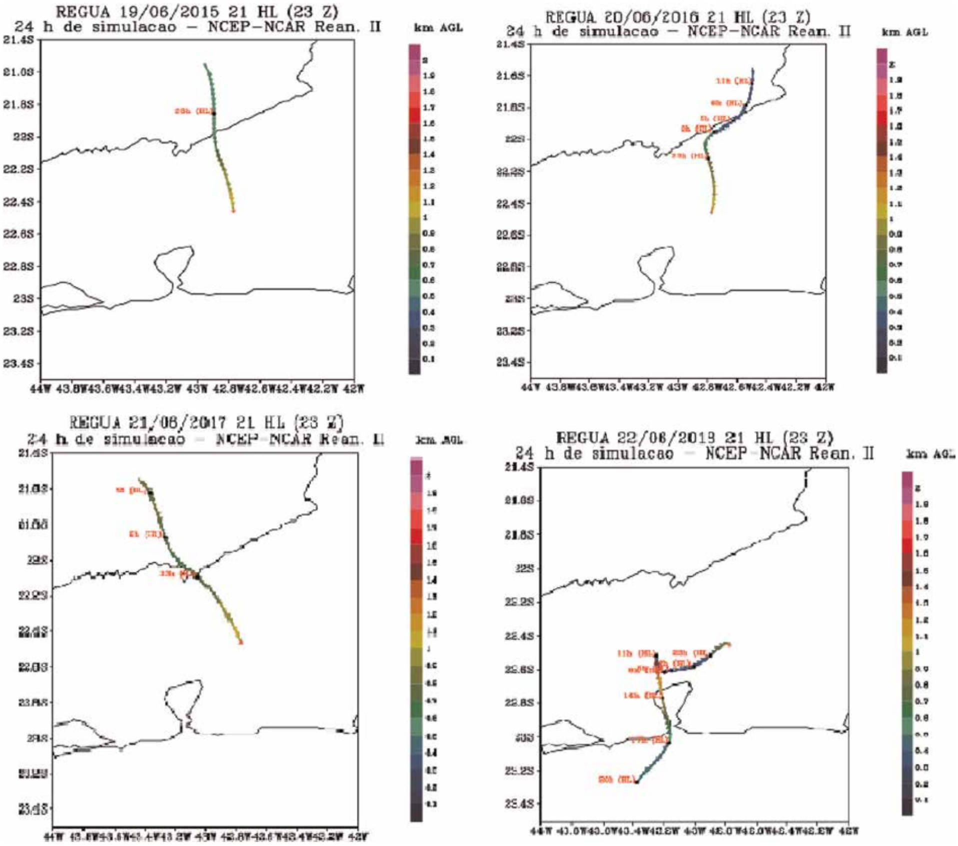

Figure 9.
Potential winter trajectories.

ocypete, *Nyceryx coffaeae*, *Nyceryx riscus*, *Pseudosphinx tétrio*, *Erinnyis alope alope*, *Xylophanes chiron nechus*, *Xylophanes porcus continentalis*, and *Xylophanes tersa tersa*. This trajectory is potentially followed by 11 out of the 13 most representative species in the area.

Analyzing the behavior of spring breezes (**Figure 10**), it is noticeable that in the years 2015, 2017, and 2018, there is a tendency toward the south-southwest direction, indicating a continental origin. Only in the year 2016, the dominance of the sea breeze occurred, with a trajectory toward the northwest and later toward the northeast.

6. Conclusions

Making an observation of all the trajectories, it becomes evident that the majority occurred in the north/south directions, which is a different pattern than expected—southwest/northeast, following the orientation of the river drainage. Only 3 out of the 16 trajectories followed this pattern. It also became evident that in the autumn and winter trajectories, there is a predominance of the sea breeze, oriented toward the north. Similarly, in spring and summer, there is a predominance of the land breeze toward the south.

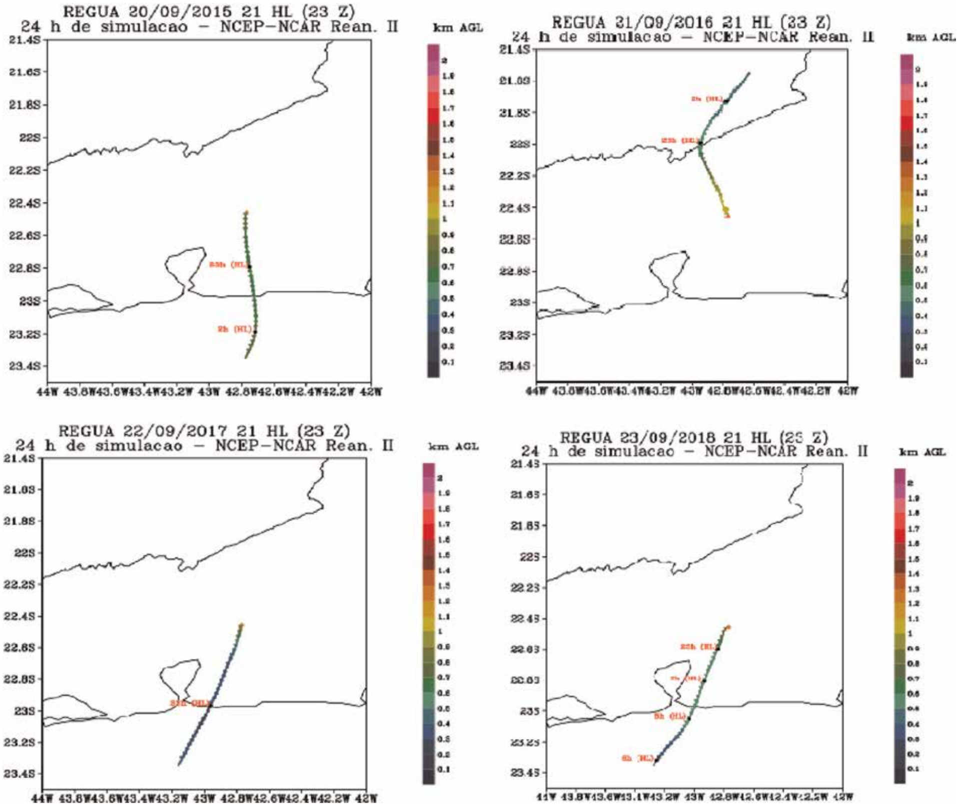

Figure 10.
Potential spring trajectories.

Observing the synthesis map (**Figure 11**), it is possible to notice that in the northern direction of REGUA, there is an abundance of small to rounded fragments and a higher number of deforested areas or abandoned pastures. This indicates that under the influence of this potential orientation of the hawkmoth pollination syndrome, there is a pathway that can serve as a basis for planning new ecological restoration projects or the implementation of new conservation units.

They can reach the state of Minas Gerais, and along their path, are able to potentially promote connectivity with the high-montane ombrophilous forests in the Serrana region of Rio de Janeiro state, where important conservation units are located. Subsequently, they can cross the Paraíba do Sul river valley, connecting the fragments of semi-deciduous stationary forests.

The trajectories in south direction extend beyond the coastal limit, reaching the ocean along their path, and they traverse landscapes of rolling hills, submontane ombrophilous forests, abandoned pastures, as well as coastal sandy areas or restingas.

The three trajectories at southwest direction would allow connectivity with mangrove ecosystems and submontane ombrophilous forests, as well as important conservation units with low- and mid-montane ombrophilous forest in the metropolitan area of Rio de Janeiro city.

At the end of the trajectories, it is noticeable the reach of the inland stationary forests, where forest cover is more sparse, with thousands of small fragments, possibly

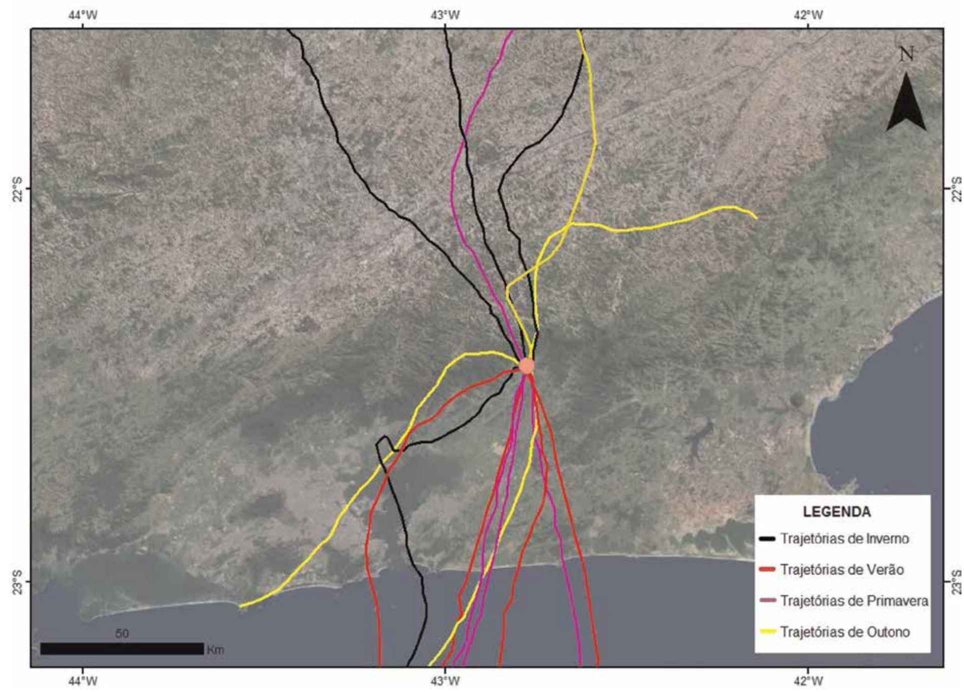

Figure 11.
Potential flight paths of hawkmoths. Legend: Circle in orange—REGUA, black lines—Winter trajectories, red lines—Summer trajectories, pink lines—Spring trajectories, yellow lines—Autumn trajectories.

disturbed and under the influence of the edge effect. It is interesting to note that such pollination syndrome via the effect of breezes is not present in the East-West directions, with basically no connectivity between REGUA and the Costa Verde and the lakes region of Rio de Janeiro (locally addressed as "Região dos Lagos").

In this sense, it is believed that the Atlantic Forest fragments in the county of Cachoeiras de Macacu, as well as their corresponding watersheds and aerial areas, potentially participate in a large dispersal pathway through the breeze system, extending from coastal ecosystems to high-altitude ecosystems in the mountainous inland areas.

The spatial arrangement of connectivity presented should still be seen as a potential, and its validation will only be possible with observations and markings of the studied fauna, which is methodologically possible, but was financially unviable for this research. At least, it is noteworthy that the species *Adhenarius daphne daphne* and the order Manduca can be seen as important bioindicators of connectivity, given their body size and occurrence throughout all of the months of the year.

The study area, as a representative sampling of the Atlantic Forest conjecture, in the context of habitat fragmentation and ecological restoration action plans, served much more than a locus of individual analyses, revealing connections between fauna and flora with distant areas. It is encompassed mountainous areas covered by fragments of the Atlantic Forest with ecosystems of semi-deciduous seasonal forests, open ombrophilous forests, mixed ombrophilous forests, advanced successional ombrophilous forests, as well as submontane coastal lowlands with ombrophilous forests, restinga ecosystems, and mangroves. This study covered a vast mosaic of landscapes through the application of integrated methods from ecology and meteorology.

Little is still known about flight trajectories of lepidopterans in Brazil, with at most the knowledge of where they may occur. Therefore, we present a possible spatial arrangement, as vast Brazilian literature demonstrates the occurrence of the species discussed in this chapter in the areas where the TC3D model simulated the trajectories.

We sincerely hope that the modeling will serve as an incentive for new researchers to seek methodologies to understand the connectivity of the forest fragments and landscapes based on pollination syndromes.

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Chapter 6

Invasive Leaf-Mining Moths (Lepidoptera, Gracillariidae) in Ukraine: Ecology, Degree of Invasion

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Abstract

This chapter is devoted to elucidating the bioecological characteristics of the existence of five invasive leafminer moth species of the family Gracillariidae Steinton, 1854 (*Parectopa robiniella* (Clemens, 1863), *Phyllonorycter issikii* (Kumata, 1963), *Phyllonorycter platani* (Staudinger, 1870), *Macrosaccus robiniella* (Clemens, 1859) and *Cameraria ohridella* Deschka & Dimic, 1986) and extent of their impact on dendroflora in Ukraine. Features of their invasion into various ecosystems were revealed. The effects of Gracillariidae caterpillar feeding on host (food) plants were studied. The caterpillars of invasive leafminer moth species affected the functional state of host plants; it was confirmed by alterations in the content of soluble proteins, activity and isoenzyme composition of benzidine peroxidase during the growing season of the host plants. The study of dynamics of highly soluble proteins showed a decrease in their content at substantial leaf damage. The restructuring of both peroxidase activity and its isoenzyme profile was revealed to be indicators of the plant biochemical adaptation to the feeding of caterpillars of invasive leaf blotch miners. The results obtained show the activation of the enzymatic antioxidant protection system in host plants in response to the damaging effect of Gracillariidae caterpillars, which ensures the survival of the plant and completes its ontogenesis program in unfavorable conditions.

Keywords: invasive Gracillariidae, leafminer moths in the fauna of Ukraine, adaptation of invasive leafminers to a novel environment, effect of leafminers on the physiological state of host plant, biochemical adaptation of trees to caterpillar feeding

1. Introduction

This chapter is devoted to elucidating the bioecological characteristics of the existence of invasive leafminer species of the family Gracillariidae (Lepidoptera) and the degree of their influence on dendroflora in Ukraine. The biological characteristics of invasive lepidopteran species were clarified. The scale and direction of invasion of five Gracillariidae species were determined in the territory of Ukraine. Features of settlement of various ecosystems were revealed.

This chapter found the effect of Gracillariidae caterpillar feeding on host (food) plants. The caterpillars of invasive insect species affect the functional state of host plants; it was confirmed by alterations of soluble protein content, activity and isoenzyme composition of benzidine peroxidase during the growing season of these plants. The study on the dynamics of highly soluble proteins showed their decrease in the case of high-level leaf damage.

The restructuring of both the activity and the isoenzyme profile of peroxidase turned out to be an indicator of plant biochemical adaptation to the feeding of caterpillars of invasive leaf blotch miners. Statistically significant differences were found for soluble peroxidase; its activity increased by an average of 2.1 times in the case of high-level damage of host plant leaves by the caterpillars. High-level leaf damage by the phytophagous insect was reflected in the alteration of the isoenzyme profile of benzidine peroxidase. The main pattern of negative effects of Gracillariidae caterpillars involves a significant increase in the activity of the most acidic molecular forms of cytoplasmic peroxidase in the leaves of host plants.

The results obtained demonstrated the activation of the enzymatic antioxidant defense system in host plants in response to the damaging effect of Gracillariidae caterpillars, which ensures the survival of the plant and completion successfully its ontogenesis program under the effect of unfavorable environmental conditions. The most significant factor in protecting the cells against caterpillar activity involves an increase in guaiacol peroxidase activity which indicates an enhancing of cell barrier properties.

It was established that the caterpillars of Gracillariidae invasive species can have a significant effect on photosynthetic apparatus of host plants. Such effect was clearly determined with the fluorescence technique which was carried out with a portable fluorometer, “Floratest.” Feeding of *Cameraria ohridella* caterpillars causes a decrease in the activity of photosynthetic apparatus in *Aesculus hippocastanum* regardless of the spatial arrangement of leaves in the tree crown. Under the influence of *C. ohridella* feeding on *A. hippocastanum* leaves, the amount of chlorophyll not involved in photosynthetic energy transfer in reaction centers increased. Conversely, during the development of only a single *C. ohridella* generation, a constant decrease in the quantum efficiency of PS II (inhibition of photosynthetic activity) was observed.

Already at the beginning of the moth mine development (first-aged *C. ohridella* caterpillars), a significant decrease in activity and an increase in the plateau coefficient were observed in the leaves in both illuminated and shaded part of the tree crown; it, in turn, indicates an inhibition of photosynthetic photophysical and photochemical processes and a reduction of electron acceptor pool in the electron transport chain. Such pathological alterations were caused by a decrease in the content of active chlorophyll (a component of PS II pigment-protein complexes) and its destruction. The values of the key parameters of chlorophyll fluorescence induction indicate a significant inhibition of photosynthesis processes and a violation of the coherence of reactions in the Calvin cycle. Being determined by local changes in chloroplast structure and functions, these metabolic transformations cause the damage of photosynthetic apparatus in plants resulted in a decrease in the content of photosynthetic pigments.

As a result of our research, no relationship was detected between the parameters of urbanocenoses in which the development of mines of invasive insects took place and the number of larvae infected by parasitoids. Only the relationship between the number of mines formed by invasive insect insects and the degree of their damage by parasitoids (Hymenoptera) has been established. The relative resistance of invasive Gracillariidae species to diseases in a novel environment was elucidated. The fact of damage to the preimaginal developmental stages by the entomopathogen was established only for *Macrosaccus robiniella*. The fact of infection of invasive caterpillars with the fungus *Lecanicillium sp.* (Verticillium) has been established for the first time in the territory of Ukraine.

2. Bioecological characteristics of Gracillariinae invasive species in the territory of Ukraine

Taxonomically, the leaf blotch miners (Gracillariidae Steinton, 1854) form a separate family. As a result of a molecular phylogenetic study performed in 2017 [1], eight following subfamilies were allocated: Acrocercopinae Kawahara & Ohshima, 2016; Gracillariinae Stainton, 1854; Lithocolletinae Stainton, 1854; Marmarinae Kawahara & Ohshima, 2016; Oecophyllembiinae Réal & Balachowsky, 1966; Ornixolinae Kuznetzov & Baryshnikova, 2001; Parornichinae Kawahara & Ohshima, 2016; Phyllocnistinae Herrich-Schäffer, 1857.

Ninety-eight genera and 1900 species of world fauna have been described. The Palearctic region possesses 42 genera with more than 500 species. In Ukraine, 94 species belonging to 18 families were described. Among Gracillariidae faunal complex, eight species have invasive status in Europe; among them, five following species were registered within Ukraine: *Parectopa robiniella* (Clemens, 1863), *Phyllonorycter issikii* (Kumata, 1963), *Phyllonorycter platani* (Staudinger, 1870), *Macrosaccus robiniella* (Clemens, 1859) and *Cameraria ohridella* Deschka & Dimic, 1986.

Almost all recent Gracillariidae species act as obligate leafminers throughout the development of all caterpillar ages. The vast majority of species are narrow oligophages or monophages, rarely polyphages, trophically associated with dicotyledons and partially with gymnosperms. Food chains of most Palearctic species were established with Fagaceae, Rosaceae, Fabaceae, Salicaceae, Aceraceae and others. They can damage fruit, ornamental and industrial plants, mainly introduced dendroflora. With mass reproduction, large-scale outbreaks of abundance can be observed. This chapter provides an assessment of the scale of modern invasion in the territory of Ukraine. The analysis of the experience of studying the bioecological features of invasive insect species in the novel range was carried out.

3. Materials and methods

3.1 Methods of field research

The studies have been conducted for 15 years (2006–2022) within the steppe zone of Ukraine in 10 stationary sample sites (the Dnipropetrovsk region: Dnipro city, Nikopol; the Dnipro-Oril Nature Reserve; Zaporizhia region: National Nature Park “Velykyi Luh”; the Mykolaiv region: Tylihulskiy Regional Landscape Park), reflecting the conditions of various types of artificial plantings of host plants that act as foodplants for invasive Gracillariidae. The ecosystems under study differed in geographical location, type and degree of anthropic load.

As an object of research, in the green areas of populated cities, various groups of model different-aged horse chestnut and black locust trees were identified with similar morphological and taxational features but with different degrees of leaf damage by invasive insect species. The degree of damage of leaf blades in horse chestnut by *C. ohridella* was evaluated visually using a modified scale proposed by Shupranova et al. [2].

All invasive species form mines on the leaves of host plants; such mines were recorded by visual examinations. Counting mines on the trees was carried out with the conventional "model branch" method [3]. A tree and a random branch with a length of at least 1 m were selected by randomization. After that, the number of leaves and mines formed by the invasive leafminer was counted. The photos of leaves damaged by miners were taken using a digital camera with a resolution of 5 megapixels. An object micrometer was applied to the leaves to calibrate the measured parameters. The measurements were made on digital photos using ToupView 3.7 software. The mine length (L) was measured along the central leaf vein; the maximum mine width (W) was measured perpendicularly to its length; also, the mine area (S) and the leaf blade area (Sl) were measured [3]. The mine length-to-mine width ratio (L/W) and the mine area-to-leaf blade area ratio (S/Sl) were calculated. Data processing was performed using Statistica 12.5 software package.

3.2 Analytical techniques for heavy metal content in plant tissues

The leaf blades were dehydrated in porcelain tiles using a drying cabinet at t° = 100°C. After being dehydrated, the dry leaves were weighed on electronic scales AXIS AD500 with an accuracy of 0.001 g (the weigh required for analysis was 0.5–1.0 g). Then, the weights were incinerated in a muffle furnace at t° = 450°C. The ash was poured into a conical flask, and 0.5 mL of concentrated nitric acid and 0.5 mL of double-distilled water were added. The resulting solution was adjusted to a volume of 10 mL with distillate water and filtered through an ash-free filter paper, then the tiles were washed with 10 mL of double-distilled water and the solution volume was adjusted to 25 mL. The content of Zn, Cu, Pb and Cd was analyzed in the samples. The heavy metal content in leaf blades was analyzed with atomic absorption spectrophotometry using an AAS-30 spectrophotometer according to the standard method proposed by Khavezov, 1983.

3.3 Morphometric methods

Photographs of the insects were taken through binocular MBS-10 equipped with 5 MP digital camera. To calibrate the measured parameters of insects, an object micrometer was applied. The measurements were made on digital photos using ToupView software. Primary data processing was performed in MS Excel 2019, and subsequent data processing was performed using Statistica 13 software package. Excess (Ex) and asymmetry (As) indicators were used to compare the detection of the morphometric characteristic deviations from the normal distribution. Coefficient of variation (CV) and standard deviation (SD) were used to analyze intrapopulation diversity. Univariate analysis of variance (ANOVA) was used to compare linear characteristics and indices to detect interpopulation polymorphism.

3.4 Methods for determining the effect of feeding of Gracillariidae caterpillars on biochemical processes in host plant tissues

To carry out biochemical analysis, the leaves were washed in water and immediately used for enzyme extraction. To isolate the enzyme preparation, tree leaves (0.3 g) were homogenized in 6 mL of 0.05 M tris-HCl buffer, pH 7.4, with 0.5% polyvinylpyrrolidone (PVP). The samples were extracted at +4°C for 1 hour and centrifuged for 15 min at 14,000 rpm. The supernatant was used to determine the activity and isoenzyme composition of benzidine peroxidase (BPOD) and guaiacol peroxidase (GPOD) activity. BPOD activity (BPOD, EC 1.11.1.7) was measured at 490 nm in a reaction mixture (0.8 mL of Na-acetic buffer, pH 5.4; 1 mL of benzidine solution and 0.2 mL of enzyme preparation) after adding 1% H_2O_2. The activity was calculated within 1-min interval when the reaction rate reached a maximum [4]. The result was expressed in absorbance units/g of raw material min.

Izoenzyme composition of BPOD was determined by isoelectric focusing (IEF) in a 5% horizontal polyacrylamide gel (PAAG) using Ultraphor (LKB, Bromma, Sweden), pH range 3.5–6.5. A benzidine technique was used to detect enzymatic activity in PAAG. Blue-colored bands appeared, which turned brown after 10–15 min. These brown-colored bands represented the BPOD activity. The gels were scanned and analyzed using the 1D Phoretix software intended for determination of the relative content (%) of each isoform in the total peroxidase spectrum.

Guaiacol-depended peroxidase (GPOD, EC 1.11.1.7) activity was evaluated according to Ranieri et al. [5, 6] by determining guaiacol oxide at 470 nm in a reaction mixture containing an acetic buffer (pH 6.0), a 2 mM guaiacol solution, 0.2 mL of enzyme preparation and 0.15% H_2O_2. The results were calculated considering the molar extinction coefficient (26.6 mM^{-1} cm^{-1}) and expressed in mM guaiacol/g*raw material.

The protein content in the samples was determined by the Bradford [7] method with Coomassie brilliant blue G 250 dye relative to the bovine serum albumin standard (Serva, USA).

The results of studies on enzyme activity were presented as mean x ± SD (standard deviation). Data obtained were analyzed using Statistica (version 8, StatSoft, USA). Tukey's Honestly Significant Difference test was used to determine the significant difference between the group means. The differences were found to be statistically significant at $p < 0.05$.

3.5 Methods for determining the effect of feeding of Gracillariidae caterpillar on the state of the photosynthetic apparatus in host plants

Diagnostics of photosynthetic disorders of native chlorophyll in fresh leaves were performed using a portable fluorometer "Floratest" developed by V. M. Glushkov Institute of Cybernetics of the National Academy of Sciences of Ukraine. The portable fluorometer "Floratest" comprises a base unit with a graphic liquid crystal display and remote optoelectronic sensor, connecting cable to the USB port of a personal computer, and a network adapter. LED, as a component of the remote optoelectronic sensor, has a maximum radiation intensity at $\lambda = (470 + 20)$ nm. Irradiation parameters in the sensor were the following: irradiation wavelength 470 + 15 nm; irradiated spot area not less than 15 mm^2; light intensity within the spot at least 2.4 W/m^2. Signal reception indicators in an optoelectronic sensor: the spectral range of fluorescence

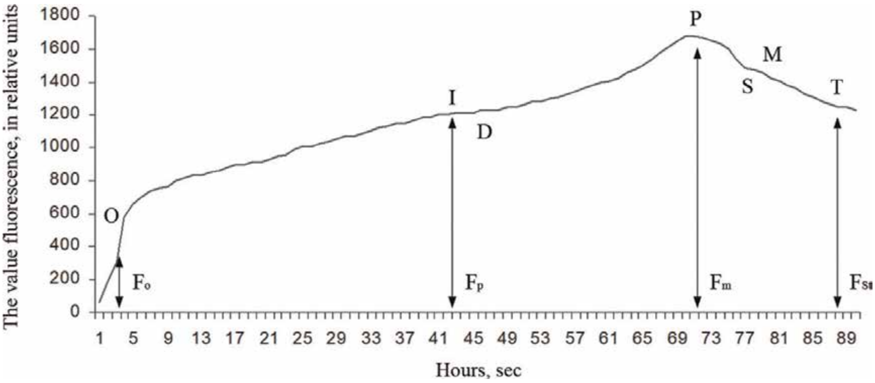


Figure 1.
Typical chlorophyll fluorescence induction curve: F_0 *is an initial value of fluorescence induction after irradiation is turned on;* F_p *is a value of "plateau" fluorescence induction;* F_m *is a maximum value of fluorescence induction;* F_{St} *is a stationary value of fluorescence induction after light adaptation of a plant leaf.*

intensity measurement was 670–800 nm; receiving window area 9 mm^2; photodetector sensitivity at λ = 650 nm was 0.45 A/W.

The observations were made on fresh *A. hippocastanum* leaves both damaged and undamaged by *C. ohridella.* After starting the light exposure, chlorophyll fluorescence intensity (induction of fluorescence or light-induced [caused] fluorescence) begins to change significantly over time. The time dependence of the chlorophyll fluorescence intensity (ChlF) has the characteristic form of a curve with one or more maximums, and it is called the ChlF induction curve, or the Kautsky curve (**Figure 1**).

The shape of this curve is quite sensitive to changes that occurred in the photosynthetic apparatus of plants during their adaptation to different environmental conditions, which has become the basis for the widespread use of the Kautsky effect in photosynthesis studies.

4. Mechanisms of invasion of different types of ecosystems by Gracillariidae species

4.1 Characteristics of invasion of field-protective forest belts

The invasion of field-protective forest belts occurs unevenly; we found the tree age to be the main factor in this process. We studied the characteristics of invasion of the black locust (*Robinia pseudoacacia* Linnaeus, 1753) plantings by the insect species because these trees occupy the most significant area among artificial field-protective plantings in Ukraine. It was found that *R. pseudoacacia* manifests itself relatively actively in almost all natural zones of Ukraine, mainly due to its spread by root growth. The forest belt surveys allowed us to distinguish three age-related variants of *R. pseudoacacia* trees (parent trees planted at the time of a forest belt creation (50–70 years), sprouting trees (15–25 years), and young trees (up to 15 years)). *Parectopa robiniella* (Clemens, 1863) and *Macrosaccus robiniella* (Clemens, 1859) were detected on black locust in all geographical areas of the country. Monitoring surveys have shown varying degrees of invasion of three age groups of trees by Gracillariidae. It was discovered that both invasive insect species mainly develop mines on the leaves of young trees (up to 15 years old).

By the results of calculating the average number of mines on a simple leaf, it turned out that the invasive insect species most often formed the mine on the lower (near the petiole) leaf segment in all three age groups of the trees. Determining the characteristics of the relative invasion of simple leaves related to the total number of damaged leaves showed that the invasion of leaf blades was the highest within the location of the middle simple leaves on the complex leaf of the black locust.

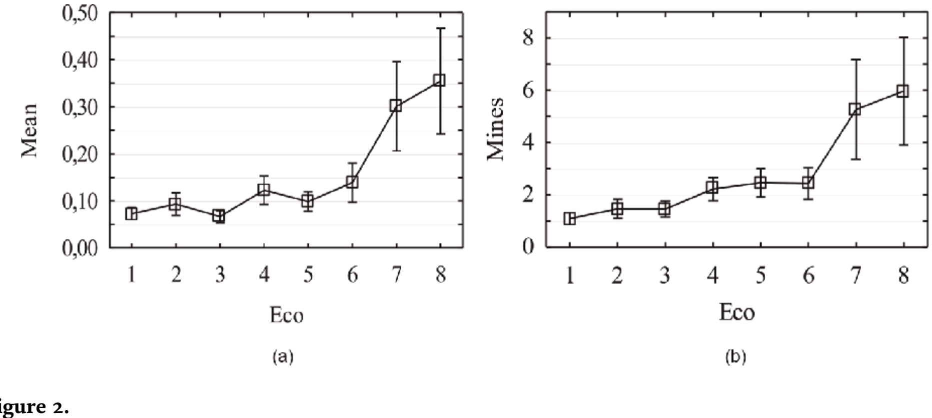
Such invasion patterns may be explained by the different growth rates of *R. pseudoacacia* leaf blades and the qualitative characteristics of leaf tissues. Thus, our survey confirmed the assumption that the topical specificity of invasion is derived from the peculiarities of divergent leaf development and acropetal development of the leaf of the host plant.

4.2 Features of invasion of urban green areas by invasive insect species

The study was conducted within Dnipro city, in seven largest green areas, and in Dnipro-Oril Nature Reserve. According to the results of statistical data processing, the largest number of mines was registered in park areas of Dnipro city (**Figure 2**), located at high terrain levels (higher than 100 m). The variability curve of the average number of mines on the leaf (a) is almost completely same to the variability curve of the absolute number of mines (b). Accordingly, the average number of mines does not depend on the number of leaves on the host plant. Based on the results of univariate variance analysis of the settlement of different green areas in Dnipro city by invasive leafminers (**Table 1**), statistically significant ($p < 0.05$) differences were found.

Morphological characteristics of mines can be considered the main indicators of successful feeding of leafminer caterpillars [3]. Assessment of the mine area determines the feeding activity of caterpillars within this mine [8]. To test these hypotheses, we conducted morphometric studies of mines formed by *Parectopa robiniella* and *Macrosaccus robiniella*. As a result of the research conducted, it turned out that both insect species studied show relatively good adaptability to the choice of suitable environmental conditions. This was evidenced by the statistical data obtained by us in morphological studies of mines formed by both insect species (**Table 2**).

According to the results of the study, both coefficient of variation and standard deviation indicated that the morphometric plasticity of mines formed by *Parectopa*



(a) (b)

Figure 2.
Variability of settlement of the model branches of R. pseudoacacia *L. by* Parectopa robiniella*:* A—*Variability of the average number of mines on leaves (Mean),* b—*Variability of the absolute number of mines on leaves (mines); the x-axis: An ecosystem number (eco), the y-axis: A value of the characteristic.*

Eco*	***x* ± *SD***	***SS***	***MS***	***F***	***P***
1	1.10 ± 0.32	1.0360	1.0360	1.2608	0.2682
2	1.47 ± 1.02				
1	1.10 ± 0.32	1.0403	1.0403	1.4135	0.2404
3	1.46 ± 0.94				
1	1.10 ± 0.32	9.8560	9.8560	6.8703	0.0120*
4	2.22 ± 1.33				
1	1.10 ± 0.32	15.7926	15.7926	4.4560	0.0387*
5	2.46 ± 2.03				
1	1.10 ± 0.32	14.2041	14.2041	5.0388	0.0295*
6	2.44 ± 1.86				
1	1.10 ± 0.32	130.2083	130.2083	6.5556	0.0146*
7	5.27 ± 5.10				

** Urbocenoses: 1—Manuylovsky Park; 2—Lazaria Hloby Park; 3—Druzhby Narodiv Forest Park; 4—Pridneprovsky Park; 5—Park 40th anniversary of the liberation of Dnipropetrovsk; 6—T. G. Shevchenko Park; 7—Oles Honchar Dnipro National University (DNU) Botanical Garden.*

Table 1.
One-way analysis of variance of invasive species settlement in different green areas of Dnipro city (n = 242).

Characteristics	***Parectopa robiniella***			***Macrosaccus robiniella***		
	CV	***SD***	***X***	***CV***	***SD***	***x***
L	0.40	5.50	13.70	0.30	4.20	15.80
W	0.60	4.90	8.40	0.20	1.30	7.40
S	0.80	3178.10	3787.60	0.30	1131.90	3628.20
Sl	0.40	11038.60	27137.70	0.30	10281.20	31877.40
L/W	0.30	0.50	1.90	0.10	0.30	2.10
S/Sl	0.80	0.10	0.10	0.40	0.01	0.10

Note: CV—coefficient of variation; SD—standard deviation; x*—average value; L—mine length; W—mine width; S—mine area; Sl—leaf blade area; L/W—the mine length-to-mine width ratio; S/Sl—the mine area-to-leaf blade area ratio.*

Table 2.
Morphometric variability of mines in populations of Parectopa robiniella *(n = 184) and* Macrosaccus robiniella *(n = 180) on the territory of DNU botanical garden.*

robiniella showed a more variable polymorphism than of mines formed by *Macrosaccus robiniella*. Average values of length of mines formed by *Parectopa robiniella* were less than of mines formed by *Macrosaccus robiniella*. At the same time, average width (W) and area (S) of mines formed by *Parectopa robiniella* were larger, and the leaf surface area (Sl) of leaves with mines formed by *Parectopa robiniella* was less than the surface area of leaves with mines formed by *Macrosaccus robiniella*. The mine length-to-mine width ratio (L/W) was greater in the mines formed by *Macrosaccus robiniella*, which indicates a higher elongation of the mines formed by this insect.

4.3 Effect of heavy metals on distribution of invasive insects in urban agglomerations

The content of pollutants of various origins in the atmospheric air is considered to be one of the leading factors of pollution in urban environments. Woody vegetation in urboecosystems is constantly exposed to such pollution, which is reflected, among other, by an increase in heavy metal content in leaf tissues. Being primary consumers, phytophages are affected by the content of heavy metals, and their certain concentrations can even influence the vital processes of larval stages of insect development.

Research of the effects of heavy metals (Zn, Cu, Pb, Cd) on urban populations of *Parectopa robiniella* showed significant differences in the types of urboecosystems (**Figure 3**). It turned out that the higher the heavy metal content (especially zinc, copper and lead) in the leaf tissues of a host plant, the greater the amount of insect mines. Results of a general linear model of the effect of heavy metal content and terrain height on the number of mines formed by *Parectopa robiniella* in the conditions of Dnipro city (**Table 3**) also demonstrated this regularity.

5. Adaptive features of invasive Gracillariidae species to the novel environmental conditions

5.1 Characteristics of morphometric variability within the population group of single urbocenosis

According to our study, the distribution of *Macrosaccus robiniella* pupae collected in Pridneprovsky Park (**Table 4**) shows among linear characteristics a significant

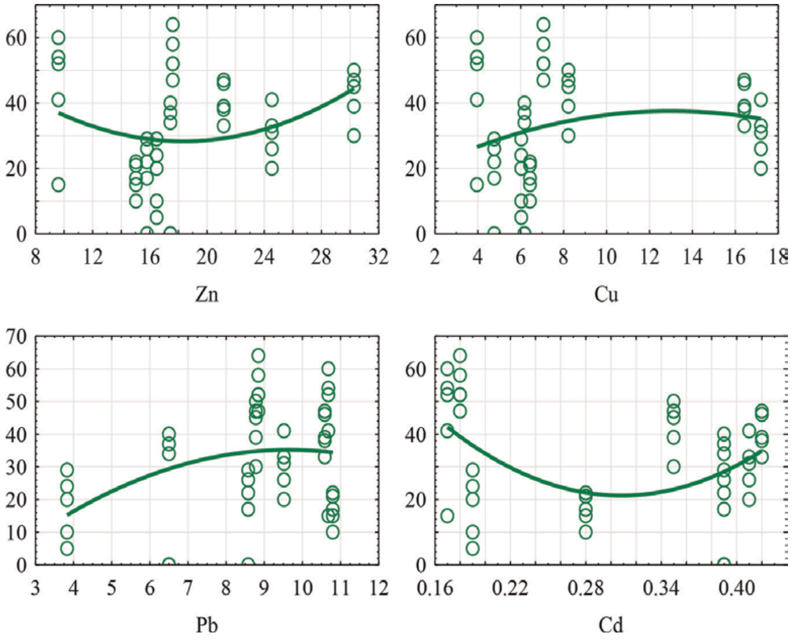


Figure 3.
Dependence of the number of mines formed by Parectopa robiniella *(y-axis) on heavy metal content (x-axis, in mcg/g).*

Effect	Sum of squares	Degrees of freedom	Mean sum of squares	*F*-ratio	*p*-level	Beta regression coefficients
Constant	2170.4	1	2170.4	14.75	<0.001	—
H	537.7	1	537.7	3.65	0.06	0.25 ± 0.13
Year	2170.7	1	2170.7	14.75	<0.001	0.42 ± 0.11
Zn	1288.0	1	1288.0	8.75	0.01	0.43 ± 0.15
Cu	0.2	1	0.2	0.00	0.97	−0.01 ± 0.17
Pb	1146.0	1	1146.0	7.79	0.01	0.34 ± 0.12
Cd	1606.1	1	1606.1	10.92	<0.001	−0.50 ± 0.15
Error	5591.4	38	147.1	—	—	—

Table 3.
Results of a general linear model of the effect of heavy metal content and terrain height on the number of mines formed by Parectopa robiniella *(R^2_{adj} = 0.48, F = 7.8, p < 0.001).*

Urbo	n	Characteristics	*x* ± *SD*	*CV*	*As*	*Ex*
Pridneprovsky Park	*16*	*Lb, mm*	3.48 ± 0.17	0.05	−0.20	0.12
		Hb, mm	0.79 ± 0.16	0.20	1.33	0.82
		Le, mm	2.12 ± 0.12	0.06	0.76	0.31
		Lb/Le	1.64 ± 0.11	0.07	0.18	−1.11
		Lb/Hb	4.55 ± 0.86	0.19	−1.09	0.05
		Le/Hb	2.76 ± 0.45	0.16	−0.97	0.02
Oles Honchar Dnipro National University Botanical Garden	*34*	*Lb, mm*	3.34 ± 0.28	0.08	−0.31	−1.15
		Hb, mm	0.82 ± 0.08	0.10	0.66	0.52
		Le, mm	1.88 ± 0.20	0.10	0.01	−1.03
		Lb/Le	1.79 ± 0.17	0.09	0.18	−0.43
		Lb/Hb	4.11 ± 0.42	0.10	0.08	−0.46
		Le/Hb	2.30 ± 0.20	0.09	−0.73	0.74
Druzhby Narodiv Forest Park	*18*	*Lb, mm*	3.30 ± 0.19	0.06	−0.91	−0.32
		Hb, mm	0.81 ± 0.09	0.11	0.79	0.73
		Le, mm	1.73 ± 0.15	0.09	0.25	−0.17
		Lb/Le	1.92 ± 0.13	0.07	−1.49	1.35
		Lb/Hb	4.10 ± 0.37	0.09	−1.37	1.75
		Le/Hb	2.14 ± 0.18	0.08	0.65	−1.08
Park 40th anniversary of the liberation of Dnipropetrovsk	*22*	*Lb, mm*	3.46 ± 0.16	0.05	−0.23	−0.80
		Hb, mm	0.76 ± 0.09	0.12	0.28	−0.17
		Le, mm	1.96 ± 0.12	0.06	0.62	0.07
		Lb/Le	1.76 ± 0.08	0.05	0.27	−0.76
		Lb/Hb	4.60 ± 0.51	0.11	0.38	−1.14
		Le/Hb	2.61 ± 0.28	0.11	0.47	−0.58

Urbo	n	Characteristics	*x* ± *SD*	*CV*	*As*	*Ex*
T. G. Shevchenko Park	*22*	*Lb, mm*	3.44 ± 0.20	0.06	−1.17	0.37
		Hb, mm	0.78 ± 0.05	0.07	0.65	−0.37
		Le, mm	1.87 ± 0.15	0.08	−0.57	−0.83
		Lb/Le	1.85 ± 0.10	0.05	0.53	0.45
		Lb/Hb	4.40 ± 0.33	0.07	0.51	−0.22
		Le/Hb	2.38 ± 0.17	0.07	0.11	−1.21
The Dnipro-Oril Nature Reserve	*28*	*Lb, mm*	2.79 ± 0.09	0.03	0.37	−0.95
		Hb, mm	0.67 ± 0.06	0.09	0.03	−0.88
		Le, mm	1.68 ± 0.16	0.10	2.19	4.82
		Lb/Le	1.67 ± 0.10	0.06	−2.32	5.39
		Lb/Hb	4.23 ± 0.36	0.08	0.40	−0.44
		Le/Hb	2.54 ± 0.27	0.11	0.51	−0.80

Note: Urbo—name of urbocenosis; n—number of pupae measured; characteristics—parameters and indices studied; x ± SD—mean ± standard deviation; CV—coefficient of variation; As—asymmetry coefficient; Ex—excess coefficient.

Table 4.
Morphometric variability of Macrosaccus robiniella *within population groups (n = 140).*

asymmetry in height of body (Hb, As = 1.33) and length of elytra (Le, As = 0.76), as well as among indices the length of body-to-height of body ratio (Lb/Hb, As = −1.09), and the length of elytra-to-height of body ratio (Le/Hb, As = −0.97). The excess values were reliable for height of body (Hb, Ex = 0.82) and the length of body-to-length of elytra ratio (Lb/Le, Ex = −1.11). Length of body (Lb) and length of elytra (Le) showed no significant asymmetry or excess. A significant coefficient of variation (CV) was also observed in height of body (Hb), the length of body-to-height of body ratio (Lb/Hb) and the length of elytra-to-height of body ratio (Le/Hb).

Among the pupae collected in Oles Honchar Dnipro National University Botanical Garden, a reliably significant asymmetry was observed only in height of body (Hb, As = 0.66), in the length of elytra-to-height of body ratio (Le/Hb, As = −0.73), while a significant excess was observed in the length of body (Lb, Ex = −1.15), the length of elytra (Le, Ex = −1.03), and the length of elytra-to-height of body ratio (Le/Hb, Ex = 0.74). The body-length-to-body-height ratio (Lb/Hb) and the body-length-to-wing-length ratio (Lb/Le) showed no significant asymmetry or excess. None of the characteristics studied showed a significant CV.

A significant asymmetry was observed among the pupae sampled in Druzhby Narodiv Forest Park: length of body (Lb, As = −0.91), height of body (Hb, As = 0.79), the length of body-to-elytra ratio (Lb/Le, As = −1.49), the length of body-to-height of body ratio (Lb/Hb, As = −1.37) and the length of elytra-to-height of body ratio (Le/Hb, As = 0.65).

A reliably positive excess was observed in height of body (Hb, Ex = 0.73), the length of body-to-length of elytra ratio (Lb/Le, Ex = 1.35) and the length of body-to-height of body ratio (Lb/Hb, Ex = 1.75); a negative excess was observed in the length of body-to-height of body ratio (Lb/Hb, Ex = −1.08). There was no significant asymmetry or excess in length of elytra (Le). The studied characteristics showed no significant indicators in CV.

In the pupae collected in Park 40th anniversary of the liberation of Dnipro, none of the studied characteristics showed a reliable asymmetry, except for length of elytra (Le, As = 0.62). However, a significant negative excess was observed for length of body (Lb, Ex = −0.80), the length of body-to-length of elytra ratio (Lb/Le, Ex = −0.76) and the body-length-to-height ratio (Lb/Hb, Ex = −1.14). Significant asymmetry and excess were not shown in height of body (Hb) and in the length of elytra-to-height of body ratio (Le/Hb). The coefficient of variation was significant in terms of height of body (Hb), the length of body-to-length of elytra ratio (Lb/Le) and the length of body-to-height of body ratio (Lb/Hb). Significant asymmetry among pupae collected in T. G. Shevchenko Park was found in the length of body (Lb, As = −1.17) and height of body (Hb, As = 0.65). Significant negative excess was observed in terms of the length of elytra (Le, Ex = −0.83) and in the length of elytra-to-height of body ratio (Le/Hb, Ex = −1.21). Significant asymmetry and excess were not shown in the length of body-to-height of body ratio (Lb/Hb).

Pupae collected in Dnipro-Oril Nature Reserve showed a significant positive asymmetry in length of elytra (Le, As = 2.19), as well as in the length of body-to-length of elytra ratio (Lb/Le, As = −2.32). The significant excess was seen in all the characteristics studied, except for the length of body-to-height of body ratio (Lb/Hb): by length of body (Lb, Ex = −0.95), height of body (Hb, Ex = −0.88) and length of elytra (Le, Ex = 4.82). Significant asymmetry and excess were not shown in the length of body-to-height of body ratio (Lb/Hb).

5.2 Characteristics of morphometric variability of population groups in different urbocenoses

Based on the results of a one-way analysis of variance based on linear characteristics, a significant difference ($P < 1*10^{-6}$) between datasets obtained in different urbocenoses. By length of body (Lb), intergroup sum of squares was greater than intragroup sum of squares. By other linear characteristics, intragroup sum of squares was greater than intergroup ones. Based on a one-way analysis of variance ANOVA of interpopulation variability of index characteristics, *Macrosaccus robiniella* (as in the case of linear characteristics) showed a significant ($P < 0.001$) difference between urbocenoses (**Table 5**) for all the indices studied. Intragroup sum of squares was greater than that of all the indices studied.

Morphological variability is one of the manifestations of adaptations that develop the accommodation of living organisms to environmental changes. Effects of factor influence can be accumulated in biological objects over a certain period of time. Morphological features of living organisms depend largely on their habitat. This is conditioned by the peculiarities of the feeding, the breeding season, adaptation to a particular environment, etc. Morphological variability is characterized by changes in weight and linear indicators; it is the result of the influence of environmental factors.

Morphological variability of the population is an instance of general genetic polymorphism and an indicator of potential population stability under conditions of high anthropogenic load on natural ecosystems. The study of morphological variability of invertebrates allows us to assess the capacity of a population to maintain its constancy, the possibility of changes within the same species and deviations from the average size, as well as to make an assessment of environmental quality.

According to the results of our study, significant differences in all linear characteristics and indices between groups of *Macrosaccus robiniella* pupae were found. Each group corresponded to the urbocenosis in which the leaves damaged by the leafminer

Characteristics	Urbo	n	x ± SD	SSi	SSb	F	P
Lb/Le	1	16	1.64 ± 0.11	2.064	1.0733	13.9359	$<1*10^{-6}$
	2	34	1.79 ± 0.17				
	3	18	1.92 ± 0.13				
	4	22	1.76 ± 0.08				
	5	22	1.85 ± 0.10				
	6	28	1.67 ± 0.10				
Lb/Hb	1	16	4.55 ± 0.86	30.7168	5.3343	4.6541	0.0006
	2	34	4.11 ± 0.42				
	3	18	4.10 ± 0.37				
	4	22	4.60 ± 0.51				
	5	22	4.40 ± 0.33				
	6	28	4.23 ± 0.36				
Le/Hb	1	16	2.76 ± 0.45	9.14	4.9003	14.3685	$<1*10^{-6}$
	2	34	2.30 ± 0.20				
	3	18	2.14 ± 0.18				
	4	22	2.61 ± 0.28				
	5	22	2.38 ± 0.17				
	6	28	2.54 ± 0.27				

Note: Urbo—number of urbocenosis; x ± SD, SSi—sum of squares within the populations studied; SSb—sum of squares between the populations studied; F—Fisher value; P—degree of confidence.

Table 5.
One-way analysis of variance ANOVA of morphological features variability of Macrosaccus robiniella *index characteristics (n = 140).*

were collected. However, on the constructed box graphs shown in **Figure 4**, it can be seen that the distribution and values of both linear characteristics and indices in the initial five urbocenoses were very different from the sixth urbocenose. The sixth urbocenose, Dnipro-Oril Nature Reserve, being located at a considerable distance from the city center and industrial areas, can act as a reference site aimed to comparison of its characteristics with urbocenoses located within the city. These differences may be caused by significantly different environmental conditions (**Figure 4a**). Pupae collected in the Dnipro-Oril Nature Reserve show less polymorphism compared to ones collected within the city in terms of length of body, height of body, length of elytra and the length of body-to-length of elytra ratio (**Figure 4a,c–e**).

Polymorphism within the individual population group in terms of two linear characteristics and three indices was greater than that between different population groups. From this, we can conclude that the diversity of pupae within a single population group or urbocenose was greater than the diversity of pupae collected in different urbocenoses. That is, environmental conditions within the city have little effect on the leafminer pupae diversity. However, morphometric characteristics can vary quite a lot in the pupae collected within the city compared to the pupae collected outside the city.

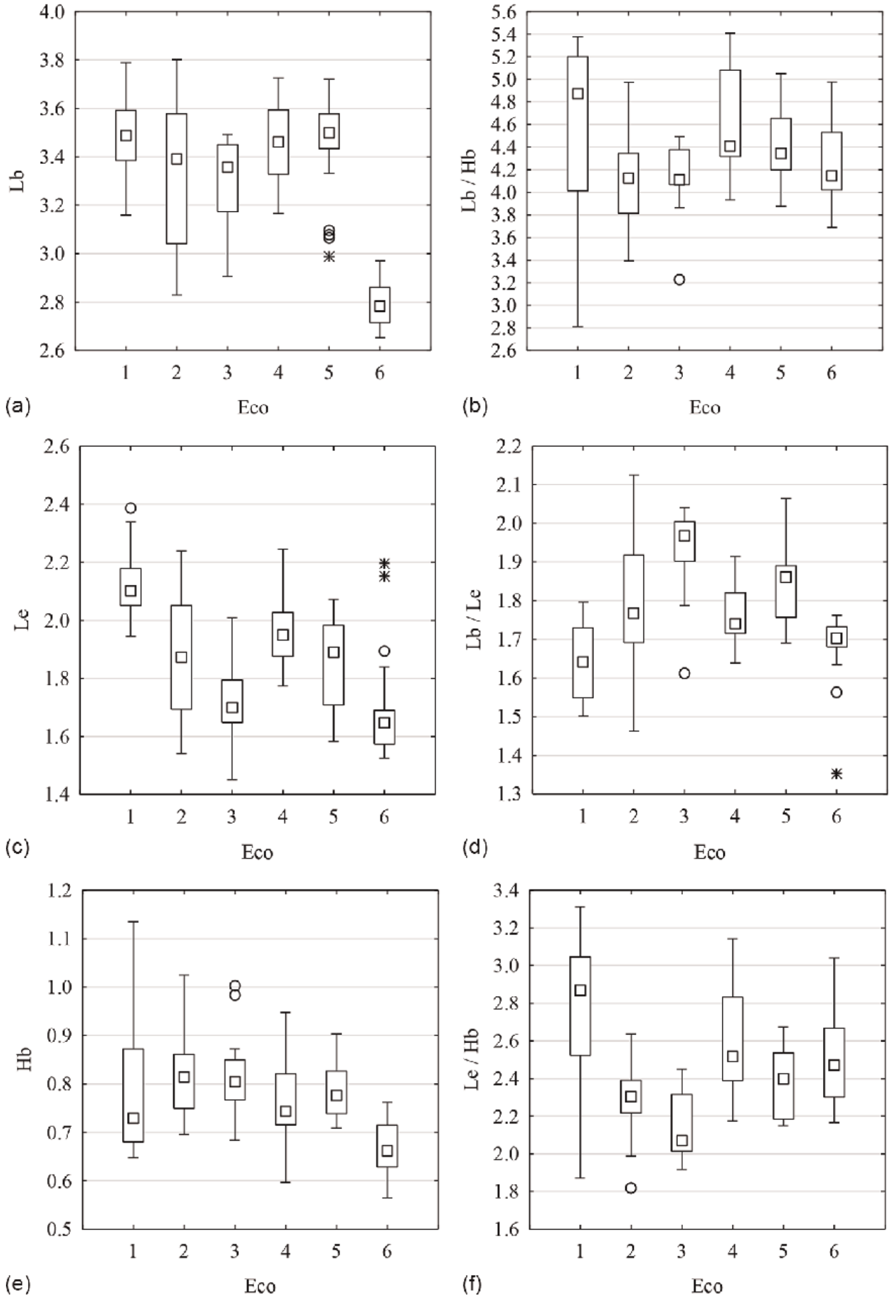


Figure 4.
Box plots of Macrosaccus robiniella *variability:* A*—length of body*, b*—the length of body-to-height of body ratio*, c*—length of elytra*, d*—the length of body-to-length of elytra ratio*, e*—height of body*, f*—the length of elytra-to-height of body ratio.*

Studies of morphometric characteristics of *Macrosaccus robiniella* pupae have shown that both linear characteristics and indices of pupae in the population were stable relative to the averages, since a significant coefficient of variation was observed only in pupae collected in Pridneprovsky Park. This may be conditioned by an insufficient dataset size, so a similar analysis of a larger dataset should be conducted in further studies. However, asymmetry and excess coefficients indicate that most of the studied population groups of *Macrosaccus robiniella* showed a tendency to deviate from the normal distribution. Moreover, in this case, the dataset obtained in the Dnipro-Orel Nature Reserve also differed greatly: the higher coefficient of excess for length of elytra and for the length of body-to-length of elytra ratio was found compared to the coefficients in other urbocenoses. The population group closest to this parameter was the group collected in Druzhby Narodiv Forest Park which also underwent less anthropogenic pressure compared to the city center. A high excess coefficient indicates a tendency to reduce the number of specimens with characteristics that differ significantly from the averages.

However, in other population groups, on the contrary, an increase in the number of individuals potentially differ greatly from the averages was observed. This may be due to the fact that *Macrosaccus robiniella* within the city limits was affected by more rigorous environmental conditions, so the specimen unable to be adapted was removed from the population. The asymmetry coefficient also indicates similar processes in populations: specimen living in the urban environment showed a tendency to a deviation of various indicators, while in the Dnipro-Orel Nature Reserve, the tendency to a deviation was manifested only in the length of elytra, but the other indicators studied were relatively stable. Such processes in populations indicated that *Macrosaccus robiniella* pupae were influenced by stressful factors to a certain extent, in particular, by anthropogenic ones.

6. Influence of feeding of invasive Gracillariidae caterpillars on biochemical processes in the leaves of host (food) plants

Studying of relationships between host plants and phytophage at the biochemical level is important for understanding of the adaptational pattern of black locust plants to the action of the leafminers *Parectopa robiniella* and *Macrosaccus robiniella*. During the staying in the host's body, complex physiological processes occur between the parasite and the plant resulted in the phytophage survival or death. When the pest insect survives, it affects the host body causing changes in metabolism that result in changes in the plant's functional state [9]. In response to plant damage by phytopathogens and pest insects, such protective reactions are initiated as increased barrier properties (lignification), gene expression, synthesis of secondary metabolites, and protective proteins [10]. The universal stress-protective systems of the plant body include an enzymatic antioxidant complex that provides neutralization of reactive oxygen species (ROS), the level of which increases under stressful effects [11–16]. Peroxidases are known to be very labile enzymes that respond to cellular homeostasis disruption under the action of stressors of various origins [17–20].

In order to find out the consequences of the impact of the leafminers *P. robiniella* and *M. robiniella* on the functional state of *R. pseudoacacia* trees, the activity and composition of the following antioxidant enzymes were determined: benzidine peroxidase (BPOD), guaiacol peroxidase (GPOD), as well as the content of highly soluble proteins. Highly soluble proteins are among the biochemical indicators that respond actively to cell damage by insects [21–24]. Our data show a slight fluctuation in the level of soluble proteins of black locust leaves between experimental samples; their values varied in the range of 1.99–2.26 mg/g (**Figure 5**).

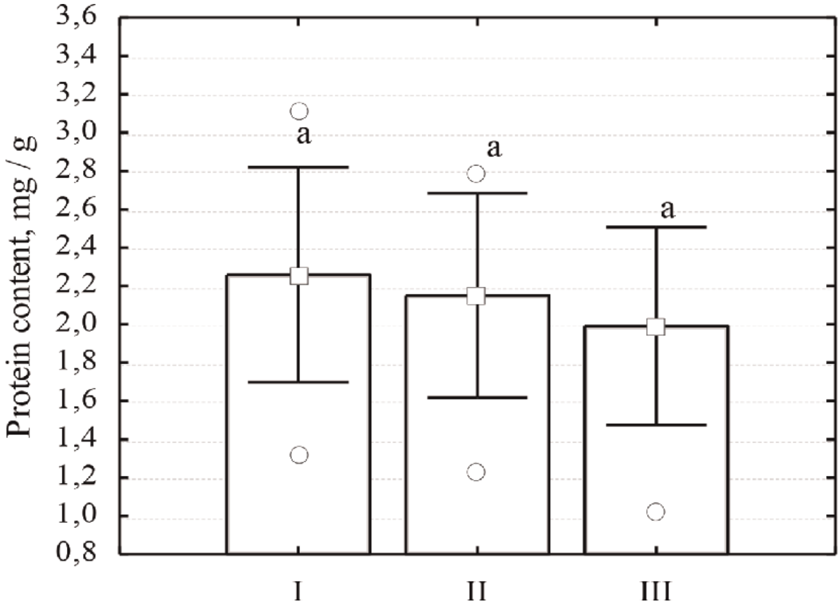


Figure 5.
Soluble protein content in leaves of R. pseudoacacia *under the action of* Parectopa robiniella *and* Macrosaccus robiniella*: I—Leaves without mines (mine-free leaves); II—Leaves with mines formed by* P. robiniella*; leaves with mines formed by* M. robiniella.

In the leaves with mines formed both by *P. robiniella* and *M robiniella,* there was a tendency to reduce the protein content under the influence of *M. robiniella* to a greater extent (the protein level decreased by 11.9%) compared to the action of *P. robiniella*. The difference between the reference and the experiment samples under the action of *P robiniella* was 4.9%. The average range of variability of this parameter was determined to be 21.5–27.1%.

Reducing the soluble protein content in the phase of active outflow of assimilates is a normal physiological process, but this process in a leaf damaged by a pest insect can be accelerated compared to reference.

The protective enzymatic antioxidant system in the cell comprises a series of enzyme systems including peroxidases (BPODs) actively involved in the mechanisms of plant adaptation to both abiotic and biotic influences [9, 17, 25]. Being regulated a number of processes directly or indirectly related to plant resistance to insect pests, peroxidases can be induced in a number of plants in response to insect attack, and they act as an important component of the direct plant response to damage [26, 27]. Our research has shown that the peroxidase system intended for detoxification of the effects of oxidative stress caused by the action of *P. robiniella* and *M. robiniella* is activated in the leaves of *R. pseudoacacia* trees (**Table 6**).

In 35–40-year-old trees with the presence of leaf blotch miners *P. robiniella*, the leaves showed a tendency to increase BPOD activity by 24.6% compared to reference (F = 2.80; p = 0.10). A significant variability (55.3%) of this indicator was found. A significant increase in benzidine peroxidase activity was noted under the action of leaf blotch miner *M. robiniella* at the level of 60.2% (F = 24.89; P = $9.11 \cdot 10^{-6}$) compared to reference and at the level of 28.6% compared to the action of *P. robiniella* (F = 4.46; p = 0.04).

Variants of invasion of leaves	n	BPOD, U/g FW min (middle-aged trees)	CV, %	n	BPOD, U/g FW min (5-year-old tree)	CV, %	n	GPOD, mm TG guaiacol/g FW min	CV, %
Mine-free leaves	24	592.13 ± 140.77^a	23.8	4	728.90 ± 3.57^a	0.49	30	6.09 ± 2.87^a	47.1
P. robiniella	34	737.59 ± 408.25^a	55.3	4	2784.81 ± 20.68^b	0.74	26	9.94 ± 5.10^b	51.3
M. robiniella	24	948.42 ± 320.27^b	33.8	—	—	—	12	15.97 ± 5.92^c	37.1

Notes: values in column marked with different letters (a, b, c) were significantly different according to Tukey's t-test $P < 0.05$. BPOD—benzidine peroxidase; GPOD—guaiacol peroxidase; TG—tetraguaiacol; U—units; FW—fresh weight.

Table 6.
Total activity of antioxidant enzymes (BPOD, GPOD) in Robinia pseudoacacia *leaves under the action of* Parectopa robiniella *and* Macrosaccus robiniella *(mean ± SD).*

Five-year-old trees showed the highest response to the damages caused by *P. robiniella*: in leaves damaged by the pest, activity of the enzyme increased by 3.8 times compared to reference ($F = 3{,}8 \cdot 10^{-5}$; $P = 1.19 \cdot 10^{-12}$). A significantly increased GPOD response to leaf damage by the invasive insects was shown in the presence of both *P. robiniella* and *M. robiniella*. In the presence of *P. robiniella*, the activity value increased by 63.2% ($F = 9.51$; $P = 3.4 \cdot 10^{-3}$), and under the action of *M. robiniella* it increased by 2.6 times ($F = 48.12$; $p = 3.44 \cdot 10^{-3}$). A significant difference in enzyme activity was also recorded between *Parectopa robiniella* and *Macrosaccus robiniella*. GPOD activity of the latter species was higher by 60.7% ($F = 8.77$; $P = 5.7 \cdot 10^{-3}$).

Peroxidases are often present in multiple isoforms, which is consistent with different physiological functions of the peroxidase family [28, 29]. Acidic and basic isoperoxidases are associated with the mechanisms of stress in plants. Most of the scientific works were devoted to study the effect of phytopathogens on activity and composition of the peroxidase system [30]. In the scientific paper was shown that activation of the basic peroxidases occurs at the beginning of infection, and alterations associated with auxin and ethylene metabolism induce intensified synthesis of acidic isoenzymes as a later stage of plant response or defense [26].

It was found that active rearrangements of the peroxidase system in the leaves occur when black locusts are affected by the leaf-mining insects. The stressful situation caused by pest attacks leads to changes in the expression of isoperoxidases in black locust leaves in the ecologically favorable area of Oles Honchar Dnipro National University Botanical Garden, where six isoforms were clearly visualized (**Figure 6**; **Tables 7** and **8**).

Benzidine peroxidase isolated from the leaf tissue of medium-aged black locust trees was divided into six fractions with Rf values from 0.08 to 0.76 (**Figure 5a** and **Table 7**). The relative content of all six isoperoxidases in leaves damaged by the pests differs from the reference samples. In trees of black locust, the proportion of 0.51 and 0.71 isoforms in the samples studied was significantly higher compared to the reference trees by 6.0 and 13.1% (*Parectopa robiniella*) and 14.4 and 34.8% (*Macrosaccus robiniella*), respectively. In the leaves of young *R. pseudoacacia* trees sampled in the Botanical Garden (in contrast to 30–40-year-olds), 10 peroxidase isoforms were isolated (**Figure 5**), and the relative content of components with R_f 0.48, 0.54, 0.71 and 0.75 was increased by 26.1, 16.5, 37.3 and 22.3%, respectively (**Table 8**).

In the leaves of *R. pseudoacacia* trees sampled in T. G. Shevchenko Park, more significant changes in the spectrum of BPOD isoforms were observed (**Figure 7**).

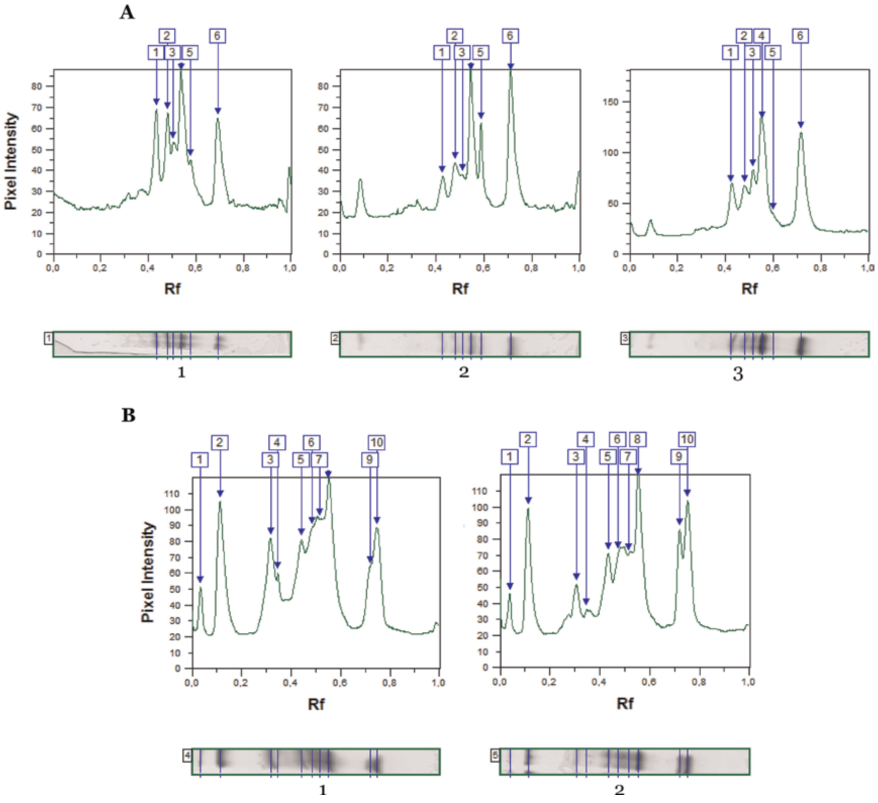


Figure 6.
IEF benzidine peroxidase profiles of leaves of 30- to 40-year-old (A) and 5-year-old (B) trees of black locust sampled in the botanical garden under the influence of invasive insects; 1—Mine-free leaves; 2—Leaves with mines formed by P. robiniella*; 3—Leaves with mines formed by* M. robiniella*; Rf—Electrophoretic mobility.*

Variants of invasion of leaves	**R_f value of isoperoxidase**					
	0.08	**0.43**	**0.48**	**0.51**	**0.54**	**0.71**
Mine-free leaves	17.89 ± 0.39^{a}	16.10 ± 0.46^{a}	10.39 ± 0.48^{a}	24.24 ± 0.66^{a}	9.74 ± 1.99^{a}	21.64 ± 0.99^{a}
P. robiniella	12.19 ± 0.57^{b}	13.63 ± 1.03^{b}	5.32 ± 0.25^{b}	25.70 ± 0.24^{b}	14.35 ± 0.56^{b}	28.81 ± 2.33^{b}
M. robiniella	13.49 ± 0.04^{c}	12.58 ± 1.15^{b}	10.82 ± 0.06^{a}	27.74 ± 0.16^{c}	6.22 ± 0.64^{c}	29.17 ± 1.14^{b}

Table 7.
The relative content of BPOD isoforms in the leaves of middle-aged Robinia pseudoacacia *trees under the action of* Parectopa robiniella *and* Macrosaccus robiniella *(n = 3; ± SD).*

Under the action of *P. robiniella*, two isoforms of peroxidase were isolated with Rf 0.09 and 0.29; these isoforms were absent in the reference samples and in the samples affected by *M. robiniella* (**Table 9**).

Under the action of *M. robiniella*, the number of components decreased by one isoform (with Rf 0.51) compared to mine-free leaves. Common areas with reference have values of Rf 0.43, 0.48, 0.54, 0.56 and 0.75. The latter isoform reduces activity under the action of the pest insects: by 27.4% (*P. robiniella*) and by 39.8%

Variants of invasion of leaves	**R_f value of isoperoxidase**									
	0.03	**0.08**	**0.30**	**0.34**	**0.43**	**0.48**	**0.51**	**0.54**	**0.71**	**0.75**
Mine-free leaves	4.13 ± 0.11^{a}	15.23 ± 0.36^{a}	12.64 ± 0.32^{a}	3.59 ± 0.09^{a}	13.03 ± 0.80^{a}	8.25 ± 0.21^{a}	9.68 ± 0.15^{a}	15.66 ± 0.52^{a}	6.01 ± 0.25^{a}	11.77 ± 0.30^{a}
P. robiniella	4.18 ± 0.08^{a}	13.43 ± 0.26^{a}	9.06 ± 0.18^{b}	3.21 ± 0.25^{a}	11.28 ± 0.72^{a}	10.40 ± 0.20^{b}	7.56 ± 0.77^{a}	18.24 ± 0.74^{b}	8.25 ± 0.08^{b}	14.39 ± 0.38^{b}

Table 8.
The relative content of BPOD isoforms in the leaves of 5-year-old Robinia pseudoacacia *trees which grow in Oles Honchar Dnipro National University Botanical Garden, under the action of* P. robiniella *and* M. robiniella *(n = 3; ± SD).*

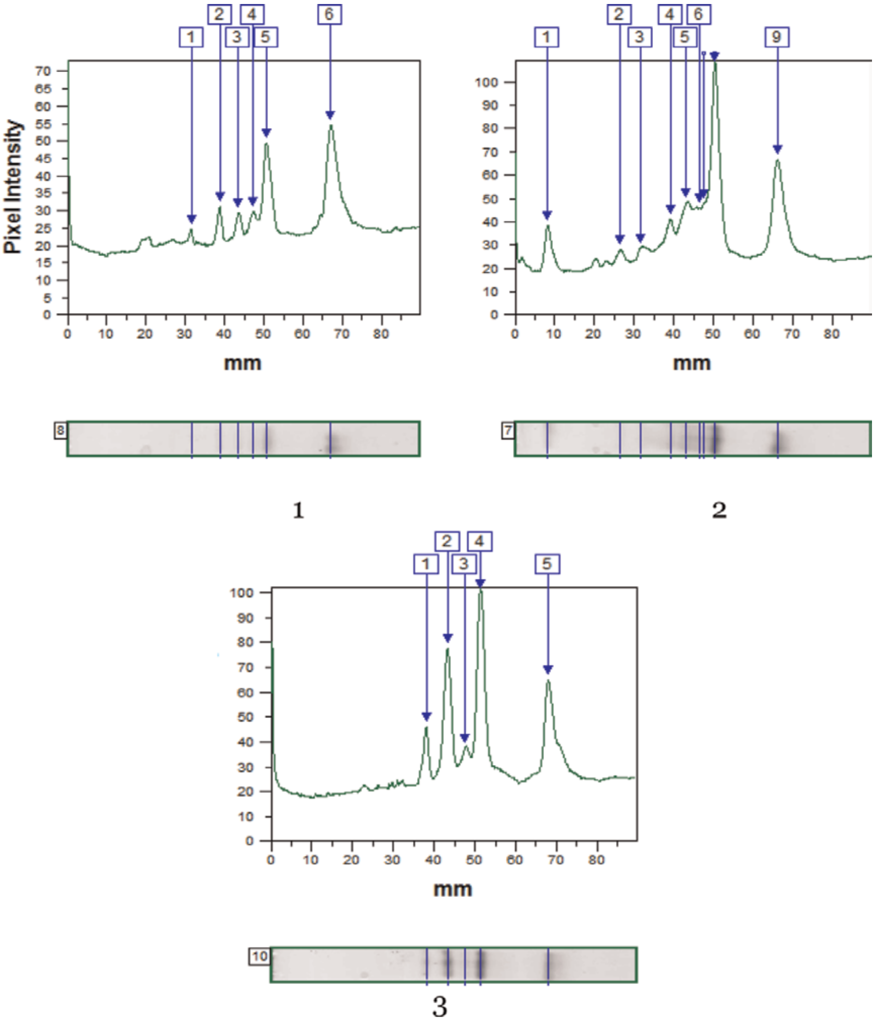


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Figure 7.
Alterations of IEF peroxidase profiles in leaves of R. pseudoacacia *trees sampled in T. G. Shevchenko Park under the action of* P. robiniella *and* M. robiniella.

(*M. robiniella*). The activity of the component with Rf 0.48 significantly increases (by 21.5 and 92.5% according to the variants affected by *P. robiniella* and *M. robiniella*). A decrease in the relative proportions under the action of the insects was noted for isoperoxidase with Rf 0.54 (by 49.4 and 20.0%, respectively, according to the variants affected by *P. robiniella* and *M. robiniella*). Study of peroxidase in leaves of black locust trees undamaged by pests sampled in Pridneprovsky Park showed only three isoforms in their composition. The main activity occurred in the component with Rf 0.75 (**Figure 8** and **Table 10**).

The leaves damaged by *P. robiniella* and *M. robiniella* showed seven active zones. Molecular forms with Rf 0.08, 0.54, and 0.75 were found to be isoperoxidases common with the reference. Compared to mine-free leaves, leaves damaged by *P. robiniella* and *M. robiniella* components with Rf 0.08 and 0.75 showed a decrease in enzyme activity by 15.9 and 5.2%, and with Rf 0.75—by 50.2 and 47.7%, respectively.

Variants of invasion of leaves	**Rf value of isoperoxidase**								
	0.08	**0.30**	**0.37**	**0.43**	**0.48**	**0.51**	**0.54**	**0.56**	**0.75**
Mine-free leaves	—	—	3.83 ± 2.52^{a}	10.08 ± 0.72^{a}	8.87 ± 3.81^{a}	—	8.36 ± 3.53^{a}	23.40 ± 10.8^{a}	34.64 ± 15.1^{a}
P. robiniella	9.14 ± 0.55	4.90 ± 0.86	6.41 ± 0.84^{b}	8.61 ± 0.35^{b}	10.78 ± 0.71^{b}	5.90 ± 0.81	4.23 ± 0.27^{b}	26.57 ± 1.05^{a}	25.15 ± 1.88^{b}
M. robiniella	—	—	—	10.62 ± 0.11^{a}	17.31 ± 11.2^{c}	—	6.69 ± 4.11^{c}	22.74 ± 14.8^{a}	20.83 ± 13.4^{c}

Table 9.
The relative content of BPOD isoforms in leaves of different-aged Robinia pseudoacacia *trees sampled in T. G. Shevchenko Park under the action of* P. robiniella *and* M. robiniella *(n = 3; ± SD).*

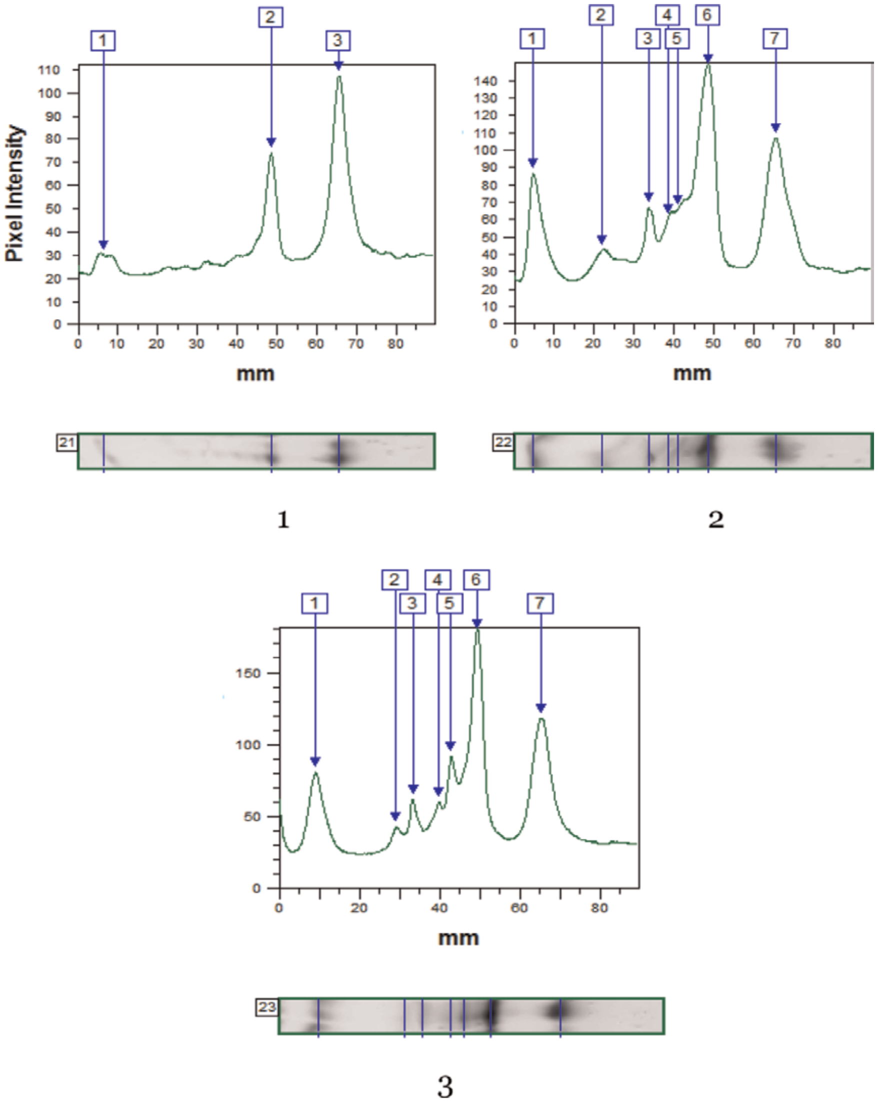

Figure 8.
Alterations of IEF peroxidase profiles in leaves of R. pseudoacacia *trees sampled in Pridneprovsky Park under the action of* P. robiniella *and* M. robiniella *(n = 3; ± SD).*

Therefore, the increase in BPOD and GPOD activity established by us under the action of leaf blotch miners on the leaves of black locusts indicates the involvement of peroxidases in mechanisms of plant defense against the attacks of phytophagous insect, which was also noted in a number of other studies [2, 22, 31–33]. The role of antioxidants consists both in neutralizing reactive oxygen species with the involvement of hydrogen peroxide (benzidine peroxidase) and in protecting cell walls by their lignification to prevent pest invasion (guaiacol peroxidase). Summarizing the data on the electrophoretic profiles of benzidine peroxidase in black locust leaves, it should be noted that peroxidase in the leaves of the trees studied has a fairly wide range of isoforms (a total of 13 molecular forms were recorded) in the acidic pH range

Variants of invasion of leaves	**Rf value of isoperoxidase**							
	0.08	**0.25**	**0.34**	**0.37**	**0.43**	**0.48**	**0.54**	**0.75**
Mine-free leaves	17.25 ± 0.41^a	—	—	—	—	—	28.46 ± 0.63^a	54.29 ± 1.05^a
P. robiniella	14.54 ± 0.50^b	7.33 ± 0.59	—	8.81 ± 0.31	3.98 ± 0.61	8.94 ± 0.74	29.47 ± 0.55^a	26.92 ± 0.94^b
M. robiniella	16.35 ± 0.64^a	—	4.72 ± 0.18	6.49 ± 0.26	5.36 ± 0.84	9.53 ± 0.77	29.13 ± 1.12^a	28.40 ± 0.52^b

Table 10.
The relative content of BPOD isoforms in leaves of different-aged Robinia pseudoacacia *trees sampled in Pridneprovsky Park under the action of* P. robiniella *and* M. robiniella *(n = 3; ± SD).*

(3.5–6.5), and changes in the level of their expressiveness and the number of components in the system may indicate their involvement in the processes of cellular defense against the complex action of stressors of both biotic and abiotic origins. We have shown that molecular forms of peroxidase located in the most acidic pH range (Rf values from 0.51 to 0.75) were subjected to the main load in the peroxidase system under the influence of stress caused by mechanical damage of leaf tissue. Thus, in middle-aged trees, the main BPOD activity was inherent in isoforms 0.51 and 0.71; their proportion in the total spectrum was 45.9% (reference leaves), 51.5% (leaves with mines formed by *P. robiniella*) and 56.9% (leaves with mines formed by *M. robiniella*). In young trees, the relative content of the components was distributed more evenly, indicating a fairly high level of complexity of the peroxidase system in young trees. In the leaves of trees sampled in T. G. Shevchenko Park, high activity was focused in areas with Rf 0.56 and 0.75. The highest activity was registered in the reference leaves. This amounted to 58.0% and was slightly lower in the damaged leaves: 51.7 (in the leaves with mines formed by *P. robiniella*) and 43.6% (in the leaves with mines formed by *M. robiniella*).

In Pridneprovsky Park, the main activity was also observed in two isoperoxidases with Rf 0.54 and 0.75, which accounted for more than 50% of all molecular forms of peroxidase. A number of studies have noted that overexpression of anionic peroxidase contributes to plant resistance to the action of lepidopteran and coleopteran insect pests [34, 35]; the peroxidase exhibits the capacity to detoxify ROS and is involved in lignin and suberin synthesis; cationic peroxidases can participate in the production of hydrogen peroxide and have NADPH oxidase activity.

The data obtained allow us to conclude that *R. pseudoacacia* is a tree species highly sensitive to the invasion by the larvae of leaf blotch miners that feed on its leaves. It is also necessary to consider the anthropogenic pressure exerted by the environment on both *Robinia pseudoacacia* and leafminers. So T. G. Shevchenko Park and Pridneprovsky Park are located in the zone of intensive sedimentation of harmful impurities emitted by a thermal power plant, a pipe rolling plant and motor vehicles. Therefore, we observed more significant changes in IEF peroxidase profiles of black locust trees growing in these areas: Depletion of enzyme spectra or almost complete decrease in the intensity of certain components of the system was recorded in addition to alterations in the expressiveness of BPOD isoforms.

7. Effect of feeding of invasive Gracillariidae caterpillars on parameters of chlorophyll fluorescence induction curves in host plants

Using a portable fluorometer “Floratest” on fresh *A. hippocastanum* leaves both damaged and undamaged by *Cameraria ohridella*, and we obtained data that clearly demonstrate the effect of the leafminer’s caterpillars feeding on chlorophyll fluorescence intensity (the Kautsky curve) (**Figure 9**).

Under normal conditions, the fluorescence level was insignificant (1–2% of the total absorbed light), which indicates the active use of absorbed light energy by cells. After lightening the leaves of plants adapted to the dark, a sharp increase in the intensity of chlorophyll fluorescence as a fast phase was observed in the first few seconds. Then, a gradual decrease occurred within a few minutes through certain stages, from the slow phase to the stationary level of F_{St} [36].

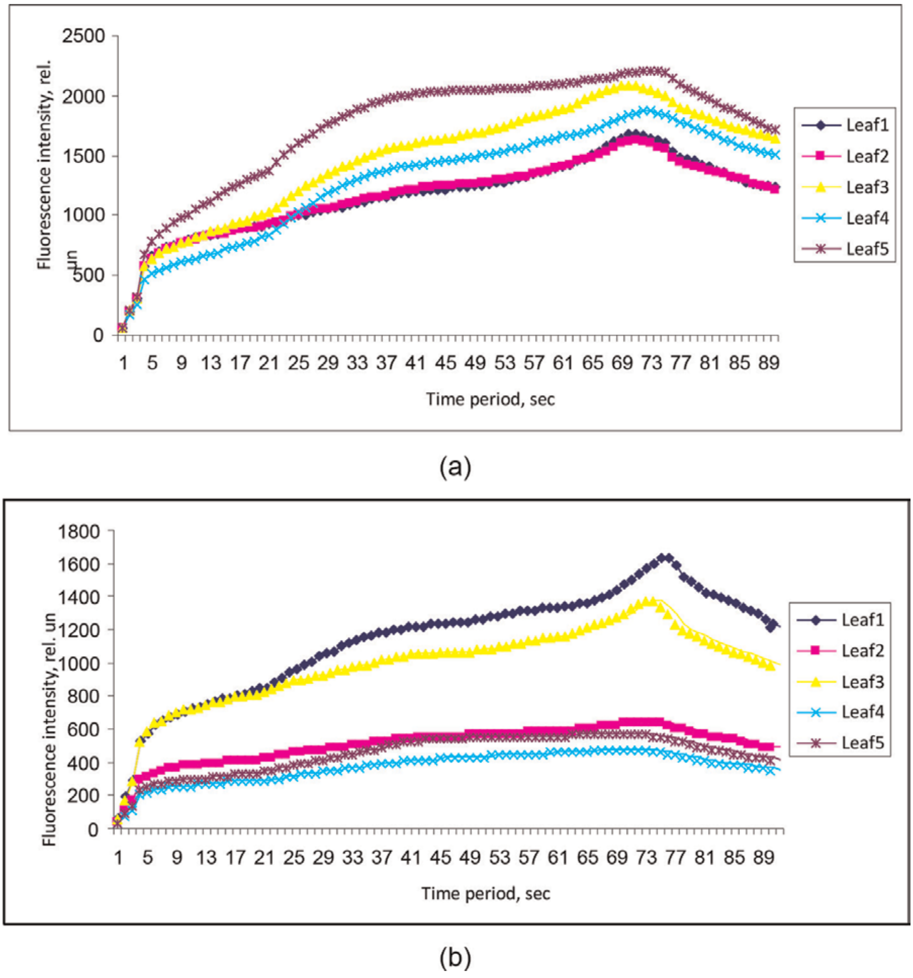

Figure 9.
Chlorophyll fluorescence induction curves (the Kautsky curves): A—Leaves undamaged by the insect (n = 5); b—Leaves damaged by the insect (n = 5).

At the initial time point, all channels of photosynthetic electron transfer are open, and the maximum energy of electron excitation goes into the photosynthetic process. During this period, chlorophyll fluorescence is minimal, and its intensity on the Kautsky curve is denoted by the letter F with the “0” index, i.e., F_0. Background fluorescence index (F_0) characterizes the amount of inactive chlorophyll that has no functional connections with the reaction centers; that is, it acts as the initial level of ChlF. It depends on the loss of excitation energy during migration along with the pigment matrix. F_0–F_p transition is associated with a decrease in electron transport. It characterizes the thermal adaptation period of the leaf. The transition is steeper when the adaptation period is short, but the transition is slower when the adaptation period is large [5]. F_m parameter indicates the highest level of fluorescence, which is recorded as the maximum on the induction curve. The entire F_0–F_m area is called the fast phase of fluorescence. The slow phase of chlorophyll fluorescence induction represents all transitions of the induction after reaching the maximum value (peak). The steady-state fluorescence level (F_{St}) is characterized by a dynamic equilibrium between the processes that cause an increase in fluorescence and the processes that lead to its decrease. During the caterpillar development (5 generations of *Cameraria ohridella* caterpillars), a gradual decrease in the values of all the main indicators (F_0, F_m, F_p and F_{St}) occur in the chlorophyll fluorescence induction curve (the Kautsky curve) (**Figure 10**) of damaged *A. hippocastanum* leaves.

A significant difference in the influence of different-aged caterpillars and leaf position in tree crowns was established for all the main indicators of the chlorophyll fluorescence induction curve. The only exception is the initial value of fluorescence induction (F_0) for which no such dependence has been established.

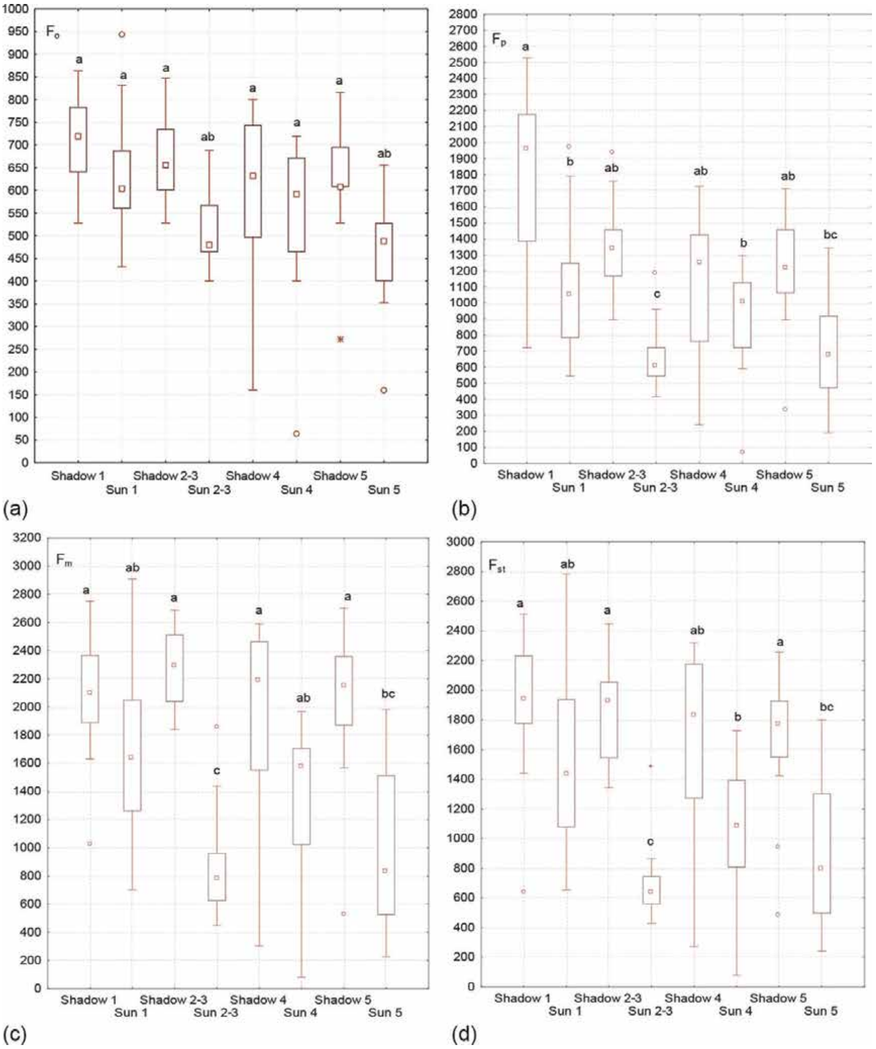

Figure 10.

Variability of indicators of the chlorophyll fluorescence induction curve (the Kautsky curve) in Aesculus hippocastanum *leaves damaged by* Cameraria ohridella*:* F_o*—The initial value of fluorescence induction after switching on the light;* F_p*—A "plateau" value of fluorescence induction;* F_m*—A maximum value of fluorescence induction;* F_{St}*—A steady-state value of fluorescence induction after light adaptation of the plant leaf.*

8. Conclusions

Long-term studies of invasive Gracillariidae as components of artificial and natural ecosystems in Ukraine allowed us to find out the scale of their invasion, mechanisms of invasion into various ecosystems, adaptive capabilities in a novel environment, features of trophic relationships and influence on the physiological and biochemical state of host plants which can be confirmed by the following conclusions:

1. According to the statistical data processing, the largest number of mines formed by invasive Gracillariidae on the leaves of host plants was recorded in ecosystems environmentally beneficial for these host plants, where the plant age and the position of tree plantation above sea level acted as key factors; for urban plantings, key factor was the content of heavy metals (Zn, Cu, Pb and Cd) in leaf tissues.

2. Statistical data obtained during the study of mines showed a high variability (40.1–100%) of morphometric indicators in most cases. According to the results of the study, both coefficient of variation and standard deviation indicated that the morphometric plasticity of mines formed by *Parectopa robiniella* showed a more variable polymorphism than that of mines formed by *Macrosaccus robiniella*.

3. Polymorphism of *Parectopa robiniella* pupae collected in green areas of the city was larger compared to the pupae collected in the Nature Reserve (reference) ecosystem. For all linear characteristics and indices, there were significant intergroup differences of *Parectopa robiniella* pupae collected in different urbocenoses by the level of anthropogenic load.

4. Polymorphism of two linear characteristics and three indexes of six studied characteristics within a single population group were greater than polymorphism established for different population groups. From this, it can be concluded that the morphological diversity of pupae within a single group or urbocenose was greater than that in different urbocenoses. That is, environmental conditions within the city have a slight effect on the variety of morphometric parameters in the pupae. However, morphometric characteristics can vary quite a lot in the pupae collected within the city compared to the pupae collected outside the city.

5. In the leaves of the host plants, an increase in the activity of both benzidine and guaiacol peroxidase was found under the conditions of invasive insect caterpillars feeding on the host plant. The enzyme activity level depended on the age of the trees and the environmental conditions in which the trees grow. In 30–40-year-old trees, BPOD activity increased by 24.6% under the influence of caterpillars on the leaves compared to the reference. A significant increase in benzidine peroxidase activity was detected under the action of *Macrosaccus robiniella* (by 60.2%) compared to the reference, and by 28.6% compared to the action of *Parectopa robiniella*. A 5-year-old tree showed the highest response to the action of *Parectopa robiniella*: the enzyme activity increased by 3.8 times in leaves damaged by the pest insect compared to the reference.

6. Active rearrangements of the peroxidase system in the leaves of host plants indicate the involvement of benzidine peroxidase and guaiacol peroxidase in plant defense mechanisms against the action of Gracillariidae caterpillars. Benzidine peroxidase is probably involved in the neutralization of reactive oxygen species with the participation of hydrogen peroxide, and guaiacol peroxidase is involved in protecting of cell walls by lignification and suberization to prevent pest invasion.

7. Caterpillar feeding affects the functional state of the host plant which was confirmed by changes in the soluble protein content. The studies have shown a decrease in the content of highly soluble proteins in leaves damaged by invasive insects. The protein content was significantly reduced in leaves affected by the invasive insects, as the plant reduced the rate of synthesis of highly soluble protein under biotic stress, and the entire translation mechanism was shifted to the production of proteins associated with the plant defense response.

8. It was established that the caterpillars of Gracillariidae invasive species affect significantly the photosynthetic apparatus of host plants. This effect was clearly determined with fluorescence analysis. Analysis of the Kautsky curve characteristics of both damaged and undamaged leaves showed that caterpillar feeding affected significantly four critical parameters.

9. Feeding of *Cameraria ohridella* caterpillars causes a decrease in the activity of the photosynthetic apparatus of *A. hippocastanum* regardless of the spatial arrangement of leaves in the tree crown. In damaged leaves, the amount of chlorophyll not involved in photosynthetic energy transfer to reaction centers increases. Conversely, a constant decrease in the quantum efficiency of PS II (inhibition of photosynthetic activity) was observed during the development of only a single *Cameraria ohridella* generation.

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Chapter 7

Population Genetic Studies of Silkworm (*Bombyx mori* L.) and Phylogenetic Relationships

Teodora Atanasova Staykova and Panomir Ivanov Tzenov

Abstract

The study aimed to evaluate the degree of genetic variability and phylogenetic relationships between 13 breeds of mulberry silkworm (*Bombyx mori* L.) from genetic resources of Bulgaria through isozyme polymorphism. PAGE was used. Among nine studied isoenzyme loci, by eight loci (Bes A, Bes B, Bes D, Bes E, Pgm A, Mdh A, Bph, and Alp A) we found intra-breed and inter-breed polymorphism. At the Hk locus, we found inter-breed polymorphism only. The number of alleles per polymorphic locus ranged from one to two. The degree of polymorphism ranged from 0% to 77.80%. Low levels of observed heterozygosity in comparison with the expected one have been calculated in all of breeds. The combined F_{IS} value over all polymorphic loci was 0.3205, which reflects a substantial deficit of heterozygotes. The value of F_{ST} showed that 49.21% of the overall genetic diversity observed was among breeds. The dendrogram constructed manifested that the two breeds of Japanese origin (Daizo and Japanese 106) were genetically most distant from other breeds. The data for isoenzyme polymorphism and genetic structure of the tested breeds can be used for genetic improvement and to develop new hybrids for silk production.

Keywords: silkworm *Bombyx mori* L., isoenzymes, polymorphism, population genetic parameters, phylogenetic relationships

1. Introduction

Until the end of the 80s of the last century, Bulgaria was one of the best producers of cocoons in Europe. For various reasons, mostly economic, this industry is now in decline. The favorable climate and the existing rich national traditions are prerequisites for its restoration and further development of sericulture in Bulgaria, because the demand for silk and its place in everyday life will continue to be highly valued due to its hygienic qualities and finesse. Bulgaria maintains a rich genetic resources of more than 250 breeds of different origins, which is also a prerequisite for the recovering of sericulture.

The selection process of the mulberry silkworm is related to the solution of some basic issues as selection of individuals with the highest productivity, reproductive ability, viability and resistance to diseases, as well as analyzing and evaluating the capabilities of the breed gene pool. The creation of new highly productive breeds requires evaluation of the promising features for selection, creation of lines with desired qualities and analysis of their gene pool, development of methods for creation of synthetic lines, and evaluation of their genotypic and phenotypic features.

A basic principle for improving breeds is the presence of genetic diversity. Genetic variability can be analyzed by different methods and markers. The method of electrophoresis provides an opportunity to analyze the genetic heterogeneity in populations by studying genetically determined protein polymorphism [1–6]. The established variability in isozyme markers can be used to characterize the genetic heterogeneity and degree of polymorphism of breeds to study the intensity of gene flow and the origin of individual breeds [7]. Application of isoenzymes and other molecular markers helps to estimate genetic diversity much more accurately than that of morphological traits [8]. Isoenzyme analysis is useful for the study of intra- and inter-breed polymorphism of mulberry silkworm and determining the level of genetic variability and genetic relationships [4, 6, 9–11]. Isoenzymes like esterase, acid phosphatase (ACP), alkaline phosphatase (ALP), malate dehydrogenase (MDH), and phosphoglucomutase have been used by various researchers to study diversity in silkworm genotypes [8, 12].

Studies for detection of polymorphic enzymatic and nonenzymatic protein systems in the breeds of *B. mori* kept in Bulgaria were made [4, 6, 10, 11, 13–15]. Data on the level of polymorphism and heterozygosity in more than 50 breeds with different origin have been established. The level of polymorphism varied between 0 and 77.8%, and the degree of heterozygosity varied between 0 and 0.280 in different breeds. Phylogenetic relationships between breeds have been identified on the basis of the established genetic distance and similarity. All these data are important for the maintenance of biodiversity in the silkworm, as well as for the selection of this species. However, there is still no information about other breeds, as the genetic resources of the silkworm maintained in Bulgaria are rich and include a large number of breeds. All this motivates the present investigation which aim was to study the degree of genetic variability and phylogenetic relationships between 13 breeds of silkworms, introduced and created in Bulgaria, on the basis of isozyme polymorphism.

2. Isoenzyme polymorphism and population genetic characteristics of silkworm breeds from the genetic resources of Bulgaria and their phylogenetic relationships

2.1 Material and methods

The silkworm resources used in the present investigation include a total of 13 breeds with different geographical origin and phenotype characteristic. They were obtained from the Scientific Center of Sericulture in Vratsa at the Agricultural Academy in Bulgaria. Breed Vratza 16 was created in Bulgaria. All others have been introduced as follow: breeds AES-1 wh and AES-1 zb originated from Spain, Tg—from Italia, Japanese 106 and Daizo originated from Japan, Mir 5—from Egypt, Mziuri 1—from Georgia, Tahvon 106—from Notrh Korea, Ukrainian 19—from Ukraine, Sh 4—from China, Line 22—from Uzbekistan, and MNB—from Madagascar. Daizo is polyvoltine, while all other breeds are mono-bivoltine. Breeds Tg, Daizo, AES-1 wh, and AES-1 zb have color cocoons. All other breeds have white cocoons. All individuals were nourished at a standard regime of silkworm breeding.

Totally 493 larvae on the fifth day of the fifth instar were studied. Larvae were selected randomly from each breed and were submitted to electrophoretic analysis of hemolymph, silk glands, and midgut tissues.

The tissue extracts were prepared according to the procedure described earlier [11, 14, 16, 17]. The individual samples were studied by 7.5% polyacrilamide gel electrophoresis (PAGE) [18] for nonspecific esterases (EST, EC 3.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), and acid phosphatase (ACP, EC 3.1.3.2)—from the hemolymph; hexokinase (HK, EC 2.7.1.1)—from the silk glands and alkaline phosphatase (ALP, 3.1.3.1)—from the midgut. The 6% PAGE was used to analyze phosphoglucomutase (PGM, EC 5.4.2.2) from the silk glands. The staining mixtures for the enzymatic activities tested were pointed previously [19].

The phenotypes of the discovered loci were recorded after the revelation of the isozyme activity regions. Allele frequencies, mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected (H_e) heterozygosity, deviation from the Hardy-Weinberg equilibrium, Nei's genetic distance (D) [20], and Wright's fixation index, F_{ST} [21] were calculated using BIOSYS-1 [22]. Dendrogram was constructed using Nei's [20] genetic distance, by UPGMA [23] method using the PHYLIP [24] software package.

2.2 Results

Isoenzyme and allozyme polymorphism of nonspecific esterases and allozyme polymorphism of phosphoglucomutase, malate dehydrogenase, acid phosphatase, alkaline phosphatase, and hexokinase were detected by polyacrylamide gel electrophoresis (**Figure 1**).

The tested enzymes recorded a total of nine polymorphic loci with 26 alleles (**Table 1**). Breed specificity of gene pools with respect to allele content and allele frequencies was established.

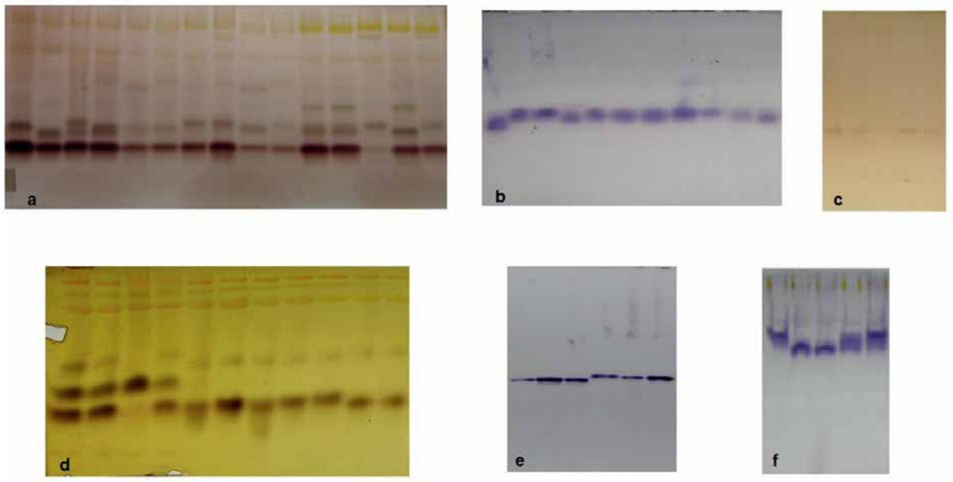

Figure 1.
PAGE spectra of: a. Nonspecific esterases of hemolymph; b. Phosphoglucomutase of silk glands; c. Alkaline phosphatase of midgut; d. Acid phpsphatase of hemolymph; e. Hexokinase of silk glands; f. Malate dehydrogenase from hemolymph of Bombyx mori L.

Locus (alleles)	**Breeds**												
	AES1 zb	**AES1 wh**	**Tg**	**Mir5**	**Japanese 106**	**MNB**	**Tahvon 106**	**Mziuri 1**	**Daizo**	**Line 22**	**Sh 4**	**Ukrainian 19**	**Vratza 16**
Bes A													
A_1	0.622	0.697	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A_0	0.378	0.303	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Bes B													
B_1	0.000	0.000	0.000	0.424	0.000	0.371	0.000	0.462	0.000	0.250	0.140	0.750	0.891
B_2	0.514	0.645	0.500	0.288	1.000	0.429	0.346	0.500	1.000	0.208	0.135	0.212	0.076
B_3	0.486	0.355	0.500	0.288	0.000	0.200	0.654	0.038	0.000	0.542	0.716	0.038	0.033
Bes D													
D_1	0.581	0.158	0.538	0.470	0.000	0.771	0.808	0.141	0.000	0.181	0.108	1.000	1.000
D_2	0.419	0.842	0.462	0.530	1.000	0.229	0.192	0.141	0.000	0.680	0.149	0.000	0.000
D_3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.139	0.527	0.000	0.000
D_0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.218	1.000	0.000	0.216	0.000	0.000
Bes E													
E_1	0.135	0.092	0.200	0.000	1.000	1.000	0.077	0.000	0.000	0.181	0.000	0.000	0.000
E_2	0.041	0.092	0.062	0.182	0.000	0.000	0.179	0.000	1.000	0.000	0.027	0.000	0.000
E_0	0.824	0.816	0.738	0.818	0.000	0.000	0.744	1.000	0.000	0.819	0.973	1.000	1.000
Pgm A													
A_1	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.013	1.000	0.167	0.000	0.000	0.000
A_2	0.338	0.474	0.550	0.697	1.000	1.000	1.000	0.577	0.000	0.389	0.473	0.700	1.000
A_3	0.662	0.526	0.450	0.288	0.000	0.000	0.000	0.417	0.000	0.444	0.527	0.300	0.000

Locus (alleles)	**Breeds**												
	AES1 zb	**AES1 wh**	**Tg**	**Mir5**	**Japanese 106**	**MNB**	**Tahvon 106**	**Mziuri 1**	**Daizo**	**Line 22**	**Sh 4**	**Ukrainian 19**	**Vratza 16**
Mdh A													
A_2	0.946	1.000	1.000	1.000	1.000	1.000	0.143	1.000	1.000	1.000	1.000	0.925	1.000
A_3	0.054	0.000	0.000	0.000	0.000	0.000	0.857	0.000	0.000	0.000	0.000	0.075	0.000
Bph A													
A	0.176	0.197	0.000	0.364	1.000	0.100	0.103	0.205	1.000	0.000	0.162	0.188	0.250
B	0.027	0.039	0.000	0.636	0.000	0.500	0.000	0.205	0.000	0.389	0.000	0.000	0.304
C	0.000	0.000	0.000	0.000	0.000	0.271	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.000	0.000	0.000	0.000	0.000	0.129	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0	0.797	0.763	1.000	0.000	0.000	0.000	0.897	0.590	0.000	0.611	0.838	0.813	0.446
Alp A													
A_1	1.000	0.566	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A_0	0.000	0.434	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Hk A													
A_1	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A_2	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 1.
Allele frequencies in breeds tested.

In the gene pool of AES 1 zb and AES 1 wh breeds (**Table 1**), we found two alleles polymorphism with "null" allele of the blood esterase A locus—Bes A_0 and A_1. In all rest breeds, Bes A_1 allele was fixed. Bes A_2 allele, which was described earlier [6] in some Egyptian breeds, was not detected in the current sample of breeds. For the Bes B locus, three alleles were recorded (Bes B_1, B_2, and B_3) in the gene pools of seven breeds—Mir 5, MNB, Mziuri 1, Line 22, Sh 4, Ukrainian 19, and Vratza 16. Polymorphism with two alleles (Bes B_2 and B_3) was established in AES 1 zb, AES 1 wh, Tg, and Tahvon 106. Bes B_2 allele was fixed in the gene pool of Daizo and Japanese 106. Among the breeds with polymorphism on Bes B locus, the allele Bes B_1 showed the highest frequency in Vratza 16, Bes B_2—in AES 1 wh and Bes B_3—in Sh 4. Polymorphism with four alleles (Bes D_1, D_2, D_3, and D_0) was found on the Bes D locus. Bes D_1 allele was fixed in the gene pool of Ukrainian 19 and Vratza 16, Bes D_2—in Japanese 106, Bes D_0—in Daizo. All four alleles were presented in the gene pool of the breeds Mziuri 1 and Sh4. Bes D_1, D_2, and D_3 alleles were presented in the gene pool of Line 22, Bes D_1 and D_2—in all the rest tested breeds. We obtained the highest frequency of the allele Bes D_1 in Tahvon 106, of Bes D_2—in AES 1wh, of Bes D_3—in Sh 4 (except for breeds with fixed Bes D alleles). Mziury 1 and Sh 4 have similar frequencies of the allele Bes D_0. Polymorphism with three alleles was found in Bes E locus (Bes E_1, E_2, and E_0) in AES 1 zb, AES 1 wh, Tg, and Tahvon 106 breeds. Two of these alleles we obtained in Line 22 (Bes E_1 and E_o), Mir 5, and Sh 4 (Bes E_2 and E_o). Bes E_1 allele was fixed in Japanese 106 and MNB gene pool, Bes E_2—in Daizo and Bes E_0—in Mziuri 1, Ukrainian 19, and Vratza 16. The "null" allele Bes E_0 demonstrated the highest frequency in all polymorphic breeds tested (**Table 1**).

Among the studied breeds we found polymorphism at the phosphoglucomutase (Pgm) locus with three alleles—Pgm A_1, A_2, and A_3 in Mir 5, Mziuri 1, and Line 22 breeds. Pgm A_2 and A_3 were presented in the gene pool of AES 1 zb, AES 1 wh, Tg, Sh 4, and Ukrainian 19 (**Table 1**). Monomorphism of phosphoglucomutase demonstrated Daizo (with fixed Pgm A_1 allele), Japanese 106, MNB, Tahvon 106, and Vratza 16 (with fixed Pgm A_2 allele). Pgm A_1 allele had the highest frequency in Line 22, Pgm A_2—in Ukrainian 19 and Pgm A_3—in AES 1 zb.

We found two alleles at the malatedehydrogenase locus (Mdh) in breeds AES 1 zb, Tahvon 106, and Ukrainian 19 (Mdh A_2 and A_3). Among these three breeds Mdh A_2 was the most common allele in AES 1 zb and Ukrainian 19, while Mdh A_3 was the most common in Tahvon 106. Mdh A_2 allele was fixed in the gene pools of the rest 10 breeds (**Table 1**). Mdh A_1 allele, which was described earlier [6, 12] in other breeds, was not detected in the current sample of breeds.

Total of five alleles of the acid phosphatase locus (Bph) were found in tested breeds (Bph A, B, C, D, and the "null" allele Bph O) (**Table 1**). Four of them were presented in the gene pool of MNB breed (Bph A, B, C, and D), three—in AES 1 zb, AES 1 wh, Mziuri 1, and Vratza 16 (Bph A, B, and 0) and two—in Mir 5 (Bph A and B), Tahvon 106, Sh 4 and Ukrainian 19 (Bph A and O), and Line 22 (Bph B and O). Bph A allele was fixed in Japanese 106 and Daizo breeds, whereas Bph O allele was fixed in Tg. The "null" allele was the most common in eight of the tested breeds. Bph B was the most expressed allele in Mir 5 and MNB.

Two alleles at the alkaline phosphatase locus (Alph A_1 and A_0) with a higher allele frequency of Alph A_1 were recorded in the breed AES 1wh, only (**Table 1**). Alph A_1 allele was fixed in the gene pool of the rest 12 breeds.

We found inter-breed polymorphism with two alleles on the hexokinase (Hk A) locus. The Hk A_1 allele was presented only in the gene pool of Japanese 106 breed, whereas Hk A_2 allele was fixed in the gene pools of all the rest 12 breeds.

The number of alleles per locus calculated with BIOSYS-1 software package in the silkworm breeds analyzed using nine enzyme loci ranged from 1.0 (Japanese 106 and Daizo) to 2.0 (Mziuri 1, AES 1 zb, and AES 1wh) (**Table 2**). The degree of polymorphism (according to the criterion 0.99) was the highest for the two Spanish breeds AES 1 zb and AES 1 wh (77.80%), and the lowest—for the two Japanese breeds Japanese 106 and Daizo (0%). The observed heterozygosity (H_o) by polymorphic loci varied from 0.000 (for Japanese 106 and Daizo) to 0.222 (for AES 1 zb). The expected heterozygosity (H_e) was higher than the observed one (H_o) in all breeds with polymorphism. Significant differences ($P < 0.05$) in genotype frequencies were seen at the most loci in breeds studied. Chi-square test (DF = 1÷3) showed that the deviations from the Hardy-Weinberg equilibrium were in result of excess of homozygotes and deficiency of heterozygotes.

The mean F_{ST} value over all loci, which is associated with the level of inter-breed differentiation, was 0.4921 and shows that 49.21% of the overall genetic diversity observed was among breeds (**Table 3**). 50.79% of genetic variations were within the breeds. The highest level of genetic diversity among breeds we found for the Hk A locus was 1.0000) and the lowest one for the Mdh A locus was 0.0571. For two loci (Bes D and Bes E), we established a level of inter-breed differentiation over than 50%. The heterozygosity in total populations F_{IT} averaged to 0.6549 and shows that there was a deficit of heterozygotes in the tested breeds and correlates with the obtained lower level of heterozygosity observed compared to the expected one and with deviations from the Hardy-Weinberg equilibrium as well. The combined F_{IS} value for all polymorphic loci was 0.3205, which also reflects a significant deficiency of heterozygotes.

The values of genetic distance [20] were calculated using the allele frequencies and ranged from 0.029 (between the breeds Vratza 16 and Ukrainian 19) to 0.730 (between Japanese 106 and Ukrainian 19).

Breed	**Mean sample size per locus**	**Mean number of alleles per locus**	**Percent polymorphic loci (P = 0.99)**	**H_o**	**H_e**
Vratza 16	46.0 ± 0.0	1.4 ± 0.3	22.2	0.085 ± 0.074	0.095 ± 0.073
Ukrainian 19	40.0 ± 0.0	1.6 ± 0.2	44.4	0.125 ± 0.059	0.141 ± 0.062
Sh 4	37.0 ± 0.0	1.9 ± 0.4	44.4	0.144 ± 0.057	0.215 ± 0.087
Line 22	36.0 ± 0.0	1.9 ± 0.3	55.6	0.164 ± 0.059	0.279 ± 0.094
Mziuri 1	39.0 ± 0.0	2.0 ± 0.4	44.4	0.157 ± 0.067	0.255 ± 0.102
Tahvon 106	39.0 ± 0.0	1.6 ± 0.2	44.4	0.097 ± 0.042	0.153 ± 0.065
MNB	35.0 ± 0.0	1.7 ± 0.4	33.3	0.156 ± 0.085	0.185 ± 0.097
Japanese 106	36.0 ± 0.0	1.0 ± 0.0	0.0	0.000 ± 0.000	0.000 ± 0.000
Mir 5	33.0 ± 0.0	1.8 ± 0.3	55.6	0.189 ± 0.074	0.264 ± 0.089
Tg	40.0 ± 0.0	1.6 ± 0.2	44.4	0.119 ± 0.063	0.214 ± 0.085
Daizo	37.0 ± 0.0	1.0 ± 0.0	0.0	0.000 ± 0.000	0.000 ± 0.000
AES 1 wh	38.0 ± 0.0	2.0 ± 0.2	77.8	0.164 ± 0.048	0.319 ± 0.066
AES 1 zb	37.0 ± 0.0	2.0 ± 0.2	77.8	0.222 ± 0.058	0.297 ± 0.070

Table 2.
Mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected heterozygosity (H_e).

Locus	F*IS*	F*ST*	F*IT*
Bes A	0.6711	0.3084	0.7725
Bes B	0.1455	0.3701	0.4618
Bes D	0.4742	0.5151	0.7450
Bes E	0.5643	0.6692	0.8559
Pgm A	0.5023	0.4191	0.7109
Mdh A	−0.0714	0.0571	−0.0102
Bph A	0.0618	0.4592	0.4925
Alp A	0.3037	0.4146	0.5924
Hk A	0.0000	1.0000	1.0000
Mean	**0.3205**	**0.4921**	**0.6549**

Table 3.
F-statistics for all polymorphic loci studied.

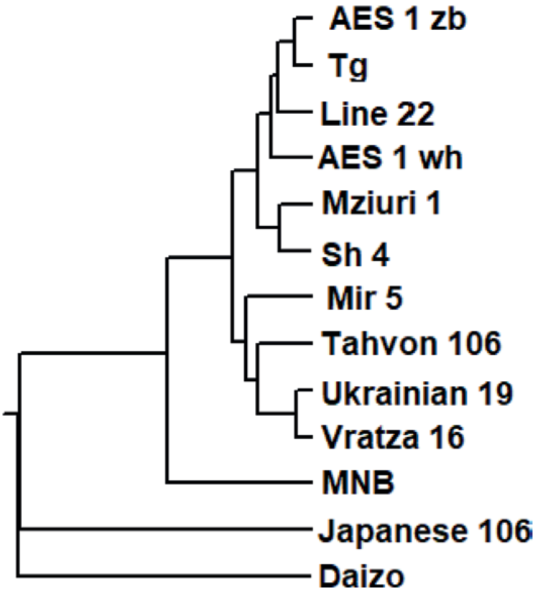

Figure 2.
UPGMA dendrogram.

Analysis of the results obtained from genetic distances and UPGMA dendrogram (**Figure 2**) revealed that all the 13 breeds were grouped into two major clusters. The first cluster included Japanese breeds Daizo and Japanese 106, while the second included the rest 11 breeds, which was distributed in two subgroups. The first of them included MNB breed. The second one included all others. This subgroup was distributed into two groups—four breeds (Mir 5, Tahvon 106, Ukrainian 19 and Vratza 16) were grouped to form one subgroup and six breeds (Sh4, Mziuri 1, AES 1 wh, Line 22, Tg and AES 1 zb) were grouped to form another subgroup.

2.3 Discussion

The study of polymorphic enzymatic and nonenzymatic proteins in mulberry silkworm is important for the selection of this species. They could serve as a kind of "passport" of the parent breeds, on the basis of which it is possible to compile optimal variants of crossbreeding and predict the effect of heterosis. Studies on proteins and enzymes in the silkworm (*B. mori* L.) have long since begun. Breeds of different origins and geographical distribution, bred in different countries as Japan, China, Korea, India, Russia and others, have been studied. Different methods of analysis have been used. A wide range of results has been obtained, which reflects not only the application of different research methods but also the existing huge variety of breeds that are used in various breeding centers around the world. Significant and positive correlation has been established between some isoenzymes and some yield parameters [25, 26]. Successes in this area of research could significantly facilitate the production of desired phenotypes and introduce elements of rigorous planning in the selection process.

In this study, we indicated a total of 12 alleles of four esterase loci. Three of them were "null" alleles. In earlier studies, [6] have reported an allele Bes A_2. We did not find this allele among the tested 13 breeds. "Null" alleles of the Bes A, D, and E loci were described in other breeds from Bulgarian germplasm resourses of silkworm [3, 6, 12, 19, 27]. Polymorphism with five alleles was determined of the acid phosphatase. One of them was found as "null" type. Allozyme polymorphism with codominant alleles of this enzyme was reported earlier [1, 6, 10, 12, 28]. We found intra-breed polymorphism with three or two alleles of the phosphoglucomutase, malate dehydrogenase, and alkaline phosphatase, as well as inter-breed polymorphism with two alleles of the hexokinase. Some of the alleles of the polymorphic loci demonstrated breed specificity. For instance, Bes A_0 allele was presented only in AES 1 zb and AES 1 wh. Alp A_0 allele was presented in the gene pool of AES 1 wh, and Hk A_1 was presented in Japanese 106 only.

The results based on population genetic analyses showed a certain degree of differentiation between the tested breeds. 50.79% of isoenzyme diversity is observed between breeds and 49.21% is maintained within breeds, which is in line with the diversity based of AFLP markers found in some Iranian breeds [29]. Larger proportion of genetic variations among *B. mori* strains (84.08%) and a relatively smaller within strains (15.92%) have been established by RAPID analysis [30]. The UPGMA dendrogram resolved 13 breeds into two main clusters. The two breeds of Japanese origin (Daizo and Japanese 106) form one cluster and all the others the other cluster. This grouping of silkworm breeds is probably related to their adaptation to the specific geographical conditions of the environment from which they originate, as well as to the founder effect in the introduced breeds. Japanese 106 and Ukrainian 19 were the most distant breeds, whereas Vratza 16 and Ukrainian 19 were the closest breeds.

Low level of heterozygosity among tested breeds has observed in this study. Heterozygote deficiencies probably results from low effective number of reproductive individuals, selection process, and inbreeding effect. Some authors [8] pointed that reduction in genetic diversity in silkworm might be mainly due to domestication, breeding systems, selection, genetic drift, and inbreeding. The effects of inbreeding can accumulate over many generations [31, 32]. Breeders use artificial selection for target characteristics which also leads to a reduction in genetic variations in the population. The import of breeds of different origins and their use in breeding programs would help maintain a higher level of genetic diversity, which is very important for selection of suitable parents required for successful development of improved breeds and hybrids of silkworm that have high adaptive potential [8]. In view of the differences found in the genetic structure of the studied breeds with different origins, the results obtained here would be useful for breeders in planning crossbreeding strategies to produce new hybrids and in the conservation programs of silkworm *B. mori*.

3. Conclusions

Our results complement the knowledge of the genetic variations among the silkworm breeds bred in Bulgaria. They confirmed that nonspecific esterases, acid phosphatase, and malate dehydrogenase from hemolymph, phosphoglucomutase and hexokinase from the silk glands, and alkaline phosphatase from the midgut are applicable to the study of genetic structure and phylogenetic relationships between breeds. The number of alleles, allelic, and genotypic frequencies at polymorphic loci show breed specificity. It is important to perform continuous evaluate the polymorphism degree of the breeds to avoid a marked increase of the homozygosity. This would result in the expression of deleterious genes that can cause high mortality or other adverse effects. The results obtained in the present study could help breeders in selecting parental pairs for crossbreeding and in determining the quality of parental forms in the early stages of development. The analysis of genetic structure on the basis of isoenzyme markers showed that the tested breeds of silkworm are genetically differentiated. Most of them have high degree of polymorphism and can be used for genetic improvement and to develop new hybrids for silk production.

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Biodiversity and Ecology of Lepidoptera - Insights and Advances presents a comprehensive overview of the order of winged insects known as Lepidoptera. It is organized into two sections and seven chapters. Section 1, “Lepidoptera: Behavioural Diversity”, explores the myriad challenges faced by Lepidoptera globally. In the twenty-first century, the most pressing issue confronting them is the decline in biodiversity, necessitating the essential restoration of ecosystems. Concurrently, Lepidoptera exhibit mastery in camouflage and mimicry, enabling them to evade detection by predators and parasites, providing a reproductive advantage. This section also delves into gynandromorphy behavior, where individuals develop a mosaic of both male and female traits, with the left and right halves of the body displaying different sexes. Additionally, it reviews recent advances in non-coding RNAs, elucidating their role in regulating gene expression through chromosomes and their significance in the overall developmental process of Lepidoptera. Section 2, “Moths: Bioecology and Genetics”, commences with the mapping of flight paths for feeding, reproduction, and pollination occurrence in hawkmoths in the family Sphingidae. The section then reveals the features of invasion into various ecosystems by five invasive leafminer moth species of the family Gracillariidae). Furthermore, it discusses the degree of genetic variability and phylogenetic relationships among 13 breeds of the mulberry silkworm (*Bombyx mori* L.).

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