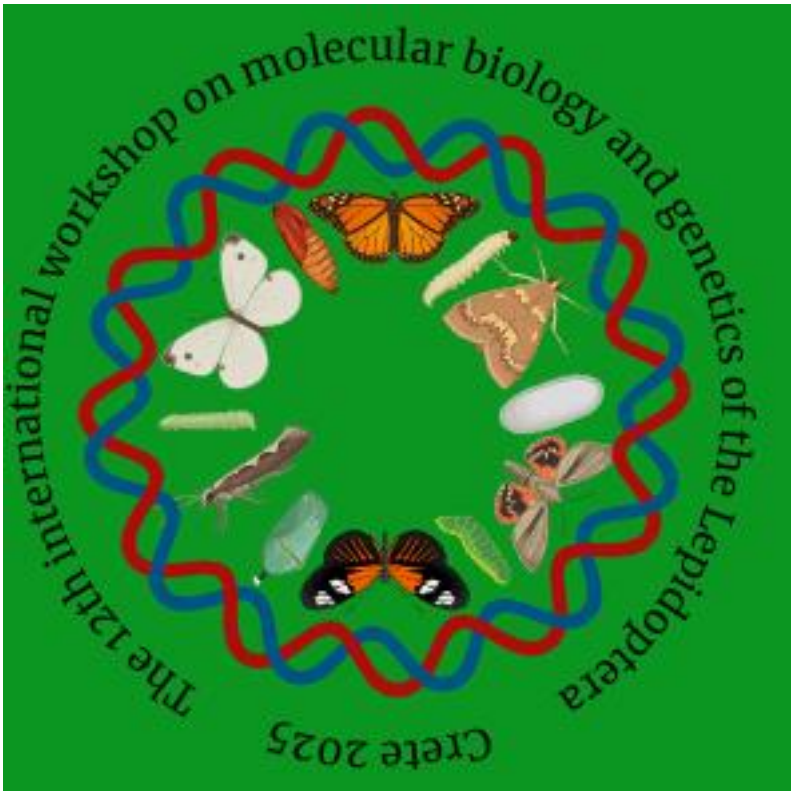
The 12th International Workshop on Molecular Biology and Genetics of the Lepidoptera



Sunday 6 July 2025 - Saturday 12 July 2025
Orthodox Academy of Crete - Kolympari

Book of Abstracts

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Monday

Keynote 1: Insights into rapid chromosome evolution in butterflies and the potential of Project Psyche for lepidopteran genomics

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Chromosome rearrangements, such as fusions and fissions, are a ubiquitous feature of eukaryotic genome evolution. However, our understanding of the evolutionary forces that shape rates of chromosome rearrangements and the effect of chromosome rearrangements on processes such as speciation and adaptation remains in its infancy. *Polyommatina* is a young subtribe of blue butterflies that evolved 23 million years ago and is characterized by two modes of chromosome evolution: extreme stability in chromosome number in the majority, with rapid chromosome number change in some clades of species. Combined with the high diversification rates of species in this group, *Polyommatina* is an ideal group to understand the forces that modulate chromosome rearrangements and their consequences on diversification. Chromosome-level genomes for 27 species of *Polyommatina* reveal a complex history of chromosome rearrangement in genus *Lysandra* and *Polyommatus* subgenera *Agrodiaetus* and *Plebicula*. Parallels between the histories of fission in each group are found. This work deepens our understanding of chromosome fission, a fundamental process that shapes the evolution of genome structure across the diversity of eukaryotes. In the second part of my talk, I will provide an overview of Project Psyche – a highly collaborative, trans-national effort to jointly sequence the genomes of all 11,000 species of Lepidoptera found in Europe – and the breadth of scientific questions that this data will make possible.

Sex chromosome evolution in Lepidoptera: insights from the *Heliconius sapho* clade

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Fusions between sex chromosomes and autosomes have the potential to resolve sexual conflict, drive local adaptation, and promote reproductive isolation between species. *Heliconius* butterflies typically exhibit a highly stable karyotype of 21 chromosomes with strong synteny across species. How- ever, three species within the *sapho* subclade show notable deviations, with up to 60 chromosomes.
We have generated chromosome-level reference genomes and corresponding gene annotations for five of the seven species within this subclade. Our preliminary findings confirm three sequential W-autosome fusions previously identified using whole-genome sequencing (WGS) and reveal an additional fusion involving chromosome 5, observed exclusively in two subspecies of *H. eleuchia*. Haplotype-resolved genomes provide further insights: a) in three species, autosomes have under- gone significant fissions, resulting in a substantially increased chromosome count; b) the Z chromo- some remains intact across all species in the clade; and c) W-autosome fusions are accompanied by additional rearrangements, including translocations, inversions, and fissions of the neo-W chromosomes. To elucidate the functional consequences of these sex-autosome fusions, we are generating RNA-seq data across all species and subspecies to investigate gene degeneration and dosage compensation mechanisms in the neo-sex chromosomes. These results will provide valuable insights into the role of sex-autosome fusions in the evolution of sex chromosomes.

Genome Structure Evolution in Ithomiini Butterflies

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Chromosome-level structural variation, such as fusion, fission, inversion, and translocation, can influence gene flow directly by disrupting meiosis and reducing hybrid fitness. Additionally, changes in linkage disequilibrium between loci can indirectly promote reproductive isolation and speciation, by affecting the efficacy of selection, and preserving coadapted gene complexes. However, the relative importance and generality of these direct and indirect effects remain poorly understood.

Lepidoptera (butterflies and moths) have holocentric chromosomes, female achiasmy, and a relatively conserved karyotype with a mode of 31 chromosomes. However, several lineages within the order show evidence of extensive chromosomal rearrangements. Among these, the Ithomiini tribe, a diverse and ecologically significant group of Neotropical butterflies, exhibits extraordinary karyotypic diversity, with haploid chromosome numbers ranging from n = 5 to n = 120 (Brown et al. 2004). This group is taxonomically rich and well studied for their role in mimicry rings, chemical ecology and tropical biodiversity. Their rapid radiation, with several genera exhibiting recent ex- plosive diversification, makes them a powerful model for investigating the link between structural genomic evolution and speciation. Here we focus on characterising the genome architecture and quantifying the extent of the rearrangements in 200 species across the Ithomiini phylogeny.

Preliminary results from a subset of assembled genomes show chromosome counts corresponding to the earlier described karyotype range, but frequent chromosomal fissions and fusions reveal more extensive rearrangements than expected. Genome sizes vary widely, from under 300 Mb to almost three-fold, mainly due to lineage-specific expansions of LINE2 transposable elements. We found a positive correlation between genome size and chromosome number, suggesting a constraint on individual chromosome size, potentially driven by cellular or meiotic mechanisms. Several genomes exhibit heterozygous fusion/fission events, indicative of ongoing chromosomal instability. Sex chromosome turnover was particularly dynamic. We identified multiple sex-autosome fusions, including Z-autosome and W-autosome fusions, with many species exhibiting multiple sex chromosomes. De- spite this variation, the ancestral lepidopteran Z-chromosome segment remained conserved across all species. In conclusion, the Ithomiini lineage is undergoing continuous chromosomal rearrangements and very dynamic neosex-chromosome formation.

The origin of zygosity-based sex determination

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Sex determination in insects is initiated by a primary trigger which sets a molecular cascade in motion leading to the development of either females or males. These triggers are known to evolve very fast, which means that a trigger found in model organisms often doesn't exist in other species be- longing to the same taxon. This is also the case in Lepidoptera, where a primary trigger was found on the W chromosome of *Bombyx mori*, while a fundamentally different mechanism was found in the butterfly *Bicyclus anynana*. In fact, as a result of convergent evolution, the butterfly mechanism is surprisingly similar to that found in the honey bee belonging to a completely different insect order. One of the remaining questions about this mechanism is its evolutionary origin and taxonomic representation. To address this, a large number of species were screened for the key characteristics of the sex determination mechanism of *Bicyclus anynana*: The presence of a hypervariable region in exon 8 of the Z-linked *Masculinizer* gene consisting of a string made up predominantly of asparagines in combination with obligatory male heterozygosity. Homozygosity is lethal in this system and females are hemizygous. Using these criteria,

the origin of the sex determination mechanism was pinpointed, and groups which subsequently lost it independently were identified. Those who lost it must have either reverted back to the previous trigger, or adopted a novel mechanism. It demonstrates that sex determination triggers evolve fast in Lepidoptera and that several if not many variants must exist.

Intersexes caused by deviations in the Z:A ratio in the eri silk-moth, *Samia cynthia ricini*

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Moths and butterflies (Lepidoptera) have sex chromosome systems with female heterogamety and two chromosomal mechanisms of sex determination have been proposed: (i) a dominant W mechanism in WZ♀/ZZ♂ species with a dominant female sex-determining factor on the W chromosome and (ii) a Z-counting mechanism in Z0♀/ZZ♂ species with W-independent sex determination. In the first case, only the presence or absence of a W chromosome affects sexual development, and the dose deviations of Z chromosomes and/or autosomes caused by ploidy changes theoretically play no role, whereas in the second case, ploidy changes can lead to a disruption of sexual development. How- ever, little is known about the actual effect of ploidy changes on sexual development in species with a Z-counting mechanism. Here, we investigated whether ploidy changes affect sexual development and dosage compensation in the eri silkmoth, *Samia cynthia ricini* (2n = 27♀/28♂, Z0♀/ZZ♂). The experiments with artificially induced polyploids showed that tetraploids with four Z chromosomes become males and tetraploids with two Z chromosomes become females. Subsequently, crosses between diploids and tetraploids produced triploids with three Z chromosomes and triploids with two Z chromosomes. Triploids with three Z chromosomes became males, while triploids with two Z chromosomes exhibited both male- and female-specific splicing of the *S. cynthia doublesex* (*Scdsx*) gene and had abnormal gonads, suggesting that triploids with two Z chromosomes are intersexes. These results showed that deviations in the ratio between the number of Z chromosomes and the number of autosome sets (Z:A ratio) caused by ploidy changes disrupt sexual development. In addition, embryonic transcriptome analyzes showed that the relative levels of gene expression are similar between samples with different doses of Z chromosomes and autosome sets, demonstrating that there are two types of dosage compensation in this species, namely sex chromosome dosage compensation and ploidy-associated dosage compensation.

Keynote 2 - EMBO Young Investigator Lecture: Evolution of segregation and genome organization

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In our lab, we integrate molecular and evolutionary biology, genomics, and biochemistry to explore the evolution of chromatin and genome architecture. One key research focus is centromeres—specialized chromosomal regions that facilitate kinetochore assembly and spindle microtubule attachment, ensuring accurate chromosome segregation during cell division.
We are particularly interested in the evolution of holocentric chromosomes in insects, with Lepidoptera as our primary model system. Intriguingly, our previous studies revealed recurrent loss of the centromere-specifying factor CENP-A in multiple holocentric insects, raising the question of how centromeres are specified in CENP-A-deficient species. To address this, we are mapping and characterizing centromeres in the silkmoth *Bombyx mori*. Recent analyses have also included biochemical and structural studies of the Bombyx kinetochore complex, leading to the discovery of novel factors critical for kinetochore stability.
In another line of research, we examine the evolutionary dynamics of spatial genome organization in holocentric chromosomes. Our studies identified unique 3D genome features in Bombyx, including a novel chromosomal compartment termed S. In current studies, we are investigating the evolutionary dynamics of compartment S to uncover conservation patterns in 3D genome organization across eukaryotes.

Non-allelic homologous recombination drives sequence loss be- tween transposable elements in smaller lepidopteran chromosomes.

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Transposable elements (TEs) drive major genome size and structural variation, yet the factors shaping their accumulation and removal remain debated. Classical models predict that higher recombination rates should lead to more efficient TE removal. However, in the painted lady butterfly (*Vanessa cardui*), smaller chromosomes were found to harbor denser TE content than larger ones despite their higher recombination rates. This unexpected pattern raises questions about whether similar trends occur across other Lepidoptera and what mechanisms might drive them. Across ten species spanning ten lepidopteran families, we investigated the relationship between chromosome size and TE organization using comparative genomic analyses. Our study reveals a striking association where smaller chromosomes consistently harbor higher densities of TEs. We also observed a positive correlation between average inter-TE distance and chromosome size for both recently inserted TEs (<5% divergence) and less recent TEs (5–10% divergence). However, the ratio of these distances (young/older TEs) negatively correlated with chromosome size in eight out of ten species, with the remaining two species showing no significant correlation. This pattern suggests that the distances between older TEs contract over time in smaller chromosomes, indicative of sequence removal between TEs, potentially due to non-allelic homologous recombination. Population genomics analyses in *Danaus plexippus* and Spodoptera frugiperda showed lower effective population sizes in smaller chromosomes, but no consistent pattern in selection coefficients, ruling out reduced purifying selection as the main driver of TE patterns. Our results indicate that chromosome size influences TE organization, with non-allelic homologous recombination likely contributing to sequence removal in smaller chromosomes.

Z chromosomal involvement in delayed exit from diapause of *Cydia pomonella* using linkage analysis

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Larvae of codling moth (*Cydia pomonella* L.) are a key pest in apple production. During spring and summer, they bore into the fruit and leave it unmarketable. The insect hibernates in a larval diapause stage and exits diapause by pupating during spring. Moths pupating early in the season have a higher chance of initiating several generations before diapause in the fall, whereas pupation later in the year reduces the chance of doing so. By repeated inbreeding and selection, two different codling moth colonies were selected from a common original population from South Tyrol, one for early and the other for late diapause termination. These show stable inheritance of diapause termination of several weeks difference (early vs. late strains), indicating a genetic basis for these phenotypes.
For both early and late strains, the signal for the delayed phenotype is dependent on photoperiod and temperature. Reciprocal single pair intercrosses between the early and late strains indicated a strong contribution of the Z chromosome contributing to the delayed pupation after diapause. Backcrosses revealed different patterns of inheritance, indicating the involvement of one or more autosomal factors as well. We are currently using whole genome sequence analyses of individuals from the early and late strains, marker selection and linkage analysis to map quantitative trait loci on those chromosomes. The results will shed light on the genetic basis and potentially the physiological mechanisms of delayed pupation after diapause. This knowledge will be crucial for understanding the worldwide spread, local pest pressure of the CM and may provide insight into the fundamental fluidity of entering the next life stage after diapause in insects.

Fading into obscurity: Rapid evolution and elusive origin(s) of the W sex chromosome in moths and butterflies.

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Sex chromosomes are shaped by evolutionary forces distinct from those acting on autosomes. The W and Y chromosomes are particularly enigmatic due to their highly repetitive content and lack of recombination. We investigated the evolution of the W chromosome across several moth and butterfly species, focusing on the accumulation and turnover of repetitive elements. We find that W chromosomes have repeat profiles distinct from autosomes and are consistently enriched for long terminal repeat (LTR) transposable elements (TEs). Although the specific TE groups vary across species, the repeated enrichment of LTRs suggests common genomic constraints or selective pressures. Notably, TE diversity on the W chromosome is lower than on autosomes, with only a subset of TE families accumulating on the W. These families often show low nucleotide diversity, consistent with recent insertion or sequence homogenization, possibly via gene conversion. Comparisons across related species of *Heliconius* butterflies revealed a striking lack of shared W-enriched repeats, suggesting rapid turnover of repeat content. This pattern was also observed among three laboratory strains of the silkworm *Bombyx mori*, indicating that W chromosome repeat content can evolve rapidly even within a species. These findings underscore the highly dynamic nature of W chromosome evolution in Lepidoptera. The lack of conserved sequence content across species complicates efforts to trace the ancestral origins of the W chromosome and highlights the risk of misinterpreting absence of shared sequence as evidence for independent origins. Our study emphasizes the importance of repetitive sequence dynamics in shaping the evolution of heteromorphic sex chromosomes and the need for caution when inferring their evolutionary history.

W sex chromosome: the last frontier in lepidopteran genomics

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Lepidoptera represent the most species rich group with a female heterogametic sex determination system (WZ/ZZ and its variants). Their sex chromosomes evolved secondarily within insects, with the ancestral constitution considered to be Z0/ZZ. The W chromosome likely originated independently multiple times. Its origin remains unresolved, with the main hypotheses including: (i) origin from an autosome that fused with the Z chromosome, (ii) origin from the Z chromosome after the emergence of a female-determining factor, and (iii) a non-canonical origin from a supernumerary B chromosome. Despite technological advances, testing these hypotheses proved difficult due to highly repetitive composition of the W chromosome, which hampers its assembly using standard approaches. The results of the early studies yielded conflicting results. While sequences generally confirm repetitive composition and high GC content of lepidopteran W chromosomes, their lengths vary greatly among species and often contradicts our expectations based on cytogenetic data. These discrepancies likely reflect the low quality and/or incompleteness of the W chromosome assemblies. Unfortunately, assessment of quality and completeness of the W chromosome sequences is not routinely performed, and standard genome-wide completeness metrics offer little insight given the W's unique characteristics. To address this, we are conducting in silico analyses to evaluate the completeness of W chromosome sequences in available butterfly genomes. By combining comparative analyses of k-mer spectra and repetitive landscape, we estimate both the size and completeness of the W chromosome from available genomic data. These approaches also provide unprecedented insight into the molecular composition of lepidopteran W chromosomes, which remain one of the last frontiers in lepidopteran genomics.

Tuesday

Keynote 3: Molecular breeding of silkworm strains for better silk and resistant to NPV virus

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The silkworm is a crucial industrial insect and serves as a model for Lepidopteran insects. Currently, the silkworm rearing market in China is valued at approximately 40*billion, contributing to a broader industrial mark* billion. However, as labor costs in China have steadily increased over the past two to three decades, silkworm rearing has undergone two significant trends. First, the primary rearing areas have shifted from the eastern to the western regions of China. Second, large-scale industrial rearing practices have emerged in Eastern China. These changes have heightened the demand for superior silkworm strains that are both high-yielding and disease-resistant. To develop better silk strains, we have concentrated our efforts on male-only strains. Our research into sex determination mechanisms has led us to construct male-only strains by inserting a toxic protein into female-specific exons of the doublesex gene. Additionally, we have established a system to separate male and female eggs using color and fluorescence markers. For disease-resistant strains, we utilized genome editing techniques to create virus-resistant silk-worms. Initially, we identified virus key genes and constructed resistant loci in a laboratory strain (multi-voltine) through the transgenesis of Cas9 and virus-targeting sgRNA. We then applied this method to industrial strains, resulting in significant resistance to the BmNPV virus. These innovative approaches are set to enhance our silkworm industry and facilitate the further expansion of large-scale rearing operations.

Genomic analysis of silkworm w1 pnd strain for the development of novel transgenic and genome editing tools

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Silkworm (*Bombyx mori*) is a lepidopteran model insect that has been utilized for the basic researches as well as for the industrial application, including protein production, silk engineering and others. *w1 pnd* is a strain that is frequently utilized for the transgenic and genome editing study, having white- egg, white-eye and non-diapause traits. Recently we carried out the time-course transcriptomic analysis of the silk gland in this strain and obtained a basic data for the entomological as well as medical research. In order to obtain the genomic information of this strain, we here carried out the whole genome sequencing analysis of this strain. The genome was extracted from one female and was applied for the long read genome sequencing using PacBio Revio system. This resulted in the production of 86.4 Gb HiFi reads, which corresponds to the x200 coverage of the silkworm genome. These reads were assembled using hifiasm and we successfully established high-quality assemble with N50 of 16Mb. We analyzed each haplotype sequence by using the output data of the assemble and found that there existed a number of structural variants. Most of them corresponded to transposons, suggesting that these transposons are highly active in the *w1 pnd* strain. We are now investigating whether they can be used as novel tools for transgenic and/or genome editing, and we expect that they can be fundamental basis for the further promotion of silkworm study.

Advancing Genetic Tools for Butterfly Research: Overcoming Challenges in Transgenesis and Fluorescent Tracking

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Butterflies offer an extraordinary reservoir of diverse traits, making them attractive systems for re- search spanning a range of disciplines. In the dynamic realm of evolutionary development, our quest to explain how diverse traits within the animal kingdom arose takes center stage. Despite major advances in genetic and genomic approaches for identifying genes and regulatory elements controlling developmental processes, and CRISPR-Cas9 editing for generating loss of function mutants in Lepidoptera, our ability to precisely track and manipulate regulatory elements in butterflies remains limited. Thus, we aim to develop transgenic tools to: 1) monitor *cis*-regulatory element activity using expression reporters, 2) manipulate gene expression in a spatiotemporally controlled manner, and 3) visualize developmental and neural processes through fluorescent imaging (e.g. epithelial morphogenesis, calcium imaging). One major hurdle has been the opacity of butterfly tissues throughout development which hampers detection of fluorescent transgene expression. Here, we present preliminary data on our efforts in developing genome editing and reporter assays in multiple butterfly species. Using CRISPR/Cas9, we've generated mutant lines of the *scarlet* gene, which display reduced pigmentation in the eyes and body. These lighter phenotypes enhance fluorescent signal detection and offer visible markers for efficient screening. We also present data on successful transgenesis using two different transposon-based approaches: *piggyBac* and *Minos*. Our work lays the foundation for expanding transgenic approaches in butterflies, opening new avenues for dissecting the genetic basis of their phenotypic diversity.

Egg cooling after oviposition extends the permissive period for microinjection-mediated genome modification in Bombyx mori

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In general, the efficiency of gene transfer in an organism depends largely on the developmental state of the fertilized egg in which microinjection is performed. Silkworm larval growth is considered to have a developmental zero point at around 11℃. Hence, we investigated the effect of refrigeration of fertilized silkworm (*Bombyx mori*) eggs during microinjection on gene transfer efficiency. First, we conducted gene transfer experiments with *piggyBac* vector at 25℃using eggs in different developmental states. As a result, the efficiency of transgenic silkworms was about 5 times higher when eggs were used 4 hours after laying eggs than when eggs were used 8 hours after laying eggs. On the other hand, no genetically modified silkworms were obtained at 12 hours. Next, the efficiency of producing genetically modified silkworms was examined using eggs stored at 10℃for 5 or 24 hours. As a result, not only 5-hour eggs but also 24-hour eggs could be used to obtain genetically modified silkworms. In addition, when the knockout experiment of the *BmBLOS2* gene was conducted using eggs stored at 10℃and the knockout phenotype was examined in the injection generation (G0), the knockout phenotype could be observed even when 48-hour-old eggs were used. These results indicate that proper refrigeration of fertilized eggs prior to microinjection can extend the suitable period of fertilized eggs for insect genome modification and dramatically improve the efficiency of genome modification. These results may be useful in other insects, especially in species with very short embryonic development periods.

Unraveling the complexity of silk glands using Hybridization Chain Reaction and RNAseq

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Silk serves many diverse and obligatory functions across lepidopteran species. The diverse use of silk among Lepidoptera includes use as cocoon materials, pre-pupal attachments, as glue to build protective leaf forts, and as an antimicrobial coating. However, little is known about the expression differences that occur within the silk gland to facilitate such diverse silk across lineages. Here we characterize gene expression differences within the silk glands of the pantry moth, *Plodia interpunctella* (Pyralidae, Phycitinae). *Plodia* is emerging as a powerful model system for functional genomics in Lepidoptera, providing an experimentally tractable alternative to silk moths for the study of silk evolution. Previous work suggests that the core silk biopolymer is secreted by the Posterior Silk Gland (PSG), and is further coated by bioadhesive proteins secreted by the Middle Silk Gland (MSG). To get a comprehensive overview of these processes in *Plodia*, I generated deep transcriptomes of the *Plodia* MSG and PSG, followed by differential gene expression analyses that depict the composition of the silkome across these two regions. I will then show that Hybridization Chain Reaction RNA fluorescent *in situ* hybridization (HCR RNA-FISH) provides a powerful method to visualize the regionalization of gene expression in whole-mount silk glands, notably corroborating the notion that the MSG is subdivided into two functional domains each expressing a specialized set of sericins. Finally, I used confocal microscopy to describe differences in the morphology of *Plodia* MSG and PSG secretory cells. Our work has established *Plodia interpunctella* as a promising model for future study of silk production and provides a foundation for further exploration of the regulatory processes underlying expression differences within silk glands.

A molecular toolkit to study host immune response in the in vivo infection model *Galleria mellonella*

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Galleria mellonella is a versatile Lepidopteran in vivo model to study host-pathogen interactions. Their ability to be maintained at 37°C, coupled with a broad susceptibility to human pathogens and a distinct melanisation response that serves as a visual indicator for larval health, positions *Galleria mellonella* as viable alternative to mammalian models for infection research. Lack of standardised molecular tools however have stymied development of the full potential of *Galleria mellonella*, and there are significant knowledge gaps on the molecular basis for host immune response and how it resembles or differs from better described models such as Drosophila melanogaster. To address this, we describe a PiggyBac, CRISPR and flow-cytometry toolkit that together enable generation of reporter and knockout lines and quantitative means to robustly interrogate *Galleria mellonella* humoral and cellular immunity. These advances increase the utility of *Galleria mellonella* in molecular research, opening the way to more widespread use as an inexpensive and ethically compatible animal model for infection biology and beyond.

Wednesday

Keynote 4: Drivers of rapid diversification in *ithomiini* butterflies

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Ithomiini butterflies, a tribe of Neotropical butterflies, are an ideal study system for drivers of rapid diversification. Some ithomiini lineages have diversified exceptionally fast, while others have done so at a much slower pace. By comparing their genetic basis of relevant traits, rates of hybridisation, chromosomal rearrangements and other factors, my group aims to elucidate the factors underlying variation in diversification rates. We find important roles of hybridisation contributing beneficial genetic variants and chromosomal rearrangements strengthening reproductive isolation. Complex rearrangements involving multiple chromosomes, often including sex chromosomes, seem to play particularly important roles in speciation.

The Most Genetically Diverse Butterfly in the New World *Junonia*: Unveiling the Haplotype Complexity of *Junonia vestina*

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Nymphalid butterflies in the genus *Junonia* are known for their vast distribution and thriving presence on remote oceanic islands, highlighting their exceptional dispersal and diversification capabilities. The New World *Junonia* carry three common major mitochondrial haplotype groups, shared between species and polymorphic within species, suggesting a possible history of hybridization and mitochondrial introgression. For these reasons, sympatric New World *Junonia* populations from different species tend to have similar haplotype frequencies in any given location. Haplotype group A subgroups A1 and A2 are most common across all *Junonia* species in South America (>80%), with haplotype group B occurring at much lower frequency (15%). *Junonia* vestina is found in the Andes (primarily between 1900m-3500m) with documented populations in Peru, Ecuador, and Bolivia. Prior studies of few specimens indicate that Peruvian populations of *J. vestina* are the sole known source of the rare mitochondrial haplotype group C, while conspecifics from other regions exhibited different haplotype groups (mostly A1). Mitochondrial haplotype group C is closely related to haplotypes found in *J. villida* from the Indo-Pacific region, hinting at potential long-distance gene flow across the Pacific Ocean. South American *J. vestina* butterfly specimens from museums and personal collections were examined. DNA from 85 samples underwent isolation, quantification, PCR amplification, and genotyping via restriction digests in *COX1*, *COX2*, *COX3*, and *ND4*. Some samples were also analyzed by Sanger sequencing. Based on data from this and previous studies, we have assigned 83 *J. vestina* specimens (from populations ranging from Colombia to Argentina and Chile) to haplotypes A1 (36%), A2 (18%), B2 (5%), B1 (1%). Haplotype C was not found to be restricted to Peru because this haplotype was also found in *J. vestina* from Bolivia, Argentina, and Chile, with an overall frequency of approximately 40%. If haplotype C was introduced into South America by *J. villida* dispersing across the Pacific Ocean, this suggests that the migrants made landfall in the southern part of the continent. The haplotype frequencies of *J. vestina* populations are very distinct from those of nearby populations of other *Junonia* species, suggesting that either selection is acting against some alleles in *J. vestina* or that hybridization events involving *J. vestina* may be less common than hybridization between other New World *Junonia* species.

The invasion process of the fall armyworm *Spodoptera frugiperda*

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The fall armyworm (FAW), is a major agricultural pest from the Americas. Since its first detection in West Africa in 2016, it has rapidly spread across Africa, Asia, and Oceania, causing significant losses in maize production.

The success process of an invasion typically follows multiple steps. First, an alien species is introduced outside its native range. Then, the invasive species overcomes competition with native species, predation, disease, or disfavorable environmental conditions to establish a stable population. Once established, the species increased its range expansion. A delay between the introduction and expansion of the distribution area has often been observed during invasions, and this period has been described as a 'lag phase', i.e. a period during which introduced populations remain at low densities before expanding. This phase is crucial, as eradicating an introduced pest species is only realistic during this period, before adaptive evolution, genetic drift, or demographic growth facilitates establishment in new environments. To better understand the dynamics of this invasion, we analyzed whole-genome sequences from 177 individuals collected across 12 locations on four continents to infer the evolutionary processes of invasion. Adaptive evolution specific to invasive populations was observed in detoxification, chemosensory, and digestion genes potentially contributing to the invasion's success. We demonstrate that the FAW invasion involved an undocumented lag phase. Invasive FAW populations have negative signs of genomic Tajima's D, and invasive population-specific genetic variations have notably decreased Tajima's D, supporting a substantial amount of time for new mutations to arrive in introduced FAW populations. Model-based diffusion approximations support the existence of a period with a cessation of gene flow between native and invasive FAW populations. Taken together, these results corroborate the presence of a lag phase during the FAW invasion and show the useful- ness of using population genomics analyses to identify lag phases in biological invasions.

Introgression Detection and Adaptive Impacts: Insights from Heliconius

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Heliconius butterflies are known for their highly diverse wing patterns and reticulate evolutionary relationships, which are a classical system for studying speciation, gene flow, and adaptation. How- ever, beyond wing patterns, the genetic mechanisms and evolutionary history of other morphological or behavioral traits remain poorly understood. By analyzing genome-wide differentiation patterns among closely related *Heliconius* butterflies, we pinpointed a highly divergent genomic locus that emerged during the early stages of species differentiation. The locus exhibited strong linkage disequilibrium, showed signatures of natural selection, and is functionally associated with insect locomotion. Further analyses revealed that the locus was an introgression hotspot and had a complex evolutionary history, which was distinct from that of the species and wing color pattern loci. These results suggested that hybridization can fuel divergence and facilitate early-stage speciation by introducing adaptive variations.

The insights from *Heliconius* butterflies also suggest that resolving the phylogenetic relationships among taxa remains a challenge due to the presence of gene flow. Thus, we have developed a deep learning-based approach, ERICA (Evolutionary Relationship Inference using a CNN-based Approach), to quantify the evolutionary relationships and identify local introgression signals via topological discordance. Evaluation of simulated datasets showed ERICA approach was effective in detecting gene flow, particularly in adaptive introgression. By applying ERICA to the real genomic data of *Heliconius* butterflies, we identified the known introgressed loci associated with wing color patterns. We also evaluated the generalization ability of the method and showed it can be applied to different taxa, including animals and plants, indicating that the method can facilitate studies on hybridization and introgression.

Inter-continental introgression in the winter pine processionary moth species complex: phylogenetic and genomic perspectives

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The pine processionary moth, *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae) is a famous defoliator of forest trees and a threat to human and animal health due to the urticating setae carried by the larvae. It belongs to a Mediterranean species complex comprising 2 (*T. pityocampa* and *T. wilkinsoni*) to 5 species (with the recently described *T. cretensis*, *T. hellenica* and *T. mediterranea*) depending on authors and studies. Notably, *T. hellenica* was described from Greece from mitochondrial data only, and found phylogenetically close to the *T. pityocampa* Eastern-North African mitochondrial clade present in Libya. Morphological characters were recently analyzed and did not bring any significant phylogenetic information at this taxonomic scale. A number of previous molecular studies based on mitochondrial information and a few nuclear sequences raised controversial results and evidenced the occurrence of mito-nuclear discordance, which tend to blur the evolutionary scenario and question the species delimitation in the complex.
Using mitochondrial and RAD-seq markers on a set of individuals sampled all over the Mediterranean range of the complex, we could propose a robust phylogeny and decipher cases of mitochondrial introgressions and nuclear-mitochondrial discordance, which raises doubts about the validity of some recently described species. In particular, our results suggested that the occurrence of *T. hellenica* in Greece could correspond to a mitochondrial introgression. To explore this situation into more detail, we sampled pine processionary caterpillars all over Greece with a special focus in the Attica region where *T. hellenica* was previously detected via mitochondrial sequencing. The sampling was completed with few individuals from Libya where *T. hellenica* is also supposed to occur. Whole-genome sequencing showed that individuals collected in the Attica region mostly have *hellenica*-like mitochondrial haplotypes, with a high haplotypic diversity. On the contrary, their nuclear genomes are mostly *pityocampa*-like, but still bear signs of past introgression from Eastern North African individuals. Interestingly, the introgressed genomic regions were different between individuals, suggesting that the hybridization event was relatively ancient. New analyses will be necessary to estimate the age of the introgression and the evolutionary forces that shaped this intriguing pattern.

Thursday

Keynote 5: The developmental origin and regulation of a complex repeated trait: eyespots on the wings of Lepidoptera

Antónia Monteiro, Suriya Murugesan, Yuji Matsuoka, Heidi Connahs, Brian Hanotte, Beatriz Willink

Lepidopteran eyespots have evolved multiple times independently on the wings of butterflies and moths perhaps due to their successful predator attack diverting role. Within nymphalid butterflies, eyespots have a single origin and are homologous. The nymphalid eyespot gene-regulatory network (GRN) appears to be derived from a partial co-option of the appendage GRN due to shared genes and shared cis-regulatory elements of genes within the network. This eyespot GRN, which was initially activated on the ventral hindwing, became activated on the dorsal surface of both wings, many millions of years later. Experiments in one species indicate that the activation or silencing of the network in different wing sectors and surfaces is due to the regulatory action of selector genes such as Hox and Tbox genes.

Characterizing the olfactory system of *Spodoptera exigua* larvae

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While the olfactory system of adult Lepidoptera has been widely studied—particularly in the genus *Spodoptera*, where the pest *S. littoralis* has served as a key model for olfaction research for over a decade—the olfactory system of lepidopteran larvae remains largely understudied. Here, we investigate the organization of olfactory sensory neurons in *Spodoptera exigua* caterpillars, a significant greenhouse pest in Europe.
First, we conducted a detailed analysis of the olfactory organs (antennae and maxillary palps) of *S. exigua* larvae using scanning electron microscopy. We then examined the expression of key olfactory co-receptors—both odorant (Orco) and ionotropic receptors (Ir8a and Ir25a)—through RNA-seq and RNA fluorescence in situ hybridization (RNA-FISH) to explore their distribution and expression patterns within olfactory sensory neurons (OSNs).
Our findings reveal the presence of OSNs expressing Orco, Ir8a, and Ir25a in both olfactory organs. Moreover, RNA-FISH demonstrated the co-expression of multiple co-receptors within the same OSN, even across different olfactory receptor families. This challenges the traditional "one OSN = one type of olfactory receptor" paradigm and aligns with recent discoveries in *Drosophila* and mosquitoes.
This study provides new insights into the olfactory biology of *S. exigua*, which could contribute to the development of novel, olfaction-based pest control strategies.

Parallel evolution of silver iridescence dimorphisms in fritillary butterflies

Luca Livraghi
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Color variations serve as compelling models for studying the genetic mechanisms of phenotypic variation and whether this process repeats itself during evolution. Wild populations of the Mormon Fritillary butterfly (*Speyeria mormonia*) are polymorphic for silver iridescence, with morphs displaying spots that are either reflective or dull. Using Genome Wide Association in two populations, we showed the dimorphism maps to the 3' region of the transcription factor gene *optix* in two distant populations. The expression of *optix* is confined to the unsilvered regions that surround the silver scales, and these patterns are transformed to a silver identity upon *optix* RNAi knockdown, implicating *optix* as a repressor of silver scales in this Neoarctic butterfly. We further show that a similar polymorphism in a related Palearctic argynnine butterfly, *Fabriciana adippe*, is also associated with the *optix* locus. This finding provides a remarkable instance of gene reuse, where distinct lineages utilize the same genetic architecture to achieve convergent adaptive outcomes. Ongoing research aims to determine whether this polymorphism in *F. adippe* and other fritillaries arises from the reten- tion of ancestral alleles across the Argynnini radiation, or through independent de novo mutations. These insights contribute to our understanding of the evolutionary dynamics that shape the repeated evolution of adaptive traits.

Exploring the genetic basis of colour polymorphism in Apamea using museomics

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Colour polymorphism in Lepidoptera has long fascinated evolutionary biologists due to its ecological importance in predator avoidance and sexual selection. Uncovering the genetic basis of this trait is essential to understand the dynamics of colour polymorphisms as it reflects both the adaptative potential as well as the constraints within this order. Historical collections represent an invaluable resource for such research, harbouring vast diversity of rare and sometimes extinct species. Moreover, modern technologies

now enable us to unlock the potential of these archives for genetic research. For this study we used the historic entomological collection of the Natural History Museum Naturalis (Leiden, NL) to investigate genomic differences between dark and light colour morphs of two *Apamea* species, A. crenata and *A. monoglypha*. DNA was extracted from 200 specimens (100 per species, 50 per colour morph) dating from 1908 to 2006. Whole genome sequencing revealed colour- associated variants for both species near the *cortex* gene. Additionally, the *cortex* gene appeared inverted between the two species. Our findings support the standing hypothesis of conservation of colour polymorphism genes in Lepidoptera while highlighting species-specific molecular mechanisms, demonstrating the value of historic collections in evolutionary research.

The genetic mechanisms of leaf masquerade mimicry in *Kallima* butterflies

Dequn Teng; Wei Zhang
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The leaf masquerade mimicry of *Kallima* butterflies represents a striking example of adaptive evolution and has long fascinated biologists. However, the genetic and developmental mechanisms underlying this remarkable phenotype remain largely unknown. In this study, we integrated multi- omic analyses and functional validation to elucidate the genetic mechanisms shaping the leaf-wing patterns of *Kallima* butterflies.

First, we investigated the genetic basis of the leaf-wing polymorphism in *Kallima* butterflies. Forward genetic analysis identified the *cortex* locus as the key regulator of the leaf-wing polymorphism. Transcriptomic analysis and CRISPR/Cas9-mediated gene editing further confirmed that multiple genetic elements within the *cortex* locus play critical roles in regulating melanic wing patterning. Second, we explored the formation of the leaf-wing framework in *Kallima* butterflies. Morphological analysis demonstrated that the leaf-wing framework aligns with the Nymphalid Ground Plan. Further transcriptomic analysis, heparin injection experiments, and CRISPR/Cas9 gene editing revealed that the Wnt signaling pathway—particularly the *WntA* gene—contributes to the organization of the leaf-wing framework.

Together, our findings highlight the pivotal roles of the genetic toolkit (e.g., the *cortex* locus and the *WntA* gene) in shaping the leaf-wing patterns of *Kallima* butterflies, providing comprehensive insights into the genetic mechanisms driving phenotypic diversity and the evolution of complex morphological traits.

Effects of parasitic infections in generalist and specialist moths

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Costly immune responses can induce a change in dietary needs, resulting in diet shifts. Our research focuses on comparative analyses between generalist and specialist moths (Lepidoptera, Noctuidae) to determine the effects of infections on larval feeding behaviors, subsequent adult reproductive behaviors and next generations, as evolutionary shifts depend on the level of gene flow between individuals and populations. We are focusing on important agricultural pests, against which there is an increased use of parasites and pathogens as biocontrol agents. We already found that immune challenges can significantly slow down the developmental time of individuals, which may result in non-random matings between healthy and immune-challenged individuals (Barthel et al. 2015, Staudacher et al. 2015, Gao et al. 2021), which likely affects the evolution of resistance. Infections may also significantly advance sexual activities to early at night (Gao et al. 2021), which may lead to the evolution of reproductively isolated sub-populations, even when in sympatry (Van Doorn et al. 2025). Here I will show our latest results on the effects on infections on host plant choice in larval and adult behaviors, and impacts on subsequent generations.

Keynote 6: Evolving Patterns: An Evo-Devo Exploration of Butterfly Wing elements

Anyi Mazo-Vargas
Duke University

Lepidoptera exhibits remarkable diversity in wing shapes and color patterns, traits often associated with adaptive roles. This diversity, combined with the relatively simple tissue structure of their wings, makes Lepidoptera a compelling model system for studying the development and evolution of morphologically complex and ecologically significant traits. Over the past decade, research on the evolution of butterfly color patterns has made remarkable strides, particularly in mapping phenotypic variation to discrete genomic loci. Notably, three loci, the ligand *WntA*, the non-coding RNA *mir-193/ivory*, and the transcription factor *optix*, have repeatedly emerged as key players, or "hotspots," in the evolution of wing pattern diversity across multiple species. This repeated association raises fundamental questions: Why do these loci serve as recurrent focal points for evolutionary change? What features of the regulatory networks in which they function confer such evolutionary flexibility? Our laboratory investigates the molecular intricacies that connect these loci to gain a mechanistic understanding of the gene regulatory networks underlying the evolution of butterfly wing patterns. I will share our recent findings, which combine advanced genomic, genetic perturbations with spatiotemporal expression analyses of key genes to elucidate the feedback mechanisms, gene interactions, and temporal dynamics that define wing patterns in two butterfly species.

An esterase affects pheromone components important for reproductive isolation between two closely related moth species

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Sexual signaling by pheromones is essential for mate finding and mate choice in moths, and plays an important role in reproductive isolation. Acetate esters produced by females of *Heliothis* (*Cloridea*) *subflexa* attract conspecific males but repel H. virescens males. A QTL (quantitative trait locus) harboring carboxylesterases and lipases was previously shown to affect acetates, and CRISPR/Cas9- induced knockouts increased acetate amounts by blocking hydrolysis of the esters as expected. A second, unlinked QTL, containing a cluster of three different carboxylesterases, unexpectedly yielded the opposite result. Decreased acetate amounts were observed in a naturally-occurring transposable element insertion in exon 8 of the esterase CXE24, as well as in a CRISPR/Cas9-induced frameshift at the same position. The paradox was resolved by a CRISPR/Cas9-induced frameshift in exon 2 of CXE24 which increased acetate amounts. The frameshift in exon 2 produced a truncated protein lacking the substrate binding site and the catalytic triad, while the frameshift in exon 8 removed only the third residue of the catalytic triad. In silico modelling showed that the exon 8-truncated protein could bind acetate ester substrates and protect them from hydrolysis by other esterases, but could not hydrolyze the esters by itself. To place our findings in an evolutionary context, we explored variation in the esterase cluster in 16 species of Lepidoptera with completely sequenced genomes. A cline in acetate levels has been observed in *H. subflexa*, and we hypothesize that variation in the frequency of the transposable element could be a possible explanation.

Single-nucleus RNA-seq analysis of dichotomous spermatogenesis in silkmoth, *Bombyx mori*

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Like in most lepidopteran species, males of the silkmoth (*Bombyx mori*) produce two distinct sperm morphs:fertilizing eupyrene sperm and non-fertilizing apyrene sperm. Apyrene sperm lack a nucleus and nuclear DNA, but are nonetheless essential for successful fertilization of ova. The molecular genetic basis governing this developmental process of dichotomous spermatogenesis remains enigmatic. Currently, only a few genes are known to differentially impact the development of the two sperm morphs. With the aim of broadly surveying the differences in gene expression associated with eupyrene versus apyrene spermatogenesis, we generated single-nucleus RNA-sequencing data from testes isolated from 5th instar larvae and 4-day old pupae. Since the transition from eupyrene to apyrene spermatogenesis occurs during pupation, these samples should capture transcriptional differences occurring between the two sperm morphs. Comparative analysis with *Drosophila melanogaster* allowed us to identify cell clusters corresponding to major cell type in spermatogenesis (e.g. spermatogonia, spermatocyte, spermatid, etc.), as well as several other testes-associated somatic cell types. We identified two distinct sets of cell clusters reflecting developmental trajectories corresponding to apyrene and eupyrene spermatogenesis. We identified >1000 genes differentially expressed between apyrene and eupyrene spermatogenesis. Notably, previous microscopic investigations suggest a major difference between apyrene versus eupyrene spermatogenesis in the role of the synaptonemal complex, but differential expression analyses between cell clusters do not support this hypothesis.

Strain-specific responses in silk-related gene expression influenced by dietary differences in *Bombyx mori*

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National Agriculture and Food Research Organization

The silkworm (*Bombyx mori*), which primarily feeds on mulberry leaves, is used industrially for raw silk production. Although artificial diets have been developed as alternative nutrient sources, silkworms reared on these diets have lower productivity than those reared on mulberry leaves. The differences in silk gland gene expression during the late fifth instar stage, when silk synthesis is most active, between silkworms raised on artificial diets and those raised on mulberry leaves, remain unclear. In this study, we analyzed the transcriptomes of the middle and posterior silk glands of fifth instar day 5 silkworm larvae reared on artificial diets and mulberry leaves using three strains: Daizo, Nichi01, and J137×C146. Our results revealed strain-dependent differential expression of silk- related genes. In the middle silk gland, fibrohexamerin (*fhx*), fibroin-light-chain (*fibL*), and fibroin- heavy-chain (*fibH*) were upregulated in Nichi01 and downregulated in J137×C146 when reared on artificial diets compared with mulberry leaves. In the posterior silk gland, ser1 was upregulated in J137×C146 under the same conditions. Co-expression analysis identified transcription factors that were co-expressed with silk-related genes. Notably, Hox genes, which were not previously reported to regulate silk-related genes, were found to be co-expressed. This suggests that the response of these genes to dietary differences may also be regulated by Hox genes, distinct from previously known
Hox genes, such as *Awh* and *Antp*. These findings demonstrate that both diet and strain significantly influence the expression of genes related to silk production in the silk glands of *Bombyx mori* during the late fifth instar stage. This study provides new insights into the molecular mechanisms underlying the nutritional regulation of silk production by different silkworm strains.

Friday

Keynote 7: Diversification in Alpine butterflies: from macroevolutionary patterns to microevolutionary processes

Kay Lucek
University of Neuchatel

The order of Lepidoptera represents one of the most diverse branches of the Tree of Life. However, the evolutionary drivers of this unique diversity are far from being understood. A potentially overlooked mechanism lies in the structure of the genome itself as Lepidoptera have so-called holocentric chromosomes that lack single centromeres. A consequence of holocentricity may be an increased potential for genomic rearrangements such as chromosomal fusions and fissions. I will present the astonishing diversity of chromosomal rearrangements and explore their potential role for species diversification, ranging from macroevolutionary patterns to microevolutionary processes. I will discuss different potential mechanisms that could underlie chromosomal rearrangements, including differences in the repeat landscape and epigenomic shifts. The focal species are Erebia butterflies, a highly diverse group of primarily cold-adapted butterflies. *Erebia* sibling species are also often strongly diverged and form very narrow zones of secondary contact, allowing to further study additional barriers to gene flow at a late stage of speciation. But what could they be?

Evolution and diversification of mimicry and masquerade in butterflies

Wei Zhang
Peking University

With over 18,700 species of butterflies, butterfly wings are relatively simple in structure but display complex patterns and functions, serving as a classic system for studying the evolutionary mechanisms of biodiversity. During the past decade, we investigated the evolution and genetic mechanisms of Batesian mimicry and Müllerian mimicry in multiple butterfly species and developed the butterfly genome editing technology and deep learning-based algorithms for genomic analysis, pro- viding a scientific foundation for us to deeply address butterfly diversification and adaptation. We recently established a research system using oakleaf butterflies in the genus *Kallima,* which have polymorphic wing phenotypes to masquerade as dead leaves. We integrated multi-omic data analyses and functional validation to infer the evolutionary history of *Kallima* species and investigate the genetic basis of their variable leaf wing patterns, providing macroevolutionary and microevolutionary insights into a model species originating from a mountain ecosystem. Currently, we are further exploring the molecular mechanisms of butterfly adaptation based on our understanding of these mimicry systems, which can provide a research paradigm for exploring solutions to promote human health based on biodiversity and biological evolution.

A climate change winner: How warmer and brighter winters trig- ger the expansion of the pine processionary moth

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The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775), is a Mediterranean species and a major defoliator of conifers. Its larvae also pose serious public health risks, as they can trigger allergic reactions in humans, pets, and livestock. This species undergoes winter larval development in conspicuous silk nests, making it highly sensitive to minimum winter temperatures. The PPM has been expanding its range northward since the 1980s due to rising winter temperatures driven by climate change.
This study aims to better understand the effects of climate on this distribution shift and to identify the environmental factors limiting the northward expansion of PPM in Europe. We calibrated three distribution models (Bayesian Additive Regression Trees, Boosted Regression Trees, and Random Forest) using both historical and modern occurrence data. We incorporated climatic variables reflecting the impact of temperature (winter minimum temperatures, summer maximum temperatures) and, for the first time, the downward shortwave radiative flux reaching the ground. This approach
allowed us to account for various thermal constraints affecting both eggs and larvae throughout their development.
Our results show that under current conditions, PPM could extend its range further north. How- ever, we hypothesize that its limited flight capabilities hinder its ability to keep pace with climate change. Future projections indicate continued northward expansion, although solar radiation is expected to constrain the northernmost limit of PPM's range. Our findings highlight regions that are likely to become suitable for PPM colonization, where proactive management measures could be implemented.
Keywords: Ecoloy, Pine processionary moth, species distribution modeling, climate change, range shift, climate-driven ecological constraint

Kynurenine 3-monooxygenase disruption by CRISPR/Cas9 in *Spodoptera exigua*: phenotype, microbial diversity, and fitness costs

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Insects have intimate relationships with their gut microbiota, where bacteria contribute to essential functions in their invertebrate hosts. Thus, maintaining gut microbiota homeostasis may be crucial for preserving host fitness.
Here, we investigate the role of the enzyme 3-hydroxykynurenine monooxygenase (kmo) in shaping the gut microbiota of *Spodoptera exigua*, a significant pest of various horticultural and ornamental plants in Europe. Using CRISPR/Cas9, we generated a *kmo* knockout (KO) and examined the im- pact on gut microbiota composition and caterpillar fitness under both artificial diet and plant-based conditions. Our findings reveal that *kmo* knockout insects exhibit a dramatic shift in gut microbiota composition, particularly a reduction in bacterial diversity. Notably, in the oral secretion of KO larvae, a single *Enterococcus* amplicon sequence variant (ASV) replaced two *Enterococcus* ASVs typically present in wild-type larvae. Despite these microbiota changes, we did not observe a clear reduction in fitness when larvae were reared under standard laboratory conditions with an artificial diet. However, under more restrictive conditions—such as feeding on pepper leaves instead of an artificial diet—KO larvae

experienced delayed growth.
Our results highlight the role of gut bacteria in supporting lepidopteran larval development and emphasize that the quality and nature of the host diet can be a determining factor in revealing microbial effects on insect fitness.

REPAT proteins as mediators of pathogen-insect-plant interactions in *Spodoptera exigua*

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Recent discoveries highlight the role of lepidopteran secreted proteins in modulating jasmonate- driven plant defense responses by interacting with repressors or transcription factors involved in the JA-signaling cascade. Some of these orally secreted proteins, identified in *Helicoverpa armigera* and *Tuta absoluta*, belong to the REPAT protein family, first discovered in Spodoptera exigua two decades ago due to their upregulation following microbial infection (hence the name REsponse to PAThogen). Here, we identify 303 REPATs across six moth genomes. Phylogenomic analyses reveal that *Spodoptera* species possess an unusually high REPAT gene content compared to Bombyx mori, *Tuta absoluta* and the noctuid *Helicoverpa armigera*. We find some REPATs exhibiting one-to-one orthology and scattered across chromosomes, whereas REPATs likely involved in plant-insect interactions are found in clusters. Spodoptera species also harbor additional REPAT clusters that lack the multi-bridge factor 2 (mbf2) domain hallmark present in all other REPATs. To unravel the physiological function of REPATs, and their link with pathogen infection and plant defense response, we focused on the orally secreted REPATs in *Spodoptera exigua*. Differential proteomics revealed a decrease in specific REPATs in the oral secretion of caterpillars infected with the *Spodoptera exigua* iflavirus 1, which ultimately correlated with an enhanced plant defense response upon herbivory by infected caterpillars. These results provide the first evidence of REPATs acting as a molecular bridge between viral infection in the insect and the plant's defense response.

Dispersal ability shapes genomic response to habitat fragmentation in widespread "non-threatened" butterflies

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Habitat fragmentation reshapes landscapes, disrupts dispersal and gene flow, subsequently increasing the extinction risk for even widespread species. Yet, the genomic consequences in widespread and non-threatened taxa remain poorly understood. In this study, I explore how innate dispersal ability modulates population genomic responses to habitat fragmentation in seven widespread Danish butterflies: *Aglais urticae*, *Pieris napi*, *Gonepteryx rhamni* (highly dispersive), *Maniola jurtina* (intermediate), and *Thymelicus lineola*, *Lycaena virgaureae*, *Coenonympha tullia* (sedentary). All are currently evaluated as of Least Concern (IUCN), yet differ markedly in dispersal ability and habitat specialization. Using whole-genome resequencing and species-specific habitat suitability mapping, I assess genetic diversity, population structure, inbreeding, and effective population size (Ne). Despite comparable habitat amounts, sedentary species show increased runs of homozygosity and greater individual-level variation in heterozygosity, while highly mobile species exhibit genetic signatures of connectivity and minimal inbreeding. All species show signs of recent Ne decline, but no current reduction in nucleotide diversity—suggesting a "drift debt" where inbreeding precedes detectable diversity loss. These results emphasize that even common butterflies may undergo subtle genomic erosion, with dispersal ability acting as a buffer against the effects of fragmentation. The study underscores the importance of integrating life-history traits with genomic and spatial data when assessing population genetic resilience. Butterflies are widely recognized as bioindicators of environmental change and as habitat loss and fragmentation continues, butterflies may serve as early genomic sentinels of biodiversity loss.

Posters

1. A characterization of piARNs, their biogenesis and their targets in *Spodoptera frugiperda* (Lepidoptera, Noctuidae)

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A defence mechanism based on small RNAs called piRNAs (PIWI-interacting RNAs) has evolved to protect the germline from the deleterious effects of transposon (Tn) mobility in genomes such as mutations, deletions or chromosomal rearrangements. Like other small non-coding RNAs (miRNAs and siRNAs), piRNAs have a specific size range (24-36nt) and interact with proteins of the Argonaute family which they guide to their regulatory targets by homology, typically leading to decrease in their expression. The Argonaute protein family consists of two clades AGO and PIWI, the latter showing genes copy number variations depending on the species. PIWI-interacting RNAs not only regulate Tns but also some genes in the germline, and a somatic piRNA pathway has been discovered and shown to be conserved in 20 species of Arthropods. This somatic regulatory pathway targets transposons but also viruses in some insect species.Biogenesis of piRNAs has been well described in *Drosophila*. Primary piRNAs are produced by slicing of large RNA precursors transcribed from genomic regions containing remnants of Tn, the piRNA clusters. These primary piRNAs guide a protein of the PIWI family (AUB) to transcripts of TE leading by cleavage to new so called secondary piRNAs. These latter guide another protein of the PIWI clade, AGO3 to cleave piRNA cluster transcripts through an amplification loop called the Ping-Pong loop. .*Spodoptera frugiperda* also called Fall armyworm (FAW) is a Lepidopteran pest of crops, described as two strains with different host-plant range (Fiteni et al., 2022; Pashley and Martin, 1987), the corn strain mostly found on corn, the rice strain associated to grasses. Since publication of their genomes (Gouin et al., 2017), miRNAs genes have been annotated (Gimenez et al., 2021; Mone et al., 2018) but almost nothing is known about the piRNA pathway except that PIWI protein homologs play a role in antiviral response (Xia et al., 2023). We will present a phylogeny of the AGO proteins annotated in Spodoptera frugiperda, their expression pattern in gonadal and non-gonadal tissues, CRISPR-Cas9 edition of one of the genes encoding proteins of the PIWI clade and its cellular localization. We have performed piRNA clusters annotation in the two strains genomes, and identified Long Non-Coding RNAs (LNCR) homologous to TE - putative sources of piRNAs. Regarding the piRNA targets, we have compared transposable elements contents in equivalent genome assemblies of the two strains as well as their regulatory patterns by piRNAs. We will also comment on a choice of cellular genes regulated by piRNAs in Spodoptera frugiperda.

2. Pattern recognition, hemolymph protease-14 activation, and enhancement of lysozyme-mediated bacteria killing by soluble peptidoglycan recognition proteins in *Manduca sexta*

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Peptidoglycan recognition proteins (PGRPs) detect invading bacteria during insect immune responses, and some can damage bacterial cell walls. We previously produced *M. sexta* PGRPs 1–5, 12, and 13, and demonstrated that the PGRP repertoire in hemolymph preferentially detects meso-diaminopimelic acid-peptidoglycans (DAP-PGs). In this study, we found that adding DAP-PGs and PGRPs to larval hemolymph significantly enhanced prophenoloxidase activation beyond the sum of their individual effects. Lys-PG of Micrococcus luteus and PGRP4/5 also displayed the synergy, but Lys-PG of Staphyloccous aureus did not. Structural modeling and ligand docking supported the preferential binding of DAP-PGs over Lys-PGs. DAP/Lys-PG, PGRP3s/3f/5/13N, and microbe binding protein

activated hemolymph protease-14 (HP14), suggesting that these PGRPs initiate the serine protease system in the same way as PGRP1. Using fluorescein-labeled M. luteus peptidoglycan as a substrate, we detected increases in fluorescence signal caused by PGRP2, 4, 13N, 12e, and 3f, suggesting that these PGRPs have amidase activity for hydrolyzing peptidoglycan, which was enhanced by Zn2+ and decreased by EDTA. Spatial locations of the catalytic residues, Zn2+ ion, and scissile bond in the models of PGRP-peptidoglycan complexes explained some of the activity differences. PGRP2 and PGRP4 had the highest specific activity. Only PGRP4 (60 μg/ml) decreased Bacillus megaterium colony-forming units (CFU) compared to controls, whereas other PGRPs did not affect CFU numbers. A mixture of PGRP1−5 or 3s (2 μg/ml) and Manduca lysozyme (20 μg/ml) significantly reduced CFU compared to lysozyme alone, even for PGRPs without amidase activity. Scanning electron microscopy revealed that lysozyme caused structural damage to the bacterial cell walls, and when combined with PGRP2, this effect was enhanced. In summary, the soluble PGRPs preferentially recognize DAP-PGs, stimulate melanization via HP14, and enhance bacterial killing by lysozyme. Mechanisms for the amidase-independent bacterial killing by PGRPs and lysozyme require further exploration.

3. Hemolymph protease-17b activates proHP6 to stimulate melanization and Toll signaling in *Manduca sexta*

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Manduca sexta hemolymph protease-6 (HP6) plays a central role in coordinating antimicrobial responses, such as prophenoloxidase (PPO) activation and Toll signaling. Our previous studies indicated that HP5 and GP6 activate proHP6 in larval hemolymph and extraembryonic tissues, respectively. Here, we report the characterization of HP17b as another HP6 activating enzyme and its regulation by multiple serpins in hemolymph. The precursor of HP17b expressed in baculovirus infected Sf9 cells became spontaneously cleaved at two sites, and these products were purified together in one preparation named HP17b', a mixture of proHP17b, a 35 kDa intermediate, and HP17b. HP17b'converted proHP6 to HP6. As reported before, HP6 converted precursors of PPO activating protease-1 (PAP1) and HP8 to their active forms. HP8 activates proSpätzle-1 to turn on Toll signaling. We found HP17b'directly activated proSPHI and II to form a cofactor for PPO activation by PAP1. Supplementation of larval hemolymph with HP17b', HP17b, or proHP17b significantly in- creased PPO activation. Adding Micrococcus luteus to the reactions did not enhance PPO activation in the reactions containing HP17b', HP17b, or proHP17b. Using HP17b antibodies, we isolated from induced plasma HP17b fragments and associated proteins (e.g., serpin-4). Serpin-1A, 1J, 1J', 4, 5, or 6 reduced the activation of proHP6 by HP17b'through formation of covalent complexes with active HP17b. We detected an activity for proHP17b cleavage in hemolymph from bar-stage pharate pupae but failed to purify the protease due to its high instability. Other known HPs did not activate proHP17b in vitro. Together, these results suggest that HP17b is a key clip-domain protease activated by an unknown endopeptidase in response to a danger signal (e.g., wounding) and regulated by multiple serpins.

4. Enhancing Europe's readiness for managing Fall Armyworm, an invasive pest threat (EUFAWREADY)

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The fall armyworm (FAW) is an invasive and highly polyphagous insect pest originally from the Americas. This species first invaded Africa in 2016 and subsequently worldwide till western Asia. Detected end of 2023 in mainland Europe (Greece, Turkey and Romania), and most recently in Bulgaria

(EPPO, 2025a), FAW represents today a major risk to European agriculture, as the pest may establish and spread in a large part of Europe, particularly in the context of global warming and extensive commercial exchanges with and within the EU.
The objective of EUFAWREADY is to empower European agricultural stakeholders (including farmers, advisors, technicians, and phytosanitary services) in enhancing their readiness in case of FAW outbreaks. This involves providing them with tools to detect the presence of the pest at the earliest possible stage and to effectively manage the situation without relying heavily on synthetic pesticides.
The specific key objectives of EUFAWREADY are to: i) Improve stakeholder awareness and preparedness on FAW risks, and enhance their engagement in FAW management efforts; ii) Generate new knowledge on the biology of European FAW populations to determine the traits enhancing their invasion potential; iii) Evaluate the economic and environmental impacts that the arrival of FAW may induce in Europe; iv) Provide European stakeholders with efficient strategies for the early detection and monitoring of FAW; v) Leverage natural enemies to broaden the range of sustainable control options available to European farmers ; vi) Explore microbial agents to enhance eco-friendly crop protection for European farmers; vii) Diversify FAW control strategies in Europe by exploring cutting-edge plant-based and natural semiochemical solutions; viii) Integrate management solutions into guidelines and toolboxes for European end-users and ix) Make the project's scientific and technical results accessible to key stakeholders, ensuring they are fully aware of FAW risks and management options.
The international and multidisciplinary consortium consists of 25 partner organisations from 13 European countries, Israel, New Zealand and South Korea, and is coordinated by INRAE (Anne-Nathalie Volkoff, DGIMI, France). The overall project approach builds upon the pillars "learn", "detect" and "manage". These components will be integrated into toolboxes tailored to the needs of targeted stakeholders. DGIMI, UvA and iEES-Paris will more particularly contribute to a better understanding of the factors that may drive FAW adaptation to EU environmental conditions, and to the identification of novel FAW management tools suitable to Europe.
Funding: EUFAWREADY is funded by the European Union, HORIZON-CL6-2024-FARM2FORK-03, Project 101212676. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Commission. Neither the European Union nor the European Commission can be held responsible for them.

5. Discovery of a Proline-Rich, Highly Regular Heavy Fibroin Chain with Unique Mechanical Properties in Leaf Roller Moths (Tortricidae)

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Insect larvae, including those of Lepidoptera and Trichoptera, produce silk fibers with exceptional mechanical properties, which they use to build cocoons, feeding tubes and nests that protect caterpillars and pupae from predators and parasites. We selected the silk of *Cydia pomonella* (Lepidoptera, Tortricidae) for a detailed analysis of its proteins and its evolutionary relationship with other Lepidopteran silks. In this study, we used an integrative approach combining transcriptomics, proteomics, Fourier transform infrared spectroscopy (FTIR) and tensile tests to investigate the molecular composition and mechanical properties of the silk. Our analysis identified key silk-associated proteins, including fibroin heavy chain (Fib-H), fibroin light chain (Fib-L), fibrohexamerin/P25, seroins, mucins, zonadhesins, and several novel proteins with no known homologs. We focused on the repetitive Fib-H sequences, which determine the mechanical properties of silk through their β-sheet structure. In addition, we analyzed the unique cocoon architecture, molecular composition and mechanical properties of *C. pomonella* silk. Our data show that *C. pomonella* fibers exhibit high tensile strength despite their relatively short crystalline motifs. Our study provides interesting insights into how sequence variations affect the mechanical properties of silk and could contribute to the development of synthetic biomaterials.

6. On the role of Masculinizer in sex determination of the speckled wood butterfly, *Pararge aegeria*

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A new mechanism of sex determination based on the zygosity of alleles of the Masculinizer gene, was recently discovered in the butterfly *Bicyclus anynana* (Satyrinae), which has raised the question whether this system is also found in other satyrid species. Preliminary data have shown that this sex determination mechanism probably also exists in other satyrid species, however *Pararge aegeria*, which also belongs into this subfamily has lost a hypervariable region which is essential for zygosity- based sex determination. Therefore, in *P. aegeria* a different sex determination mechanism must have recently evolved. In the genome of *P. aegeria*, three slightly different copies of the Masc gene were found instead of one. These copies are all clustered together on the Z chromosome and one of them (MascB) contains seven genomic copies of exon 6, that are tandemly repeated between exons 8 and 9 and alternatively spliced with 0-6 copies per transcript. In the first step of our research, we are investigating whether this constitution of the Masc gene is unique to *P. aegeria tircis*, a subspecies in which this constitution was discovered, or whether it is also present in other subspecies such as *P. aegeria aegeria* and species of the same genus such as *P. xiphia* and *P. xiphioides*, or in other closely related species belonging to the subtribe Parargina such as Lopinga, Lasiommata, Kirinia, Tatinga and Chonala.
Our initial results show that *P. xiphia* hasn't lost the hypervariable region which suggests that the loss of this region occurred in the Pararge genus and the remaining Parargina species have retained it. This suggests that the *P. aegeria* sex determination mechanism evolved very recently. In a next step, we will investigate whether all Masc copies are involved in the sex determination of *P aegeria*.

7. Evolution of gustatory receptors and their role in ecological speciation

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Understanding the genetic basis of ecological speciation is still limited. It is however clear that in many phytophagous insects host specialization plays an important role. In Lepidoptera, there are many complexes of closely related species that feed and oviposit only on particular hosts. Change in host acceptance is one of the very first steps in the evolution of such species complexes.
Small ermine moths (*Yponomeuta*) provide a good example of host specialization and speciation. In this group, a major shift from Celastraceae (which contain the sugar alcohol dulcitol) to Rosaceae (which contain the stereoisomer sorbitol) occurred. The gustatory receptor sensitivity in the different species reflects this change. It has been suggested that a "phytochemical bridge" formed by the presence of a small amount of dulcitol - in addition to sorbitol- in *Prunus padus* (Rosaceae) facilitated the host shift from Celastraceae to Rosaceae.
To investigate this hypothesis, we made an inventory of the gustatory receptor genes in 5 species of small ermine moths from both host specialization types. Although around 30 receptor genes are present in each species, the actual ligands for these newly found receptors are still unknown. However, recent progress in receptor folding, molecular docking, and molecular dynamics simulations now makes it possible to predict candidate ligands. Here we report a proof of concept for this approach. The results will guide future heterologous expression experiments to confirm activity of the ligands. This is turn will form the basis of a reconstruction of the evolutionary changes in receptor repertoire that allowed the host shifts and subsequent ecological speciation.

8. Developing transgenic *Galleria mellonella* for the study of bacterial infections

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G. mellonella is acquiring an important role as a replacement model for infection studies due to similarities between mammalian and their innate immune response and has been reported to be a suitable model for several pathogens.This animal offers multiple benefits such as short lifecycle, abundant offspring and easy and unexpensive maintenance, breeding, and handling.
Currently the only readouts used for this infection studies with *G. mellonella* are survival assays and observable melanisation over time.
At the *Galleria mellonella* Research Centre (GMRC), we use our own lab grade reared larvae, we use quantitative readouts on phagocytosis through flow cytometry, and have stable transgenic lines of *G. mellonella*.
The aims of my project are to assess the interaction between intracellular bacteria *Burkholderia thailandensis* with the immune system of *Galleria* larvae, understand time-course and distribution of infection through bioimaging and quantitative readouts of phagocytosis using flow cytometry and to develop transgenic lines responsive to infection using PiggyBac mediated transgenesis.

9. Female remating behavior in a polyandrous butterfly: the effect of male colour pattern and age

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In polyandrous insects, females can remate with multiple males to improve their fitness. Multiple mate choice decisions can be influenced by phenotypic traits of the male, and may depend on some attributes of the last male she mated with. To increase their fitness, females may become choosier and 'trade-up' male quality with successive matings. In this work, we investigated how male age, size, and wing color pattern affect female remating decisions, specifically the likelihood and delay of remating, in *Heliconius numata*, a butterfly species with remarkable variation in wing coloration within populations. In *H. numata*, mating between individuals with the same wing coloration(assortative) produces less viable offspring than matings between individuals with different coloration patterns (disassortative). We found that females of the two morphs (*H. n. bicoloratus and H. n. euphrasius*) assessed preferred to remate with *H.n. euphrasius* males, with a trend for a higher preference if the first mating was disassortative, suggesting that remating preference might depend on the first mating type. Moreover, we observed that the remating delay was influenced by their first mating and by the morph of the second male. Additionally, male age also influenced female remating behaviour, since females that first mated with older males remated sooner than those that mated with younger males. These findings suggest that male wing color pattern and age may influence female multiple mating behaviour in *H. numata*, and that females may adjust their sequential mating decisions based on traits of the male they previously mated with.