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WORKING MATERIAL

Dormancy management to enable mass-rearing and increase efficacy of sterile insects and natural enemies.

Report of the First Research Coordination Meeting of an FAO/IAEA Coordinated Research Project, held in Vienna, Austria, from 21 to 25 July 2014

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BACKGROUND

Insect pests cause significant and widespread damage worldwide, and insecticides remain the predominant method of control. According to FAO, 30-40 % of agricultural production is lost to pre-harvest and post-harvest infestations, mainly caused by insect pests. Despite growing worldwide dependence on agrochemicals, suppression of pest populations is frequently inadequate. In addition, due to regulation, pest resistance, and environmental and human health concerns, there is an increasing demand for the replacement of the intensive use of these chemicals by environmentally friendly, effective and sustainable methods within integrated pest management approaches. Chief among these are biological control applications based on the use of sterile insects and natural enemies. Enhancing the efficacy of biological control requires an understanding of life-history phenology of the target pest or beneficial species.

Most insects face times of the year when reproduction or development are suppressed due to a lack of resources or unfavourable environmental conditions. Dormancy responses have evolved to mitigate the stresses of these unfavourable times and to synchronize insect life cycles with favourable periods. Dormancy responses can include both pre-programmed, hormonally mediated diapause, and also quiescence induced directly by the environment (e.g., low temperatures, drought, lack of hosts, etc.). Quiescence is a state of developmental arrest that can occur in any life stage. In contrast, diapause is a stage-specific developmental arrest that can be either facultative (determined by token stimuli) or obligate (occurring regardless of prevailing environmental conditions).

Many univoltine pest species have an obligatory diapause that synchronizes them with resource availability. For such univoltine insect pest species, sterile insect technique (SIT) and augmentative natural enemy control have not been either practical or possible due to obligatory diapause responses that prevent or interfere with continuous mass rearing. Examples include the European cherry fruit fly, apple maggot fly, Chinese citrus fruit fly, Russian melon fly, and processionary moths. Although obligatory diapause has been a major roadblock to developing biological control programs for many pests, current research suggests that there are approaches that can potentially disrupt obligatory diapause and facilitate mass rearing. Four approaches that appear particularly promising for circumventing the challenges of obligate diapause include: 1) simple environmental manipulations, such as thermal shock, 2) chemical or hormonal treatments, such as application of organic solvents, 3) choosing geographical populations without diapause or artificial selection for non-diapausing strains within populations, and 4) genetic modification by mutagenesis or transgenesis of critical genes for diapause. Successfully circumventing obligate diapause with any of these approaches, or a combination thereof, would provide new opportunities for effective mass rearing of important pest species.

Beyond dormancy there is a spectrum of responses that may increase the stress tolerance of insects, and many of these responses could possibly be exploited for mass rearing and biological control. For example, rapid cold-hardening responses can be induced in active insects. Similarly, variations in the storage environment (for example, via fluctuating thermal regimes) can significantly improve low temperature tolerance. Although insects stored at low temperatures are likely in a quiescent or dormant state, there remain opportunities to increase the duration and reduce the impacts of low temperature exposure via manipulations of storage conditions or physiological states.

While obligate diapause is an obstacle in some cases, two aspects of dormancy and other physiological tolerance responses can be effectively exploited to improve the efficacy of biological control programs.

- First, dormancy can be used to stockpile mass-reared insects and to time the supply of biological control agents to coincide with seasonal demand for release. The ability to synchronize the supply of control agents with demand is critical for the growing biological control industry. Furthermore, an enhanced understanding of dormancy responses could improve phenological models for both pest species and beneficials. Inducing dormant states or other physiological-conditioning treatments opens up new opportunities for either enhancing classical cryopreservation of embryos (in liquid nitrogen) or developing new methods for long-term cold storage of other life stages, such as larvae or pupae. Development of such techniques from an organismal or biochemical perspective could make it feasible to maintain strains over the long term without compromising the genetic integrity of those strains, while avoiding the efforts and costs involved in continuous rearing. This ability to maintain stocks without continuous rearing is especially important when considering the rapid accumulation of mutant and transgenic strains in entomological research laboratories.
- Second, increased stress tolerance is often a hallmark of dormancy, a feature that could be exploited in biological control applications. The efficacy of biological control, including sterile insect programs and natural enemy releases, is affected by the quality of insects released into the field. Poor performance of insects used in field releases can be a product of stresses experienced at multiple points during the production, marking, irradiation, shipping, and release process. The ability to specifically induce dormant states, including either diapause or quiescence, could potentially reduce the above stresses, thereby improving the performance of individuals in field releases. For example, some diapausing insects are known to be resistant to low-level irradiation. Perhaps diapause could be exploited to reduce off-target irradiation damage, outside of germ-line genomic DNA, and improve the performance of sterile insects. Similarly, insects are frequently exposed to mechanical disturbance, hypoxia, and thermal stress during shipping, stresses that may be mitigated by inducing dormant states prior to shipping.

Beyond applications to existing biological control tactics, there is an opportunity to develop the knowledge base needed to generate novel strategies for controlling pest populations by managing diapause and dormancy responses. For example, new approaches to prevent diapause, terminate diapause, or prolong diapause could be exploited to desynchronize insects from favorable environmental conditions, thus inducing “ecological suicide” in pest populations.

Dormancy management and biological control

Developing dormancy management tools could be important for biological control involving sterile insects or natural enemy releases. Eight key questions should be addressed:

1. Can dormancy, physiological conditioning, or storage conditions be used to manage insect life cycles to enable or improve the efficacy of mass rearing?
2. Can dormancy, physiological conditioning, or storage conditions be used to maintain the genetic integrity of laboratory strains?
3. Can an understanding of how environmental variation influences dormancy and physiological tolerances enhance our ability to build phenology models that forecast life-history transitions in pest and beneficial populations?

4. Can dormancy responses, physiological conditioning, or storage conditions be used to enable or enhance the shelf life of sterile insects and natural enemies while making them available for release upon demand?
5. Can dormancy responses, physiological conditioning, or storage conditions be used to reduce radiation injury and enhance sterile insect performance?
6. Can dormancy responses, physiological conditioning, or storage conditions be used to decrease shipping-related damage and enhance post-shipping performance of biological control agents?
7. Can manipulating the insect microbiome enhance or reduce stress tolerance and dormancy?
8. Can a greater understanding of dormancy responses or physiological conditioning foster the development of novel approaches for insect pest management, for example "ecological suicide"?

These questions are expanded in the following paragraphs:

Can dormancy responses, physiological conditioning, or storage conditions be used to manage insect life cycles to enable or improve the efficacy of mass rearing?

A critical challenge for mass-rearing efforts is that many pest species undergo an obligate diapause that prevents continuous rearing. Many obligate diapause responses necessitate a long period of cold storage to fulfil the biological requirements for continuation of development. This requirement for prolonged cold exposure introduces substantial time gaps that either present an obstacle for mass rearing or decrease the efficiency of the process. Reducing or circumventing the requirement for prolonged cold storage would substantially enhance the efficacy of mass rearing for such species. Several strategies may be used to manage diapause responses. First, diapause may be prevented by environmental, hormonal, or chemical manipulations. Second, insects may have their dormancy terminated prematurely through environmental, hormonal, or chemical manipulations. Third, genetic manipulations may be used to generate non-diapausing strains. These genetic manipulations include a variety of approaches including: artificial selection of strains, exploitation of natural geographic variation for strain foundation, and mutagenic or transgenic approaches for strain development.

Can dormancy responses, physiological conditioning, or storage conditions be used to maintain the genetic integrity of laboratory strains?

The loss of genetic variation and inadvertent artificial selection are challenges for all insect mass-rearing. Long-term rearing over many generations often leads to animals adapted to the mass-rearing environment, but leaving them less able to perform in the field. Dormancy responses may be used to preserve the original genetic integrity of strains, including heterozygosity and rare allelic diversity, as well as the specific genetic architecture of selected strains. Both diapause and other forms of physiological conditioning may be used to exploit new strategies for cryopreservation at very low temperatures in liquid nitrogen (-196^{o}C). Although most cryopreservation has focused on embryos, new strategies focusing on other life stages, such as larvae or pupae, may be effective. Another complementary strategy may be low-temperature cold storage (+10^{o}C to -10^{o}C) of either diapausing or non-diapausing embryos, larvae, pupae, or adults to maintain genetic integrity and to reduce the high costs of strain maintenance by continuous rearing.

Can an understanding of how environmental variation influences dormancy and physiological tolerance enhance our ability to build phenology models that forecast life-history transitions in pest and beneficial populations?

A fundamental aspect of biocontrol programmes is that the release of control agents within a field setting is synchronised with the appropriate developmental stage of the target species, e.g. adult (reproductive) stage for SIT, or host developmental stage for control with parasitoids. This is also relevant to synchronise pollinator release with flowering time. Being able to predict seasonal life history transitions under different environmental scenarios is therefore extremely valuable and will be greatly facilitated by the development of phenology models. The programming of diapause represents a pivotal point in the timing of life history events, and our investigations of how environmental variability influences the decision to enter diapause, diapause duration, the transition to post-diapause quiescence, and subsequent emergence will provide key parameters for these models.

Can dormancy responses, physiological conditioning, or storage conditions be used to enable or enhance the shelf life of sterile insects and natural enemies while making them available for release upon demand?

For both sterile insects and natural enemies, efficient deployment of these insects is achieved when their release coincides precisely with the appearance of pest populations in the field. Unfortunately, there is often a timing gap between the production of sterile insects or natural enemies and the demand for these agents in field releases. The ability to stockpile insects in a dormant state without loss of performance and to mobilize them quickly upon demand is essential for a viable biological control industry using sterile insects or natural enemies.

Can dormancy responses, physiological conditioning, or storage conditions be used to reduce radiation injury and enhance sterile insect performance?

While irradiation effectively induces the dominant lethal mutations necessary for germ-line sterility, irradiation also produces off-target somatic cellular damage that can decrease performance of sterile males in the field. The efficacy of sterile insects would be enhanced substantially if off-target effects of irradiation could be reduced. Increased stress resistance is a characteristic of dormant states and physiological conditioning in insects, thus applying radiation in the dormant state or after physiological conditioning may protect against off-target effects generated during irradiation.

Can dormancy responses, physiological conditioning, or storage conditions be managed to decrease shipping-related damage and enhance post-shipping performance of biological control agents?

Long-distance shipping from mass-rearing facilities to release sites is a common element encountered in the deployment of biological control agents. Shipping can be stressful because mass-reared insects are exposed to multiple stresses including hypoxia, altered barometric pressure, mechanical disturbance, and extremes of high and low temperature. The enhanced stress resistance displayed by dormant insects and insects after physiological conditioning may mitigate some of these perturbations and prevent shipping-related declines in performance of both sterile insects and natural enemies.

Can manipulating the microbiome of insects enhance or reduce stress tolerance and dormancy?

Insects carry a diverse array of microbes, both eukaryotic and prokaryotic, including symbionts, commensals, and pathogens. These microbes can occur externally, in the gut, and internally, including those microbes that are housed intracellularly. This microbiota can enhance nutritional efficiency, modify physiological responses to a range of stresses, and alter interactions with pathogens. In addition, immune responses constitute part of the generalised stress response, can be activated by a range of physiological stressors, and are likely essential for survival in the field, particularly in high-diversity tropical environments. Beyond the general stress response, there may be components of immunity that are specifically triggered by dormancy, physiological conditioning, or low-temperature storage. Composition of the microbiome in mass-reared insects is an area of growing research interest, yet we know little about how artificial diets, many of which contain antimicrobial compounds, affect insect microbiomes. Thus, understanding the role played by the microbiome in dormancy, physiological conditioning, and cold storage may open new avenues to improve mass-rearing techniques via microbiome manipulation, but this approach has not been explored.

Can a greater understanding of dormancy responses or physiological conditioning foster the development of novel approaches for insect pest management, for example "ecological suicide"?

Insect development is finely tuned to exploit favorable seasons of the year. Altering developmental patterns at the population level by preventing dormancy, breaking dormancy prematurely, or by delaying dormancy termination disrupts synchronization of the insect with its environment, making it vulnerable to unfavorable environmental conditions. This critical seasonal synchronization of dormancy with environmental conditions may be disrupted using hormonal, chemical, or genetic techniques. This knowledge could serve as a base for developing new strategies for pest insect population control.

CO-ORDINATED RESEARCH PROJECT (CRP)

This Coordinated Research Project (CRP) is based on a Consultants Meeting that was held from 7-11 May 2012 in Vienna, Austria (report available) to assess the potential for conducting co-ordinated R&D in dormancy management and cold tolerance of insects with the potential use in SIT application, and to formulate a proposal for a CRP on *Dormancy Management to Enable Mass-rearing and Increase of Sterile Insects and Natural Enemies*.

The overall objective of this new **CRP D4.10.25**, approved for the **period 2014-2019**, is to understand and harness dormancy management, physiological conditioning, and cold-storage approaches to enable mass-rearing of insect species previously difficult to rear and to enhance current mass-rearing efforts for biological control, specifically using sterile insects and natural enemies as part of an environmentally friendly, area-wide integrated pest management approach.

FIRST RESEARCH CO-ORDINATION MEETING (RCM)

Twenty-eight scientists from 17 countries attended this first RCM, held in Vienna, Austria from 21-25 July 2014. The list of participants, which included CRP contract and agreement holders, as well as 10 additional observers, is given in **Annex 1**. The agenda for the meeting is attached in **Annex 2**.

During the first two days of the meeting RCM participants presented research relevant to the CRP, as well as their research plans for the first year of the CRP.

During the last three days of the meeting, general discussions were held to define and review the thematic areas of the CRP, the review of the general and specific R&D objectives to be addressed during the 5 years of the CRP (**Sections 1 and 2 of this document**), and the CRP Logical Framework, in order to agree on minimum outputs to be achieved at the end of the CRP. Furthermore, participants were divided into two working groups (**Annex 3**) to develop more detailed R&D plans to be conducted during the first 18 months of the CRP.)

Abstracts of the presentations are compiled in **Annex 4** and a copy of all presentations was made available to all participants at the end of the RCM.

A glossary of technical terms frequently used in this field was revised and updated with the objective of harmonizing the exchange of information among participants, develop protocols and to be used in publications during the CRP (**Annex 5**).

1. MANIPULATING DORMANCY RESPONSES

1.1. Background Situation Analysis

When considering dormancy responses there is both diapause and environmentaly induced quiescence. The seasonal biology of dormancy in any particular species may include both diapause and quiescent periods. Manipulating seasonal biology to facilitate mass rearing will require understanding the three phases of diapause, induction, maintainance and termination, plus any related quiescence responses - all of which can be influenced by temperature (Figure 1). Our long-term goals are to:

- Eliminate the obligate diapause of target species to facilitate mass-rearing programs to support the development of new biological-control programs.
- Induce diapause to stockpile biological control agents, and then terminate diapause on demand to synchronize availability of agents with need in the field.
- Provide insights that would improve modelling the field phenology of pests and beneficial organisms.

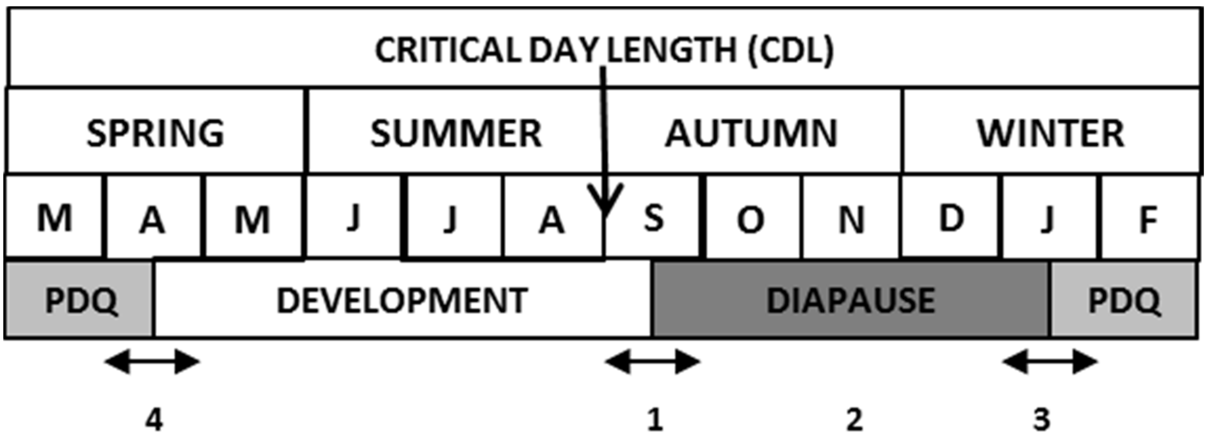

Figure 1. Schematic representation of a ‘typical’ seasonal phenology (life cycle transition) for a species with facultative diapause induced by critical day length (CDL) in late summer. Stages of development/activity, diapause and post-diapause quiescence (PDQ) are shown. Arrows indicate that the timing of transition points between these stages can shift in either direction depending on temperature. While overwintering diapause is typically induced by CDL, this response can be masked by warm temperatures in several species. Developmental rate between induction and diapause initiation (1) is also influenced by temperature. This means the seasonal timing of diapause initiation can be strongly temperature dependent. The relationship between temperature and diapause development/the duration of diapause maintenance (2) is complex (Bale and Hayward, 2010), but in virtually all species, the thermal conditions experienced during diapause will influence the timing of diapause termination (3). Once in PDQ, the organism is able to resume development (4) as soon as temperatures cross the appropriate developmental threshold. Thus, the resumption of development and timing of the subsequent adult stage is also temperature dependent. We propose to determine the effects of temperature on the timing of events 1-4 in a range of species within controlled lab environments. This will allow us to determine key thermal thresholds that influence life history transition points and so develop much more accurate phenology models than are currently available. The purpose of these models will be to better predict field population responses to different environmental scenarios and so enhance the efficacy of biocontrol, SIT and pollinator release.

Achieving these outcomes will require substantial advances in our knowledge of the regulation of dormancy responses and exploitation of that knowledge to artificially manipulate dormancy. We will address these long-term goals through research on four sub-themes.

1. Genetics of dormancy responses
2. Environmental treatments that may affect dormancy responses
3. Pharmacological manipulations that may affect dormancy responses
4. Performance outcomes of dormancy management

1.1.1. Genetics of dormancy responses.

Current Knowledge

Decades of research has shown that many dormancy responses have an underlying genetic basis, dormancy responses tend to be polygenic, there is substantial genetic variation within and among populations, and this variation is maintained by local adaptation due to spatial and temporal variation. We lack a detailed understanding of the genetic architecture of many dormancy responses and specifically diapause. This understanding of dormancy genetic architecture could facilitate the development of improved strains with respect to dormancy characteristics by selective breeding or genetic modification.

Gaps identified:

- Dormancy responses are typically polygenic, but multigenic models of diapause have not been well developed particularly with respect to pleiotropic and epistatic genetic interactions.
- Few studies have applied modern genetic tools to characterizing the genetic architecture of dormancy, leaving us with poor resolution of genetic architecture that hinders our ability to identify mechanisms of diapause regulation, or to selectively breed strains with diapause characteristics of interest.
- Interactions between genetic, epigenetic, and environmental factors require substantially more study to predict the life-history stage and environmental conditions in which dormancy may more effectively be manipulated.

1.1.2. Environmental treatments may affect dormancy responses

Current Knowledge

Environmental cues play critical roles in the induction, maintenance and termination of facultative diapause responses. Although less obvious, obligate diapausing species may also respond to environmental variation. For example, holding temperature prior to diapause or during diapause may affect the proportion of individuals that terminate diapause early versus late. Environmental inputs including temperature, photoperiod, host and enemy cues, and physical stimulation can affect diapause initiation and termination. Novel environmental manipulations may be a particularly useful route to manipulate dormancy. Obligate diapause can be eliminated by either preventing diapause induction or by driving early termination.

Gaps identified

- The mechanisms triggering induction in both facultative and obligate diapausing species are poorly known, but represent promising targets for manipulation.

- The underlying mechanisms of diapause termination in both facultative and obligate species remain poorly characterized, but represent promising targets for manipulation.
- To understand how environmental factors are translated through physiological machinery to govern dormancy responses we must explore: modes of action of environmental manipulations, periods of sensitivity within the lifecycle, the effective magnitude and duration of manipulations, and whether manipulations that are used at the research scale are deliverable to larger scales, such as in a mass-rearing facility.

1.1.3. Pharmacological manipulations of dormancy responses.

Current Knowledge

Pharmacological manipulations offer a promising set of tools for facilitating mass rearing of obligate-diapausing species and for regulating facultative dormancy to enhance shelf life of biological control agents and other beneficial insects, like pollinators. Although some pharmacological manipulations have been very successful in either averting diapause completely or terminating diapause, for example applications of ecdysteroids, juvenoids, or hexane, several gaps in our knowledge are currently preventing wide-scale use of pharmacological manipulation of diapause across species.

Gaps identified

- The mechanisms triggering induction in both facultative and obligate diapausing species are poorly known, but represent promising targets for pharmacological manipulation.
- The underlying mechanisms of diapause termination in both facultative and obligate species remain poorly characterized, but represent promising targets for activation to facilitate mass rearing of obligate-diapausing species, and to promote synchronization of biological control agents and beneficials with demand using pharmacological manipulations.
- To understand how pharmacological agents affect the physiological machinery to affect dormancy responses we must explore: modes of action, periods of sensitivity within the lifecycle, the effective doses and durations of manipulations, and whether pharmacological agents that are used at the research scale are deliverable to larger scales, such as in a mass-rearing facility.

1.1.4. Performance outcomes of dormancy management

Current Knowledge

Dormancy responses are often beneficial in promoting survival of organisms through stressful seasons and for synchronizing organismal lifecycles with favourable seasons, like times of resource availability. However, there can be substantial costs of undergoing dormancy. Across many systems with facultative dormancy, individuals that have undergone dormancy show reductions in important life-history parameters compared to non-diapause individuals when held in the same, benign conditions including: survival, energetic reserves, fecundity, fertility, flight, etc. Furthermore, there can be strong ecological costs of averting diapause when needed, or disrupting phenologies by terminating diapause too early or too late. Biological control agents must retain adequate performance after any environmental or pharmacological manipulations to remain effective. Thus, the performance impacts of any manipulation of dormancy must be carefully parameterized.

Gaps identified

- We know little about the effects of averting diapause in obligate-diapausing species on subsequent performance and timing of other life-history events like sexual maturation.
- We know little about the effects of novel environmental or pharmacological manipulations that induce or terminate diapause on subsequent performance and life-history traits.
- We know little about how multiple stressful portions of an integrated biological control program will interact with dormancy manipulation. For example, will dormancy management techniques improve or degrade the response of an individual to other stressors in a biological control program, such as the stresses of shipping and field release, or irradiation in the context of SIT.
- We know little about how altering the genetic architecture of dormancy responses by selecting for non-diapause or facultatively diapausing lines may affect other life-history and performance traits, in addition to inbreeding effects.

1.1.5. Critical knowledge gaps across projects

Across projects we will take several approaches for filling critical knowledge gaps, including:

- Selection of non-diapause strains. Even obligate-diapausing species will often have some small portion of the population that fails to enter diapause and instead continues development, or portions of the population that will terminate diapause early relative to the rest of the population. Selective breeding experiments using these non-diapausing or short-diapausing individuals may be particularly useful for addressing both genetic architecture and physiological mechanisms of diapause regulation. Production of non-diapause or short-diapausing lines could facilitate our understanding of the genetic architecture of diapause through breeding designs to estimate heritability and segregation ratios, and QTL mapping to identify candidate genome regions. Furthermore, these lines and the progeny of crosses between non-diapause and diapausing lines may facilitate dissecting the physiological architecture of diapause responses. Selection of non-diapause lines may also provide a near-term benefit by providing lines to be used in mass-rearing programs.
- Exploiting naturally segregating variation in diapause induction or termination timing. Variation in diapause has been demonstrated to segregate among individuals both within and among populations in numerous species of arthropods. Characterizing this variation phenotypically and through associations with population-genetic markers will provide insights into the genetic architecture of diapause and additional opportunities for artificial selection of non-diapause or short-diapause strains.
- Applying environmental manipulations. Changes in the ambient environment, including exposures to high heat and long photoperiods, among others, are well known to affect whether insects enter diapause and the duration of diapause (Figure 1). Dose-response curves for the magnitude and duration of environmental manipulations affecting diapause induction or duration will be estimated for several species. Dose-response experiments should carefully consider the ecological relevance of parameters used and the applicability of manipulations to eventual mass rearing. Beyond traditional single-factor, single-exposure manipulations, some treatments should explore synergistic effects of multiple exposures and exposure to multiple environmental factors, as well as environmental varibility.

- Applying pharmacological manipulations. Pharmacologial manipulations that either trigger endogenous physiological pathways, such as endocrine signals for diapause, or cause suifficient stress may dissuade the induction of diapause or promote the early termination of diapause. Critical periods for compound application, dose-response curves, and estimations of effects of pharmacological agents on performance of treated insects must be considered carefully. Investigators should bear in mind that there may be synergistic effects between different pharmacological treatments or between pharmacological treatments and environmental manipulations for diapause regulation.

2. LOW TEMPERATURE BIOLOGY

2.1. Background and situation analysis

Low temperature storage or treatment is an integral part of the rearing or release protocols in many biological control programs. In addition, although insects may be reared under optimal thermal conditions, they may be released into the field under thermal conditions that may differ substantially from the conditions under which they were reared. Therefore, an understanding of insect thermal biology has the potential to enhance the performance of insects reared for release. We identify four main areas in which thermal biology can contribute to rearing and storage.

1. Treatment conditions and manipulations that improve insect cold tolerance.
2. Facilitation and enhancement of survival and performance under long-term cold storage.
3. Enhancement of the thermal performance of insects destined for release.
4. Manipulation of thermal biology to cause 'ecological suicide' in a pest control context.

We recognise that there may be a gap between the development of potential methods and their practical implementation. The purpose of the CRP is to identify key parameters that could enhance storage and release; the translation of these findings to practical use will be a problem for the future.

2.1.1. Treatment conditions and manipulations that improve insect cold tolerance

<u>Current knowledge</u>

Insects use a few main strategies to tolerate low temperatures: most insects can not survive body freezing and use mechanisms to prevent their bodies from freezing when they are exposed to sub-zero temperatures (freeze intolerant) while a number of other species can tolerate being frozen (freeze tolerant). Insect thermal tolerance is highly plastic, and responds to a range of external cues during development, after exposure within a single life stage, and after brief exposures within a short period of time (Table 2).

Table 2: List of endogenous and exogenous factors that may affect phenotypic plasticity in cold tolerance. After Colinet and Boivin (2011, *Biological Control* 58, 83-95).

Exogenous Factors	Endogenous Factors
Temperature	Mass and body reserves
Duration of exposure	Life-history startegy
Rating of cooling or heating	Nutrition
Gradual acclimatation	Mode of reproduction
Rapid acclimation	Age/stage
Acclimatization	Dormancy status
Developmental temperature	Gender
Constant and fluctuating cold exposure	Physiological history
Combined cold exposure	
Humidity	
Photoperiod	
Chemicals	
Oxygen concentration	
Handling	

Cold injury has a range of causes, including disruption of ion balance, injury to cell membranes, metabolic perturbations, and mechanisms underlying plasticity include variation in the production of cryoprotectants, antifreeze proteins, membrane composition, alterations in a number of metabolic pathways, and behavioural modifications that ultimately affect cold tolerance (Figure 2). In addition, cold tolerance can be transferred between generations, and from host to parasitoid, although the mechanisms underlying this information transfer are unknown. Cold tolerance can also be improved by more artificial interventions, such as hyperprolinemia via dietary loading.

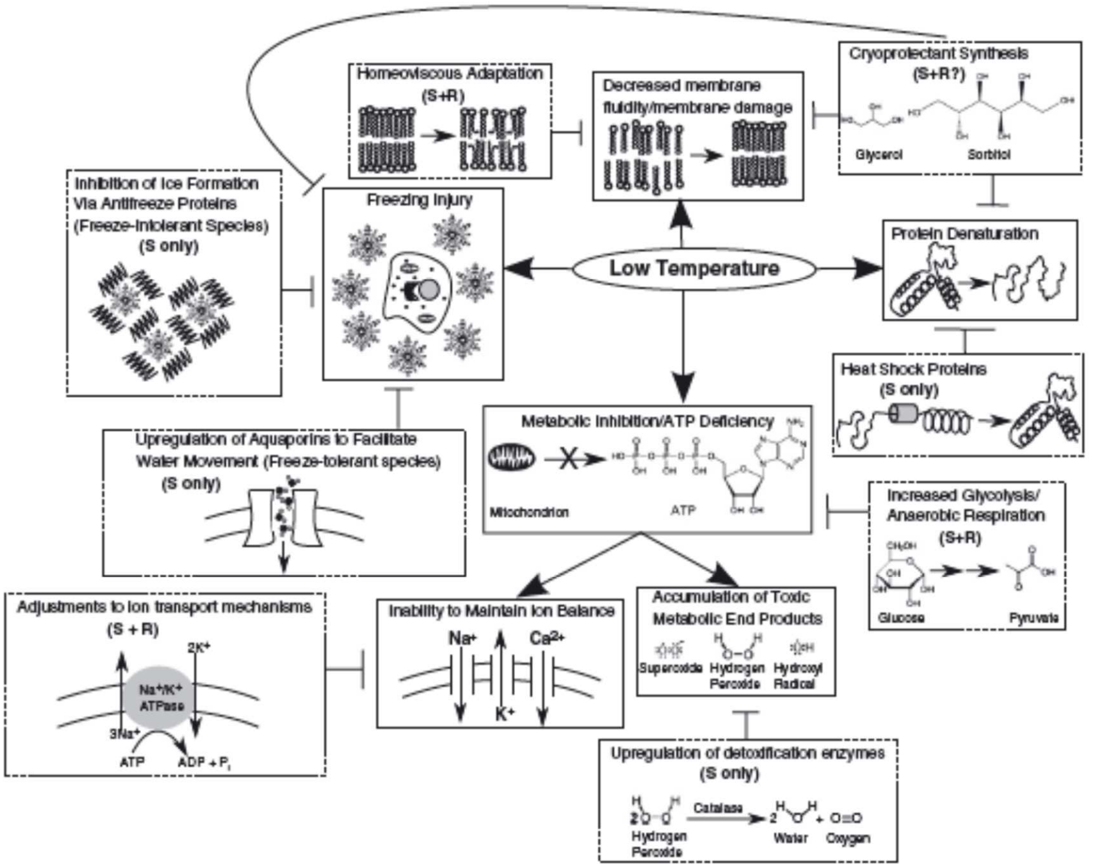

Figure 2: Mechanisms of low temperature damage and repair in insects. From Teets and Denlinger (2013 *Physiological Entomology* 38, 105-116).

During a rearing-and-release protocol, insects may be exposed to sub-optimal temperatures at several points: at collection, during radiation exposure, during packing for transport, during transport, and as part of the release.

<u>Gaps Identified</u>

- What are the mechanisms underlying cold injury, recovery and repair of injury, and plasticity of thermal tolerance?
    - o Understanding the mechanisms of cold injury may allow us to prevent damage, to enhance the speed of recovery from cold exposure, to specifically induce responses that improve thermal tolerance, or to identify novel approaches for enhancing performance after cold exposure during the protocol.

- Can physiological conditioning be used to improve survival?
  - Physiological conditioning can be used to increase cold tolerance of insects prior to exposure to cold. This can include thermal treatments, photoperiod changes, and exposure to other stresses that may induce cross tolerance or hormesis.
  - In addition, diet manipulation in preparation for cold exposure has the potential to shorten recovery time, improve survival, or enhance performance
- Can cold tolerance be transferred among generations or between trophic levels?
  - It is increasingly apparent that the environmental history of parental generations can affect the thermal tolerance of their offspring, and that thermal tolerance can be passed from host to parasitoids. However the prevalence and mechanisms of these relationships are poorly understood.
- What is the role of epigenetic processes in determining thermal tolerance?
  - Epigenetic mechanisms, involving small RNAs, DNA acetylation, DNA methylation, and histone modification can drastically alter patterns of gene expression, transduce information between life stages and generations, and alter responses to environmental cues. There is evidence for epigenetic regulation in diapause regulation, and while cold-specific microRNAs have been identified for goldenrod gall flies, epigenetic regulation has not been well-explored in other systems.
- Wat is the role of the microbiome and immunity in cold hardiness?
  - There is a wealth of recent literature that suggests that the microbiome has significant impacts on the biology of their host animals, across a wide range of taxa. In insects, we know that endosymbiotic bacteria (and even some pathogens) can enhance thermal tolerance. Although there is some evidence that insect immune systems respond to cold, the role of the microbiota in cold tolerance has not been explored.
- How do dormancy processes interact with cold tolerance?
  - Although dormancy and quiescence are closely associated with the development of cold tolerance in many species, understanding how much the genetic and physiological architecture are shared between traits could allow diapause or cold hardiness to be independently modified.

2.1.2. Facilitate and enhance survival and performance under long-term cold storage.

Current Knowledge

During cold exposures of days to weeks (or even months), insects can accumulate indirect chilling injuries. In some insects, these injuries can be prevented by pre-treatments that include exposure to mild cold. In addition, it is now well-established that fluctuations in the thermal environment, for example, periodic bursts of exposure to permissive temperatures (Fluctuating thermal regimes, FTR), can enhance cold survival and even abate the accumulation of injury. The mechanisms by which FTRs reduce cold injury are not well-known, but are subject to ongoing investigation. In addition, many insects are capable of surviving long exposures to very low temperatures, and there is the potential to use understanding of those mechanisms to optimise long-term storage (for example, to stockpile reared insects for future release), or to identify novel molecules and processes to enhance existing cryopreservation protocols (used to maintain genetic diversity in populations reared for release).

Gaps Identified

- What are the mechanisms by which FTRs improve survival?
  - Understanding these mechanisms will allow us to develop treatments that use FTRs to enhance survival during long-term cold storage and improve post-storage performance.
  - Understanding the mechanisms underlying chilling injuries may be particularly useful for identifying treatments that ameliorate long-term chilling damage.

- Can FTRs be optimized to improve cold storage of mass-reared insects?
  - Currently most FTRs utilise a daily pulse at permissive temperatures. Practical concerns about warming and cooling make less frequent interruptions of cold exposure attractive. What are the minimum and maximum durations and intervals for FTRs that can elicit enhanced chilling tolerance?
  - The fitness and metabolic costs of the FTR responses have not been well-explored, and the consequences for field performance must be considered, as must the potential for FTRs to elicit deacclimation or development during the FTR regime.

- Can fluctuations in environmental parameters other than cold during storage enhance survival?
  - Currently, most fluctuating regimes that affect cold tolerance primarily use daily exposures to permissive temperatures. The potential for fluctuations in photoperiod, humidity, and other environmental factors to improve chilling tolerance has not been well-explored.
  - Other physical, diet, or chemical manipulations, for example dietary amino acid loading or hypoxia, could also act in a manner similar to FTRs to abate chilling injury.

- Can reliable markers for insect quality be identified?
  - There exists a general need for biomarkers to determine the effects of cold storage on the quality of insects destined for release. The development and implementation of such markers requires not only the identification of the relationships between cold-induced damage and fitness-related parameters such as flight and reproductive performance, but also the development and dissemination of standard protocols.

- Can cryopreservation techniques be enhanced and extended to more species?
  - Cryopreservation procedures have been very successful for a small number of species. There is a need to expand the range of taxa for which cryopreservation is available, and to increase the throughput of existing cryopreservation protocols.
  - Exploration of the mechanisms underlying insect cold tolerance might reveal novel mechanisms and molecules that could be used to enhance or modify existing cryopreservation protocols, or extend cryopreservation to additional life stages or taxa.

2.1.3 Enhancing the thermal performance of insects destined for release.

Current Knowledge

Insects may often be released into environments that contrast significantly with those under which they were reared and stored. It is well-known that there are tradeoffs between upper and lower thermal tolerances (mediated, for example, by modifications to cellular membrane fluidity). In addition, there is a considerable empirical and theoretical understanding of the evolutionary and ecological implications of acclimation, although the mechanisms underlying cold acclimation in insects are less well-understood.

Gaps Identified

- Can we identify mechanisms underlying thermal acclimation and deacclimation?
  - Understanding these mechanisms may allow high-low temperature tradeoffs to be ameliorated, or for laboratory selection to allow decoupling of high temperature tolerance (necessary for field survival) from survival of low temperature storage.
- Can we optimize the timing of release to enhance performance?
  - Thermoperiod, photoperiod, atmospheric pressure, and endogenous circadian rhythms all affect insect performance. There exists capacity to use these rhythms to optimise the performance of the insects upon release.
  - Enhancement of thermal performance could also be season-specific, for example thermal conditioning could be used to enhance low temperature performance of early-season releases, where insects will encounter lower temperatures than at mid-summer.
- Can we develop treatments that increase field competitiveness?
  - A key component of all thermal manipulations is that they can affect performance in the field. There exists a need to extend measurement of post-cold performance beyond survival to include field performance (see also measures of quality, above).

2.1.4 Manipulating thermal biology to cause 'ecological suicide' in a pest control context.

Current Knowledge and identified gaps

We have a reasonable knowledge of the scope and range of plasticity of insect cold tolerance, as well as the capacity to use this knowledge to enhance thermal tolerance (see sections 1-3). This knowledge could also be harnessed to disrupt thermal tolerance in field populations of pest insects. This approach has been explored for ice-nucleating bacteria to reduce overwinter cold tolerance (albeit with limited success), but has not been widely explored in other systems.

3. LOGICAL FRAMEWORK

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
Overall Objective: The objective of the project is to understand and harness dormancy management, physiological conditioning, and cold-storage approaches to enable mass-rearing of insect species previously difficult to rear and enhancing current mass-rearing efforts for biological control, specifically using sterile insects and natural enemies as part of an environmentally friendly, area-wide integrated pest management approach.	N/A	N/A	Member States will continue to suffer major losses to endemic and introduced insect pests. The demand for area-wide integrated insect pest management approaches, including SIT and augmentative biological control as non-polluting suppression/eradication components, continues to increase, mandating expansion and improvement in cost-effectiveness of these environment-friendly, sustainable approaches. International trade in agricultural commodities will continue to increase and be disrupted by pests requiring expensive post-harvest and quarantine measures.

Specific Objectives:	**Verifiable indicators**	**Means of verification**	**Important Assumptions**
1. Develop and assess new methods to manage dormancy responses, physiological conditioning, and storage conditions to facilitate mass rearing.	Research and development focused on generating new methods to manage dormancy to facilitate mass rearing.	Scientific reports and peer-reviewed publications.	Dormancy responses, physiological conditioning, and storage conditions can be managed in many systems to avert dormancy or shorten the length of the dormancy period.
2. Develop and assess new methods to use dormancy responses, physiological conditioning, and storage conditions to maintain the genetic integrity of laboratory strains.	Basic and applied research focused on the use of dormancy responses in cryopreservation and long-term cold storage.	Scientific reports and peer-reviewed publications.	Dormancy responses, physiological conditioning, and storage conditions can be used to enhance the capacity for cryopreservation and long-term cold storage.
3. Develop methods to incorporate dormancy into phenological models to improve the timing of field releases.	Research and development focused on improving phenological models.	Scientific reports and peer-reviewed publications.	Dormancy responses can be incorporated into phenological models to improve the timing of field releases of biological control agents.
4. Develop and assess new methods to use dormancy responses, physiological conditioning, and storage conditions to enable or enhance the shelf life of biological control agents, including sterile insects and natural enemies.	Research and development focused on the use of dormancy responses, physiological conditioning, and storage conditions to enhance shelf life.	Scientific reports and peer-reviewed publications.	Dormancy responses, physiological conditioning, and storage conditions can be used to enable or enhance the ability to stockpile and mobilize biological control agents upon demand.

5. Assess whether dormancy responses, physiological conditioning, and storage conditions can be used to reduce radiation injury and enhance sterile insect performance.	Research and development focused on the use of dormancy responses, physiological conditioning, and storage conditions to reduce injury and enhance post-irradiation performance.	Scientific reports and peer-reviewed publications.	Dormancy responses, physiological conditioning, and storage conditions can be used to reduce radiation injury to somatic cells of biological control agents while maintaining the sterility of germ-line cells.
6. Assess whether dormancy responses, physiological conditioning, and storage conditions can be used to decrease shipping-related damage and enhance post-shipping performance.	Research and development focused on the use of dormancy responses, physiological conditioning, and storage conditions to reduce shipping-related injury and enhance post-shipping performance.	Scientific reports and peer-reviewed publications.	Dormancy responses, physiological conditioning, and storage conditions can be used to reduce injury incurred during shipping and improve post-shipping performance.
7. Explore the role of the microbiome on dormancy responses, physiological conditioining, and cold storage to enhance mass rearing and shelf life of biological control agents.	Basic research focused on charachterizing associations between the microbiome and dormancy responses, physiological conditioning, and storage conditions	Scientific reports and peer-reviewed publications.	The microbiome contributes to dormancy, physiological conditioning, and cold storage responses and that the microbiome can be manipulated to improve the quality of biological control agents.
8. Explore the potential for dormancy responses to generate novel approaches for inducing “ecological suicide”.	Basic research focused on developing new techniques and theoretical perspectives for disrupting dormancy responses to control pest insect populations.	Scientific reports and peer-reviewed publications.	Disruption of natural dormancy responses can be used to control insect pest populations by desynchronizing seasonal pests with their environments.

Outcomes:	Verifiable indicators	Means of verification	Important Assumptions
1. Mass rearing of pests enabled or enhanced by exploiting dormancy or physiological conditioning.	Protocols applied to mass rear at least 2 species with dormancy or physiological conditioning.	Mass-rearing reports	There is a need to mass rear insects with fixed periods of dormancy and/or improved physiological tolerances.
2. Genetic integrity of strains maintained through dormancy management or physiological conditioning in cryopreservation and long-term cold storage.	Protocols applied for cryopreservation or long-term cold storage for at least 2 species.	Colony management reports.	Facility staff struggles to maintain the genetic integrity of strains; dormancy management or improving physiological tolerances may increase strain maintenance capacity and decrease costs.
3. Better prediction of phenologies to boost the efficacy of release programs.	Phenology models that include dormancy responses applied to at least 2 species.	Phenology modelling reports.	The ability to synchronize the release of biological control agents with the phenology of targets itn eh field will improve the efficacy of control programs.
4. Dormancy management and/or physiological conditioning in use to enable or enhance the shelf life of biological control agents, including sterile insects and natural enemies.	Protocols applied to stockpile at least 2 biological control strains and at least 1 pollinator.	Colony production and storage reports.	The ability to stockpile insects and mobilize them on demand is important for the viability of biological control and pollination programs.

5. Dormancy management and physiological conditioning used to reduce radiation injury and enhance sterile insect performance.	Protocols applied for evaluation in at least 2 species of biological control agents.	Evaluation reports under operational conditions.	Enhanced stress resistance is a facet of many dormancies and physiological-conditioning treatments, so treated individuals may experience reduced somatic radiation damage during sterilization, while maintaining germ-line sterility.
6. Dormancy management or physiological conditioning used to reduce shipping-induced injury and enhance the performance of sterile insects and natural enemies.	Protocols applied for evaluation in at least 2 species of biological control agents.	Evaluation reports under operational conditions.	Damage incurred during shipping due to hypoxia, mechanical disturbance, and exposure to temperature extremes limits the efficacy of biological control agents in field releases and inducing dormant states or physiological conditioning may reduce such damage.
7. Microbes demonstrated to impact dormancy and cold responses.	Associations between tolerances and microbial communities evaluated in at least 2 species.	Scientific publications and technical reports.	Microbial manipulation could open up new avenues to enhance the performance of sterile insects and natural enemies.
8. The potential for dormancy responses to generate novel approaches for inducing "ecological suicide" evaluated under laboratory conditions.	New perspectives evaluated under laboratory conditions.	Scientific reports and peer-reviewed publications.	There is a need for novel environmentally friendly tools to control pest populations.

Outputs:	**Verifiable indicators**	**Means of Verification**	**Important Assumptions**
1.The potential to terminate or avert unwanted dormancy responses using thermal, hormonal, and chemical manipulations evaluated.	Thermal, hormonal, and chemical manipulations tested in at least 3 species.	Reports and peer-reviewed publications.	Thermal, hormonal, and chemical manipulations that have been successful in other models will be applicable to species important for biological control.
2.The potential for dormancy management to maintain the genetic integrity of important strains, cryopreservation and long-term cold storage techniques assessed.	Protocols developed and assessed in model species and extended to selected pest species.	Reports and peer-reviewed publications.	Recent successes in cryopreservation and long-term cold storage of model species can be extended to species important for biological control.
3.The potential to enhance biological control and beneficial insect release practices with new phenology models evaluated.	Phenology models developed and assessed in at least 2 species.	Reports and peer-reviewed publications.	Phenological models that incorporate dormancy responses will more effective than existing models
4. The ability to enable or enhance storage and mobilization of biological control agents with dormancy management evaluated	Protocols developed and assessed in at least 3 species.	Reports and peer-reviewed publications.	Dormancy responses can be exploited to increase the shelf life of biological control agents while maintaining performance.
5.The potential for dormancy to reduce radiation injury and enhance sterile insect performance determined.	Protocols developed and assessed in at least 2 species.	Reports and peer-reviewed publications.	Stress resistance is often enhanced in the dormant state and this can be exploited to reduce radiation-induced injury while maintaining sterility.

6.The potential for dormancy to reduce shipping-related damage and enhance post-shipping performance evaluated.	Protocols developed and assessed in at least 2 species.	Reports and peer-reviewed publications.	Stress resistance is often enhanced in the dormant state and this can be exploited to reduce shipping-related damage while maintaining performance.
7.The potential of the microbiota to improve dormancy responses, physiological conditioning, or cold-storage evaluated	Associations between the microbiota and insect performance assessed in at least 2 species	Reports and peer-reviewed publications.	Understanding and manipulating associations between insects and their microbiome will enhance dormancy, physiological conditioning, and cold storage.
8.Novel perspectives on the role of dormancy disruption in controlling pest populations explored.	Novel theoretical perspectives explored.	Reports and peer-reviewed publications.	Hormonal and chemical manipulations currently under investigation for dormancy regulation could be translated into novel methods for control.
ACTIVITIES:	**Verifiable indicators**	**Means of Verification**	**Important Assumptions**
1. Hold Consultants Meeting.	Consultants meeting held May 2012.	Consultants Meeting resulted in CRP proposal.	Consultants meeting approved.
2. Announce project amongst established entomologists working on seasonal pests and establish CRP.	CRP announced and research contracts and agreements submitted, evaluated, and forwarded to IAEA committee.	Issued contracts and agreements.	Proposals submitted and approved by IAEA committee
3. Organize first RCM to plan, coordinate and review research activities (3nd quarter 2014).	1st RCM held in July 2014.	Working material printed and distributed for 1st RCM.	Research activities commence. Reports published and distributed following each RCM.
4. Carry out R&D.	Research carried out by contract and agreement holders.	Reports and publications.	Research activities continue, progress satisfactory.

5. Second RCM to analyse data and draft technical protocols as required (early 2016)	2nd RCM held in 2016.	Working material printed and distributed for 2nd RCM; Research published in scientific literature and disseminated to member states and scientific community.	Renewal requests and continued funding of RCM's and CRP.
6. In conjunction with the second RCM meeting 3-4 day hands-on workshop on "Tools and techniques for quantifying insect performance in relation to dormancy, physiological conditioning, and cold storage".	Workshop held in 2016. Harmonized procedures and trainees capable of implementing novel techniques.	Workshop report integrated as an appendix to the report of the Second RCM.	There is need for training; techniques and instructors are available.
7. Continue R&D.	Research carried out by contract and agreement holders.	Reports and publications.	Renewal requests and continued funding of RCM's and CRP.
8. Review the CRP after its third year.	Mid-CRP review carried out.	Report of mid-CRP review.	Mid-CRP review by Agency committee is positive.
9. Convene third RCM to evaluate and standardize protocols (late 2017).	3rd RCM held in 2017.	Working material printed and distributed for 3rd RCM; Research published in scientific literature and disseminated to member states and scientific community.	Mid-CRP review approved by IAEA committee. Research activities continue, progress satisfactory.
10. Continue R&D.	Research carried out by contract and agreement holders.	Reports and publications.	Renewal requests and continued funding of RCM's and CRP.
11. Hold final RCM to review data and reach consensus (early 2019).	Final RCM held in 2019.	Final CRP report.	Research and dissemination activities concluded.
12. Evaluate the CRP and submit evaluation report.	CRP evaluation carried out.	CRP evaluation report.	CRP evaluation by Agency committee is positive.

13. Summarize and publish advances of CRP in a series of joint publications.	CRP members submit papers summarizing activities.	Publication in scientific literature.	Manuscripts submitted, edited, peer reviewed and published.

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ANNEX 1: List of participants

First RCM on "Dormancy Management to Enable Mass-rearing and Increase Efficacy of Sterile Insects and Natural Enemies"

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ANNEX 2: Agenda

FIRST FAO/IAEA RESEARCH COORDINATION MEETING ON

Dormancy Management to Enable Mass-rearing and Increase Efficacy of Sterile Insects and Natural Enemies

21-25 July 2014 Vienna, Austria

Vienna International Centre (IAEA Headquarters), Building M - Room M4

AGENDA

MONDAY, 21 JULY 2014

08:00 – 09:00	Identification and registration at VIC Gate (next to subway station U1)
09:00 – 09:15	**Marc Vreysen and Rui Cardoso Pereira:** Welcome statement and goals of the CRP and the meeting
09:15 – 09:30	**Rui Cardoso Pereira and Nima Mashayekhi:** Agenda and administrative issues
09:30 – 09:45	**Rui Cardoso Pereira:** Coordination Research Projects: Scientific and implementation mechanisms
09:45 – 10:15	**Stephanie Bloem:** Mass rearing codling moths through diapause: Successes, challenges and methods development opportunities – what peer-reviewed publications don't tell you.
10:15 – 10:30	Bank Issues **(Nima Mashayekhi)**
COFFEE BREAK	
	SESSION I: Dormancy management of tephritid fruit flies and other insect pests (Chairperson: David Denlinger)
11:00 – 11:30	**Bo Wang:** A new "bridge" monitoring method: Female population dynamics of *Bactrocera dorsalis* between the colder winter and warmer spring in China
11:30 – 12:00	**Changying Niu:** Mechanisms of 20-hydroxyecdysone break pupal diapause in the Chinese citrus fruit fly, *Bactrocera minax*
LUNCH	
13:00 – 13:30	**Nikos Papadapoulos:** Obligatory and facultative diapause responses in *Rhagoletis cerasi* and associated costs
13:30 – 14:00	**Juan Rull:** Dormancy management for fruit flies in the genus *Rhagoletis* in Mexico

14:00 – 14:30 **Dan Hahn:** Sleeping beauties: mechanisms of developmental arrest, seasonal timing, and speciation in the apple maggot

14:30 – 15:00 **Mahbub Hassan:** Mass-rearing and control strategies for Indian Meal Moth *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) minimizing their dormancies

15:00 – 15:30 **Nikos Kouloussis:** Manipulation of diapause development for improving control of *Eurytoma* species

COFFEE BREAK

SESSION II: Dormancy management of biocontrol agents (Chairperson: Juan Rull)

16:00 – 16:30 **Karina Fisker:** Optimizing performance of biological control agents used in Integrated Pest Management

16:30 – 17:00 **Mariana Viscarret:** Study of factors inducing dormancy on *Trichogramma nerudai* Pintureau and Gerdind and *T. pretiosum* Riley under rearing conditions

17:00 – 17:30 **Martin Wohlfarter:** Colony establishment of the egg parasitoid *Trichogrammatoidea lutea* Girault (Hymentoptera: Trichogrammatidae) for dormancy management studies, in order to improve integrated control of codling moth [*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)].

TUESDAY, 22 JULY 2014

SESSION III: Cold tolerance and dormancy (Chairperson: Dan Hahn)

08:30 – 09:00 **Astrid Bryon:** Cold tolerance and diapause of beneficial and harmful arthropods

09:00 – 09:30 **Dave Denlinger:** Can cold tolerance be transferred from hosts to parasitoids?

09:30 – 10:00 **Brent Sinclair:** New approaches to understanding insect freeze tolerance

COFFEE BREAK

10:30 – 11:00 **Hervé Colinet:** Fluctuating thermal regimes: an efficient method to extend the shelf life of insects during cold storage

11:00 – 11:30 **George D. Yocum:** Use of fluctuating thermal regimes and decreased oxygen during storage improves survival of post-diapause *Megachile rotundata*

11:30 – 12:00 **Vladimír Koštál:** Overwintering and cold tolerance in the codling moth (*Cydia pomonella*)

LUNCH

	SESSION IV: Genetics and dormancy (Chairperson: Nikos Papadopoulos)
13:00 – 13:30	**Bin Chen:** Molecular regulation mechanism of winter and summer diapause in *Delia antiqua* for the control of insect pests
13:30 – 14:00	**Christian Stauffer:** Population genomics of diapause phenotypes in european *Ips typographus* (Coleoptera, Curculionidae) using high-throughput RADSeq
14:00 – 14:30	**Gregory Ragland**: Genetic basis of rapid evolutionary shifts in polygenic diapause regulation across development
14:30 – 15:00	**Scott Hayward:** Molecular profiling of the diapause programme to identify mechanisms underpinning diapause regulation, enhanced stress tolerance and post-diapause fitness: applications in optimising mass culturing, SIT and biocontrol

COFFEE BREAK

15:30 – 16:00	**Shin Goto:** Roles of the circadian clock genes in photoperiodic regulation of diapause in the bean bug
	SESSION V: General discussion (Chairperson: Rui Cardoso Pereira)
16:00 – 17:00	General Discussion
17:00 – 17:30	Selection of Working Groups (including Group Leaders)

WEDNESDAY, 23 JULY 2014

	SESSION VI: Review of the individual proposals (Chairperson: Rui Cardoso Pereira and Group Leaders)
08:30 – 10:00	Working Groups: Background situation analysis, baseline knowledge at start of CRP and review of research gaps that need to be addressed (Rooms available M4, A2311, A2172)

COFFEE BREAK

10:30 – 12:00	Working Groups: Continued review of research gaps that need to be addressed

LUNCH

13:00 – 15:00	Working Groups: Continued review of research gaps that need to be addressed

COFFEE BREAK

15:30 – 17:30	General Discussion: Review of baseline knowledge at start of CRP and review of research gaps that need to be addressed (Room M4)

18:30	**GROUP DINNER (Strandcafe)**

THURSDAY, 24 JULY 2014

	SESSION VI (cont.): Review of the individual proposals (Chairperson: Rui Cardoso Pereira and Group Leaders)
08:30 – 10:00	Working Groups: Review of individual research proposals for the different working areas
COFFEE BREAK	
10:30 – 12:00	Working Groups: Continued review of individual research proposals for the different working areas
LUNCH	
13:00 – 15:00	Working Groups: Continued review of individual research proposals for the different working areas
COFFEE BREAK	
15:30 – 17:30	General Discussion: Review of individual research proposals for the different working areas (Room M4)

FRIDAY, 25 JULY 2014

	SESSION VII: RCM report (Chairperson: Rui Cardoso Pereira and Group Leaders)
08:30 – 10:00	Review and adjustment of the logical framework
COFFEE BREAK	
10:30 – 12:00	Agreement on content of RCM report, and drafting and compiling of RCM report
LUNCH	
13:00 – 15:00	Finalization of draft RCM report
COFFEE BREAK	
15:30 – 17:30	Agreement on information exchange mechanisms, on location of 2^{nd} RCM, and closure of the RCM

ANNEX 3: Working groups

Manipulating Dormancy Responses	Low Temperature Biology
Dan Hahn	Dave Denlinger
Changying Niu	Karina Vincents Fisker
Nikos Papadopoulos	Mariana Viscarret
Juan Rull	Martin Wohlfarter
Bo Wang	Brent Sinclair
Mahbub Hassan	Hervé Colinet
Nikos Kouloussis	George Yocum
Bin Chen	Vlad Kostal
Christian Stauffer	Rui Cardoso Pereira
Greg Ragland	
Scott Hayward	
Shin Goto	
Vid Bakovic	
Jingli Gao	
Martin Schebeck	
Astrid Bryon	

ANNEX 4: Abstracts of presentations

Mass rearing codling moths through diapause: Successes, challenges and methods development opportunities – what peer-reviewed publications don't tell you

Stephanie Bloem

USDA-APHIS-PPQ, Center for Plant Health Science and Technology, Plant Epidemiology and Risk Analysis Laboratory, Raleigh, NC 27606 (Stephanie.Bloem@aphis.usda.gov)

Desirable behavioral attributes in mass-reared insects should include the ability to perform favorably under the various environmental conditions they encounter upon release in the field. This presentation will tell the story of using diapause as a strategy to improve the quality of mass-reared codling moth (*Cydia pomonella*) used in a sterile insect release program in British Columbia, Canada.

A new “bridge” monitoring method: Female population dynamics of *B.dorsalis* between the colder winter and warmer spring in China

Bo Wang

Beneficial Insects Research Institute, Fujian Agriculture University, 350002 Fujian, China (wangbofjfz@163.com)

Firstly, the female’s population dynamics of *B. dorsalis* has been tested as early as possible in the spring 2014.

Secondly, some important time points between pre- and post-overwintering will be examined in the field cages. For example, when the females stop/start to lay fertile eggs, when the temperature rise above the developmental threshold for maturation, when the first adult emerge, etc.

Thirdly, some developmental parameters will be also tested during this period, such as the mortality rate of stage-specific fruit fly, average fecundity per female.

Mechanisms of 20-hydroxyecdysone break pupal diapause in the Chinese citrus fruit fly, *Bactrocera minax*

Yongcheng Dong, Zhenzhong Chen, Rui Pereira, Changying Niu

College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China (niuchangying88@163.com)

Diapause is an adaptive strategy with a crucial role in the synchronization of seasonal life cycles and adaptation to environmental change. The termination of diapause is the critical time point, when the insect rapidly increases its metabolic rate and resumes development. It can be achieved by changes in environmental conditions and artificial stimulation. To date our understanding of diapause process in insects focuses largely on facultative diapause, little on obligatory diapause. Nevertheless, molecular mechanisms involved in termination of obligatory diapause in insects remain poorly understood. In the present study, we used, *Bactrocera minax*, a temperate univoltine pest in Citrus spp., as a good model insect to investigate the mechanism underlying pupal diapause termination. High-throughput RNA-seq technology will be employed to characterize the *B. minax* transcriptome and identify differentially expressed genes during pupal diapause development. Using 20-hydroxyecdysone (20E) as a tool to break pupal diapause in this tephritid fly, we will construct four digital gene expression (DGE) libraries in different stages of pupal diapause: early diapause (ED), late diapause (LD), post-diapause (PD), and individuals treated with 20E for diapause termination (DT). On the basis of these results, our immediate goals are to: (1) identify the early response genes triggered by 20E in signaling cascades responsible for pupal diapause termination; (2) analyze the functional characterization of genes using RNA interference or inhibitor techniques; and (3) investigate physiological pathways promoting diapause termination through 20E stimulation. The transcriptome and DGE profiling data will provide comprehensive genetic information for *B. minax* and greatly improve the understanding with regard to pupal diapause development. It also provides new insights into the mechanism for pupal diapause concerning stress resistance and metabolic depression in this species. The results will also suggest potential tools for the development of artificial rearing and subsequent integrated pest management, like sterile insect technique, for the control of *B. minax* and other similar pest insects.

Obligatory and facultative diapause responses in *Rhagoletis cerasi* and associated costs

Nikos T. Papadopoulos, Cleopatra Moraiti, and Stella Papanastasiou

Laboratory of Entomology and Agricultural Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly (nikopap@uth.gr)

The European cherry fruit fly, *Rhagoletis cerasi* (L.) (Diptera: Tephritidae) undergoes an obligatory diapause at pupal stage that allows adults to emerge next spring when local fruit are available and susceptible for oviposition. However, some individuals within populations may extend their dormant period for one or more years. This paper reports on the assessment of the genetic and environmental effects on diapause termination and the expression of prolonged dormancy in *R. cer*asi pupae, and fitness costs associated with pupae prolonged dormancy at adult stage. We used two Greek (Dafni, Kozani; Kala Nera, Magnesia) and one German (Dossenheim, Karlsruhe) populations from ecological different habitats with various rates of gene flow to determine diapause termination responses. When adult emergence ceased, dead pupae and pupae still in dormancy were recorded. In additional trials we compared the demographic traits of adults from one population (Dafni) that emerged from pupae with annual and prolonged dormancy. Pupae from K. Nera terminated diapause almost one month earlier than those from Dafni and Dossenheim, suggesting an adaptive response to habitat heterogeneity (mainly differences in phenology patterns of local host cultivars). Regardless of the population, insufficient chilling (short duration (≤5 months) or warm temperatures (10-12^{o}C) triggers the expression of prolonged dormancy. Interestingly, extended chilling (longer than required for terminating diapause) makes pupae to 'return' to another (facultative) cycle of dormancy. Prolonged dormancy did not affect female longevity but dramatically decreases fecundity rates. Our data demonstrate that *R. cerasi* has evolved a mixed life-history strategy for terminating diapause, based on a combination of local adaptation and diversified bet-hedging strategies. The latter are expressed either as prolonged dormancy or as facultative dormancy; a strategy first time reported for univoltine insects. However, prolonged dormancy is associated with significant fitness cost for the resulting adults. In additional trials we determined the six most representative developmental stages within the puparium and the developmental progression after the end of the chilling period, which lasted 7 months at 3°C before pupae being transferred back to 25° C. Within 20 days post chilling there was a gradual progress from stage I to stage VI (pharate adult). However, approximately 30% of pupae failed to progress development, after 20 days at 25°C (optimum temperature for development). This suggests that a proportion of pupae (that had already terminated diapause) remain at a dormant stage for an extended period of time (at least 20 days post chilling) in an optimum temperature for development.

Dormancy management for fruit flies in the genus *Rhagoletis* in Mexico.

<u>Juan Rull</u> and Eduardo Tadeo

Intituto de Ecología A.C., Xalapa, Veracruz, México

The genus *Rhagoletis* includes several pest species of temperate fruit that cause significant damage to crops in North America and Europe. In North America, common ancestors of these pest species took refuge in the highlands of central Mexico during Pleistocenic glaciations, diverged in isolation, and re-expanded their range during postglacial periods. These episodes of isolation and gene flow produced large genetic variation related to diapause regulation allowing flies in the genus to quickly adapt to host plants with different fruiting phenology. Several of these plants include species of commercial value (apples, cherries, walnuts, hawthorns). Understanding of diapause regulation in the genus *Rhagoletis* has therefore application for pest management. Among other things, we have worked on establishing the distribution, host range and basic biology of flies in the genus *Rhagoletis* in Mexico for the past 12 years. Such studies include flies in the *pomonella*, *cingulata*, *suavis* and *striatella* species group. Overall, we have observed large variation in diapause duration within and across populations, host races, species, and species groups. For most species that we have studied a small proportion of the populations forgoes dormancy, adults emerge over a time span lasting several months, and a small proportion of the population can engage in dormancy periods including two, three or four years. Parasitoids associated to *Rhagoletis* have also evolved dormancy management strategies that enable them to synchronize with their hosts. Understanding of the factors that affect dormancy can aid in predicting pest emergence, managing flies for research purposes, or rearing biocontrol agents. Additionally, the possibility of selecting individuals to establish non-diapausing strains could result in development of SIT and augmentative biological control that could be highly successful in isolated fruit growing high elevation areas in Mexico. We therefore propose to study dormancy of several species in the genus *Rhagoletis*, and attempt to select a non-diapausing strain of *R. pomonella* with aims at developing SIT for flies in this important genus.

Sleeping beauties: mechanisms of developmental arrest, seasonal timing, and speciation in the apple maggot.

Thomas H.Q. Powell, Qinwen Xia, Gregory J. Ragland, Stewart W. Berlocher, Jeffrey L. Feder, and Daniel A. Hahn.

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The apple maggot, *Rhagoletis pomonella*, a text-book model for sympatric speciation and adaptive genetic divergence, has recently (~200ya) expanded its host range from native hawthorns to domesticated apples. Apples flower and fruit earlier in the summer than hawthorns and to synchronize themselves with the novel plant apple flies have shifted the timing of their seasonal dormancy. Thus, the mechanisms that 1) maintain animals in the dormant state, and 2) elicit the termination of dormancy and the resumption of development are critical for driving speciation in this group. First, we use respiratory gas exchange to describe the transition from the dormant state into active morphogenesis. Second, we use our respiratory data to select important developmental time points and use microarrays to characterize the tanscriptome of individuals from deep in pupal dormancy through dormancy termination and through adult development to identify sets of candidate genes for maintaining pupal dormancy and initiating adult development. We identified six clear transcriptional patterns over our developmental series, some of which are enriched in candidate genes for cell cycling, chromatin remodeling, stress resistance, and endocrine signaling. Among those of most interest is a series of genes in the calcium-dependent branch of the *Wnt* signaling network that appear to be the earliest molecular indicators of dormancy termination. This work bridges the gap between developmental and genetic mechanisms underlying the evolution of dormancy timing, host plant shifts, and diversification. Furthermore, this work has the potential to uncover evolutionarily conserved mechanisms that, in the long run, could allow us to initiate or terminate dormancy artificially in economically important insect taxa, including pests and natural enemies.

Mass Rearing and Control Strategies for Indian meal Moth *Plodia interpunctella* (Hübner)(Lepidoptera: Pyralidae) Minimizing their Dormancies

Md Mahbub Hasan

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Indian meal moth (IMM), *Plodia interpunctella* is an insect pest of stored products, and is found throughout the world. It causes infestations in a wide array of commodities. This species undergoes larval diapause as a pre-pupa. Diapause induction in *P. interpunctella* depends on a number of factors. In the present experiment, the Indian meal moth was collected from the different food facilities in northern region of Bangladesh prior to establish their mass cuture. They have been cultured under the laboratory condition for finding out the most suitable food media. The life cycle of IMM has been studied prior to enable or improve the efficacy of mass rearing utilizing their dormancy. The parasites were also collected from the different food faclities prior to investigate their efficacy against IMM under dormancy conditions. Morover, the incidence of IMM has been conducting in diffeent food facilites using the pheromone traps. However, the over all project should be focused on the improvement of mass rearing of *P. interpunctella* and their control strategies utilizing dormancy management that should enhance the efficiency of sterile insect and natural enemies.

Manipulation of diapause development for improving control of *Eurytoma* species

Nikos A. Kouloussis and **Dimitris S. Koveos**

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Eurytoma amygdali and *Eurytoma plotnikovi* (Hymenoptera: Eurytomidae) are the two major pests of almond and pistachios trees respectively with high economic importance. Their control mainly relies on the application of cover insecticide sprays. These are often ineffective, thus creating the need for new control approaches. Both species are univoltine with similar life cycles and an obligatory larval diapause. The adults lay their eggs in the mesocarp of their host fruit and the hatching larvae destroy the seed and enter diapause as mature larvae. Diapausing larvae remain inside the fruit for a very long period of about 10 months (from June through summer and winter till the next year spring when the adults emerge). Therefore the intensity of diapause in both species is very high.

Previous experiments conducted in our lab have shown that diapause termination is affected by low temperatures and long day photoperiods. In further experiments we aim to try to manipulate larval diapause development and either prolong or shorten diapause duration. In particular we aim to test (1) low temperatures, photoperiod and night interruption in different phases of diapause development and determine the sensitive phases of diapause larvae, (2) the effect of various shocks such as short period exposure to very low subzero temperatures, or wounding which may result in acceleration of diapause development and (3) the effect of various chemicals applied on the surface of the infested fruits in various concentrations in the laboratory on diapause termination. Overall, the aim is to have post-diapause adults available for use at any time needed and explore possibilities of managing these insects with new methods.

Optimizing performance of biological control agents used in Integrated Pest Management

Karina V Fisker, Stine Slotsbo, Martin Holmstrup, Johannes Overgaard, Torsten N Kristensen, Jesper G Sørensen

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The use of arthropod predators and parasitoids as Biological Control Agents (BCAs) is well established in greenhouse horticulture and has recently received increasing attention for pest control in agriculture and fruit production. Phenological models have been developed optimizing the timing and frequency of releases of 'pest controllers', depending on presence of pests and the prognosis regarding the growth of these pest populations. However, a continued effort is needed to further improve and develop the use of BCAs in order to ensure efficiency and financial competitiveness of this approach. One aspect of the pest-BCA interaction that may have promising and unexploited benefits relates to the physiological and evolutionary fine tuning of the BCAs.

Our main idea is that effective selection (adaptation) and optimization of rearing conditions (acclimation) of BCAs can significantly increase their effectiveness against insect pests. Numerous studies have shown that the performance of insect species is highly affected by local adaptation as well as thermal and nutritional "history" of the animal. Based on this ecological paradigm we hypothesize that existing management using insect BCA systems would benefit greatly if these parameters were considered more actively when allocating BCA to plant production systems. We propose that even small improvements of the effectiveness of biological control of pest species may have a large potential to reduce the use of pesticides and therefore also the associated negative side-effects of pesticide use. Studies on thermal acclimation of the parasitic wasp *Trichogramma achaeae* and the ladybird *Adalia bipunctata* will be used to illustrate the potential.

Study of factors inducing dormancy on *Trichogramma nerudai* Pintureau and Gerdind and *T. pretiosum* Riley under rearing conditions

Viscarret, M. M.; López, S. N.; Medina, M del P.; Castro, F.

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Factors to induce diapause have been studied in many insect species. The alternative between dormancy (diapauses or quiescence) and normal development in insects depends on broad endogenous and exogenous factors. The knowledge and use of these factors to induce dormancy could be valuable to develop storage methods in the mass-rearing of biological control agents. *Trichogramma* spp. have been used in several biological control programs against lepidopteran pest. Two species of *Trichogramma* genus, *T. nerudai* and *T. pretiosum,* are rearing in our laboratory. The first one is a bi-parental species found on *Rhyacionia buoliana* and evaluated as natural enemy of, *Helicoverpa zea*, *Cydia pomonella*, *Tuta absoluta*, etc. *T. pretiosum* is a thelytokous species that parasitizes different lepidopteran eggs such as *Argyrotaenia sphaleropa*, *Bonagota cranaodes*, *Trichoplusia ni*, *Helicoverpa zea*, etc.

Our work group evaluated the effect of cold storage on *T. nerudai* using a uniform lower temperature. The results showed that storing *T. nerudai* pupae at low temperature could be useful to manage the rearing and release this natural enemy. However, the duration of storage would affect the biological quality of the species in a critical way.

For the first year of the project our goal is to study the effect of cold storage of pupae of *T. pretiosum* on the adult emergence time, sex ratio, deformed adult proportion and adult flight ability. Likewise, the effect of maternal photoperiodic conditions will be studied on the offspring of *T. nerudai* (maternal effect), recording the adult emergence time, adult emergence proportion, deformed adult proportion and female proportion. Parasitized host eggs from which no adult parasitoid emerges will be dissected to register the preimaginal stage of the dead parasitoid, both for the F0 and F1 generation.

The lipid and protein contents in the different stages of the dormancy will be evaluated. Recently, in *T. pretiosum* pupae some studies about contents of lipids, carbohydrates and glycogen were carried out. The wasps used in theses preliminaries studies were kept under rearing conditions of temperature, humidity and light/dark cycle.

Based on the results of this first phase of our research, we expect to determine a regular methodology of storage of these species.

Colony establishment of the egg parasitoid *Trichogrammatoidea lutea* Girault (Hymentoptera: Trichogrammatidae) for dormancy management studies, in order to improve integrated control of codling moth [*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)].

Wohlfarter M

Entomon Technologies (Pty) Ltd, P.O. Box 12669, Die Boord, 7613, South Africa (martin@entomon.co.za)

In order to expand the pest management service provided by Entomon Technologies (Pty) Ltd to apple & pear growers in the Elgin valley, Western Cape Province, South Africa, the polyphagous indigenous egg parasitoid, *Trichogrammatoidea lutea* (TL), was selected as an additional control agent for integrated management of codling moth (CM).

CM which is considered a key pest locally on apples & pears, is currently largely managed by the use of synthetic insecticides, pheromone mediated mating disruption (MD) and to a very limited extent bio-control by use of granulovirus and Sterile Insect Technology (SIT). Deciduous fruit growers employing SIT, do so in an effort to reduce reliance on insecticides, thus aiding the natural occurring predators, parasites and parasitoids to provide a bio-control service. Due to decades of harsh and plentiful pesticide applications, presence of such bio-control agents in commercial plantings is low. Mass rearing and augmented releases can therefore aid in the re-introduction of these predators, as well as, sustaining them throughout adverse weather- or production conditions.

The hanging of sentinel CM egg sheets provided a reliable measure for determining presence/absence of TL in commercial plantings, dispersal patterns after introduction into clean orchards and survival after orchard treatments with pesticides. TL's high susceptibility to a variety of pesticides stressed the need of maintaining a laboratory colony and continuous reintroductions in such conventional commercial plantings. Temperatures below 21°C and above 32°C have been found unfavourable for TL host parasitism and development, explaining their field absence during cooler early season conditions. Overwintering mechanisms, how these can be initiated or terminated and long-term storage of infected mass reared host eggs, have not yet been analysed, however are future aims of this study.

Since the establishment of a laboratory TL colony which ensures a constant supply of test insects, further work on the sequential and concurrent temperature conditioned rearing of CM can and will be conducted, for releases during the coming fruit growing season. Releases of TL under CM SIT can allow rapid TL population increases, due to the high number of host eggs present, therefore improving their overall efficiency.

Cold tolerance and diapause of beneficial and harmful arthropods

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Previous research has focused on the cold tolerance and diapause responses of both beneficial and harmful arthropods. For beneficial species, this relates to: 1) cold tolerance and diapause as key traits for the use of arthropod natural enemies in biological control in north Western Europe, 2) cold storage of mass reared beneficial arthropods, and 3) cold tolerance as part of the establishment potential of non-indigenous biological control agents in the framework of a risk assessment procedure. For instance, storage capabilities of predatory heteropterans and phytoseiids have been studied in order to rationalize their mass production and optimize their release. The overwintering potential of non-indigenous coccinellid predators used in biological control, including *Harmonia axyridis* and *Cryptolaemus montrouzieri*, has been explored yielding information relevant to the risk assessment of these biological control agents in northwestern Europe. Further studies indicated the role of bacterial endosymbionts in the cold tolerance of the mirid predator *Macrolophus pygmaeus.*

For harmful arthropods, part of the research relates to the establishment potential of invasive pests. The overwintering strategies of the invasive tomato leaf miner *Tuta absoluta* have been studied, in order to assess its establishment potential in northwestern Europe. This was done by investigating diapause responses using climatic chambers, and by determining supercooling points, lethal temperatures, and lethal times.

Furthermore, we investigated essential physiological processes in diapausing *Tetranychus urticae* by studying genome-wide expression changes. This experiment compared active female spider mites with diapausing females that were both cultured in diapausing conditions (17°C, 8L:16D) and was carried out using a custom built microarray. The results displayed that 11% of the total predicted *T. urticae* genes were differentially expressed of which 50% was downregulated. Genes related to detoxification, cryoprotection, cytoskeletal organization, carotenoid synthesis and signal transduction were all greatly influenced during diapause. This study further adds to the understanding of the overall strategies of diapause in arthropods.

Can cold tolerance be transferred from hosts to parasitoids?

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Increasing cold tolerance is a goal that would enhance the storage, shipment and usage of insect parasitoids for biological control. We have tested methods for doing this using the fly parasitoid, *Nasonia vitripennis,* and one of its favored hosts, the flesh fly *Sarcophaga crassipalpis.* The diapause status of the parasitoid had a profound impact on cold tolerance: diapausing larvae were far more cold tolerant than their nondiapausing counterparts. The host's diapause status also significantly influenced the parasitoid's cold hardiness, but this effect was much smaller than the effect generated by the parasitoid's diapause status.

The success of the Kostal laboratory in enhancing cold tolerance in several species of Diptera by supplementing the fly diet with proline and other potential cryoprotectants prompted our attempt to increase cold tolerance in *N. vitripennis* by augmenting the host fly's diet with alanine, proline and glycerol, to thus determine whether we could observe a tri-trophic effect on cold tolerance in the parasitoid. A series of experiments first defined concentrations of putative cryoprotectants that could be tolerated by the fly host. Parasitoid cold hardiness was enhanced when any of the three cryoprotectants were added to the host fly's diet, but the most pronounced effect was achieved with proline. These results thus suggest that cold tolerance of parasitoids can indeed be enhanced by manipulating the diet of the host.

Experiments currently underway, in association with the Kostal laboratory, are using metabolomics to identify shifts in metabolites elicited in the host and parasitoid in response to diapause status and in response to alternations in the host diet. Results from such experiments may identify additional metabolites that could be used as dietary supplements to increase cold tolerance.

New approaches to understanding insect freeze tolerance

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The ability of some insects to survive internal ice formation was first described over 200 years ago, yet we still do not understand the underlying mechanisms, nor are we able to induce robust freeze tolerance in non-freeze tolerant insects. I will briefly discuss what is known about insect freeze tolerance, and how the approaches we have taken in the past may have led to dead-ends. I will then describe new approaches being taken in my laboratory to elucidate the mechanisms underlying freeze tolerance in insects. These are 1) the development of new comparative models that allow variation in freeze tolerance to be studied on seasonal, geographic, and evolutionary scales; 2) the utilization of –omics technologies (primarily RNA-Seq) to identify new candidate molecules and pathways; 3) an hypothesis-testing approach to critically examine the existing models of insect freeze tolerance. I will present a selection of data derived from each of these approaches, particularly focusing on whether freeze tolerance has an underlying set of common mechanisms in spite of having evolved independently on multiple occasions. I will also discuss new data about the processes associated with thawing in insects that survive freezing, and discuss the identity, function, and synthesis of acetylated lipids, which may function as intracellular cryoprotectants. I conclude that these new approaches signal an exciting time in the study of freeze tolerance, and the likelihood of identifying novel biochemical and physiological mechanisms of cold tolerance in ectotherms.

Fluctuating thermal regimes: an efficient method to extend the shelf life of insects during cold storage

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Insects used in biological control or sterile insect technique programs have to be reared in very large numbers for mass release. It is thus essential to have efficient storage methods during the processes of mass-rearing, distribution and release. Storage of insects is not limited to industrial rearing of beneficial insects, it is also required for maintaining species or lines collections for research purpose. Low temperature is the main method used to increase the shelf-life of insects. In a few model organisms, such as yeasts or nematodes, individuals may be stored frozen at ultralow temperatures. However, cryopreservation has very limited applications in insects. A potential alternative strategy to prolong insect's generation time arises from consideration of overwintering strategies. For species that exhibit a diapause, a long-term cold storage (i.e. months) may be realized, assuming that induction and termination can be controlled. However, the ability to enter diapause is not present in all species. For many species, only a short-term cold storage (i.e. days to weeks) can be realized with non-dormant and often chill-susceptible phenotypes.

Usually, constant low temperatures (CLTs) are used to extend the shelf life of insects during storage. Exposure to CLTs leads to the accumulation of chilling damages in a time- and temperature-dependent manner. However, in nature the temperature fluctuates and insects may exploit daily bouts of warmer temperature to recover from chilling injuries. Based on this assumption, we have previously shown in several insect taxa that interrupting the cold period with periodic bouts of warmer temperatures (i.e. fluctuating thermal regimes, FTRs) deeply reduced cold-induced mortality. This suggests that insects possess the ability to quickly recover from cold and to switch between injury and non-injury state in a very quick fashion. An increasing number of studies have found a positive impact of FTRs on insect's cold tolerance, which reinforces the need to explore cold tolerance (or storage) under thermally-variable settings.

I will first review the early observations on the promoting effect of FTRs on insect cold tolerance. I will highlight how survival gain under FTRs varies according a range of parameters. The mechanisms underlying the higher cold tolerance of insects submitted FTs during the cold exposure are currently under deep investigation. In the second part, I will review the current knowledge on physiological mechanisms that are involved in the positive effect of FTRs. So far, physiological data on the response to FTRs have been merely correlative. Thus, to fully appreciate the physiological consequences of FTRs, manipulative experiments would be desirable (e.g. genetic manipulations targeting particular genes/functions or pathways of interest). Because of the technical advantages and the range of physiological and genetic tools available for drosophila, we are now starting research on FTRs using drosophila as “work horse” for understanding the underpinning of FTRs. I will present the early stages of this research. I will show that drosophila flies robustly respond to FTRs, which offers great opportunity for exploring the mechanisms of FTRs.

Use of fluctuating thermal regimes and decreased oxygen during storage improves survival of post-diapause *Megachile rotundata*

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The alfalfa leaf cutting bee, *Megachile rotundata,* is the world's most intensively managed solitary bee. *M. rotundata* is used primarily for alfalfa seed production in the northwestern United States and western Canada, but also provides pollination services for carrot, hybrid canola, onion, various legumes and other specialty crops. Under normal management practices, *M. rotundata* are overwintered as diapausing prepupae using a constant temperature (4-6°C) regime from late September until early spring of the following year. Those prepupae not used or sold are an economic loss to the producers. An improved storage protocol for diapausing and post-diapausing quiescent prepupae would decrease economic loss to the producers and help stabilize year-to-year cost fluctuation for customers. Our working hypothesis is: Any protocol that delays the onset of mortality also delays the onset of sublethal effects that degrade pollinator quality.

Our first objective was to determine if diapausing prepupae could be stored for more than one year. It has been clearly demonstrated that insect tolerance to low-temperature exposure can be significantly increased by a fluctuating thermal regime (FTR): short high temperature pulses during the low-temperature exposure. Storing diapausing and post-diapausing prepupae under FTR extended their survival into the second post-wintering growing season. We achieved further gains in storage duration by decreasing the concentration of oxygen the prepupae were exposed to during storage. Post-diapausing prepupae stored under FTR and under 10% oxygen survived 23 months without significant decrease in survival.

To further our understanding of storage physiology of *M. rotundata,* we conducted two RNA-seq experiments. In the first, post-diapausing prepupae were stored under either constant 6°C or under FTR. Within the FTR treatment, transcripts involved in ion homeostasis, various metabolic pathways, and oxidative stress response were upregulated. In the second experiment, two groups of prepupae were collected depending on when they entered diapause (early and late season). The two groups were further subdivided according to how they were overwintered (constant 6°C or under field conditions). Samples were collected from the four treatments from October to June. Approximately 6400 differentially regulated transcripts were isolated. One notable observation was that there were environmental- (laboratory versus field) and seasonal- (early versus late) specific differentially regulated transcripts. These results indicate that diapause offers unique opportunities for significant improvement in storage of *M. rotundata* as well as for other agriculturally important insects.

Overwintering and cold tolerance in the codling moth (*Cydia pomonella*)

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The codling moth (*Cydia pomonella*) is a major insect pest of apples worldwide. Fully grown last instar larvae overwinter in diapause state. We observed that codling moth larvae rely on extensive supercooling, or freeze-avoidance, as on their major strategy for survival of the winter cold. The supercooling point decreases from approximately -15.3°C during summer to -26.3°C during winter. Seasonal extension of supercooling capacity is assisted by partial dehydration, increasing osmolality of body fluids, and the accumulation of a complex mixture of winter specific metabolites. Glycogen and glutamine reserves are depleted, while fructose, alanine and some other sugars, polyols and free amino acids are accumulated during winter. The concentrations of trehalose and proline remain high and relatively constant throughout the season, and may contribute to the stabilization of proteins and membranes at subzero temperatures. In addition to supercooling, overwintering larvae acquire considerable capacity to survive at subzero temperatures, down to -15°C, in partially frozen state (freeze-tolerance). We analyzed fatty acid composition of triacylglycerol (TG) depots in the fat body and relative proportions of phospholipid (PL) molecular species in biological membranes. In addition, temperature of melting (T_m) in TG depots was assessed by using differential scanning calorimetry and the conformational order (fluidity) of PL membranes was analyzed by measuring the anisotropy of fluorescence polarization of diphenylhexatriene probe in membrane vesicles. Significant increase of relative proportion of linoleic acid (C18:2n6) at the expense of palmitic acid (C16:0) in TG depots during the larval transition to diapause was observed, which was accompanied with decreasing melting temperature of total lipids, which in turn, might increase the accessibility of depot fats for enzymatic breakdown during overwintering. The fluidity of membranes was maintained very high irrespective of developmental mode or seasonally changing acclimation status of larvae. The seasonal changes in PL composition were relatively small. We will discuss the results in light of alternative survival strategies of codling moth larvae (supercooling vs. freezing) and variability / low predictability of environmental conditions.

Molecular regulation mechanism of winter and summer diapause in *Delia antiqua* for the control of insect pests

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To escape harsh environments such as the cold of winter, the high temperatures and drought of summer, one of the strategies adopted by insects is diapause. Diapause is a form of developmental arrest that occurs at a specific stage in the insect life cycle and is usually programmed by photoperiod and temperature. Insect diapause involves intricate molecular regulation mechanisms, and is regulated by hormones including juvenile hormone, ecdysteroids, and peptide hormones. The onion maggot, *Delia antiqua*, a major underground agricultural pest, is widely distributed in Asia, Europe and North America. The pest larvae damage bulb onions, garlic, chives, shallots, leeks and the bulbs of tulips. Onion maggot naturally enters diapause in the pupal stage in winter or summer seasons just after the head evagination occurs.

To insight into the molecular mechanisms governing diapause and how they could be utilized beneficially for agriculturally-important pest control, we have established a model system for the comparative study of winter and summer diapause in *Delia antiqua*, and have conducted preliminary studies on winter and summer diapause-associated genes in recent years. Based on this model system, our preliminary studies shows that winter and summer diapause performs inconsistency in physiological processes. While the winter diapause-associated genes have been moderately investigated, the molecular regulation mechanism of summer diapause is little understood. The ongoing research in the first 18 months on the subject will aim to: 1) identify both the shared and the specific genes associated with both diapauses at the different diapausing stages of the species using RNA-seq; 2) confirm the association of these genes with diapause using PCR array; 3) clone and sequence the full-length cDNA of those genes associated with diapause using 5'RACE and 3'RACE; 4) characterize them through bioinformatics analyses, and analyze their regulatory pathways. The further study in the next step will aim to: 5) study their function in diapause regulation using RNAi.

In the long run, our ultimate aims are: 1) to determine how the neuropeptide diapause hormone exerts its diapause-induction and -terminating in *D. antiqua*; 2) to unravel the mechanism of photoperiod influence on diapause induction and the function of clock genes; 3) to investigate the molecular mechanism between diapause and corresponding increasing high- and low-temperature tolerance through genome sequencing and comparative genomics; 4) to probe insect population manipulation techniques through preventing, terminating, or prolonging diapause, and develop the new strategies for the control of pest insects using key diapause-regulating genes identified in this research.

Population Genomics of Diapause Phenotypes in European *Ips typographus* (Coleoptera, Curculionidae) Using High-Throughput RADSeq

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We will present the aims of a project funded by the Austrian Science Foundation.

Insects use diapause to synchronize their life cycles. In this project, the genetic variation of diapause of the European spruce bark beetle, *Ips typographus* will be analyzed. Increases in temperature and progressively warmer springs permits more rapid rates of development in this spruce pest with the consequence of an increase in the number of generations per year in populations dominated normally by univoltine individuals. The method double digest restriction site associated DNA sequencing, ddRADSeq, will be applied in order to investigate 1) the genetic basis of the evolution of facultative diapause from an ancestral condition of obligate diapause and 2) the phylogeography of European *I. typographus* populations emphasizing functional genetic variation associated with the diapause phenotype. Ecopyhsiologically defined individuals reared in the laboratory will be genetically screened via ddRADSeq and information shall be obtained on the genetic basis of alternative diapause developmental pathways. As diapause is a complex developmental phenotype only the analysis of a large number of loci or single nucleotide polymorphisms covering the entire genome will distinguish genome-wide phylogeographic effects among loci from genetic divergence driven by selection on the diapause phenotype. Distinguishing between phylogeographic structure and local selection will allow the identification of genomic regions subject to adaptation. Consequently, ddRADSeq will be applied on European populations studying how populations from Europe are genetically structured. Besides demographic information, this screening will bring insight how single populations are phenotypically structured, i.e. an estimate how many obligate *vs.* facultative individuals are present in each population.

Genetic basis of rapid evolutionary shifts in polygenic diapause regulation across development.

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Though in some species genes of major affect appear to underlie naturally segregating variation in diapause, the extreme evolutionary liability of diapause suggests that the trait must often be under polygenic control. I will present data consistent with an evolutionary shift in allele frequencies at many loci underlying divergent diapause timing between two recently diverged host races of the apple pest *Rhagoletis pomonella* (Diptera: Tephritidae). *R. pomonella* historically infested North American Crataegus (hawthorn) fruits, but a host race infesting Malus (apples) has formed sometime in the ~300 years since apples were introduced. The flies are univoltine, short lived, and overwinter in a pupal diapause. Adult emergence must tightly coincide with host fruits, and as a consequence the derived, apple-infesting host race terminates pupal diapause earlier than the ancestral, haw-infesting host race. Earlier seasonality also changes environmental conditions during diapause initiation, so both phases of diapause appear to be under strong selection. We performed ddRADseq (Genome-wide SNP genotyping) on several phenotypes associated with diapause initiation and termination, and we demonstrate that 1) variation in both diapause phases is highly polygenic, 2) that host races are divergent at diapause-associated loci, and 3) that similar loci affect both initiation and termination, constraining the independent evolution of these ontogenetically distinct traits.

Molecular profiling of the diapause programme to identify mechanisms underpinning diapause regulation, enhanced stress tolerance and post-diapause fitness: applications in optimising mass culturing, SIT and biocontrol

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Diapause is crucial to insect survival of seasonally recurring chronic forms of environmental stress, and to coordinate growth, development and reproduction with annual cycles of favourable environmental conditions. Despite the fundamental role of diapause in determining the spatial and temporal distribution of insects, as well as its importance in the mass culturing of insects for SIT and biocontrol, our understanding of the molecular processes underpinning diapause regulation is extremely limited. Many ecologically relevant species are yet to be sequenced, which limits our ability to characterize genetic changes associated with the diapause programme. Metabolomics and lipidomics allow unbiased screening of metabolic changes associated with diapause, and have the advantage in representing an end-point measure of molecular adaptation downstream of any changes in gene or protein expression. In this regard, metabolomics also serves to validate hypotheses about pathways generated from transcriptomic or proteomic studies, and pin identities on the metabolites involved. This facilitates the identification of potential mechanisms with a direct contribution to diapause-associated phenotypes. Here I describe initial results of a detailed metabolomic and lipidomic analyses of diapause in the blue bottle fly *Calliphora vicina*. We provide evidence of diapause up-regulated metabolites and lipids linked to stress tolerance, energy metabolism and membrane adaptation. Recently, we have identified cross-generational plasticity in cold hardiness that is uniquely associated with diapause. Ongoing work with *C. vicina* and *Nasonia vetripennis* now seeks to elucidate whether information about the environmental gradients that programme diapause is maternally transferred to the progeny via metabolic signals.

Roles of the circadian clock genes in photoperiodic regulation of diapause in the bean bug

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A photoperiodic response in insects comprises a sequence of several events: (i) photoreception; (ii) measurement of day or night length by a photoperiodic time measurement system; (iii) simultaneous counting of the number of photoperiods by a counter system; and (iv) action of the endocrine effectors that determine seasonal events like diapause. Classic physiological experiments revealed that a circadian clock, an endogenous time-keeping system generating rhythmicity with an about 24-h period, is causally involved in the photoperiodic time measurement. However, molecular validation of this hypothesis has been awaited.

The RNAi technique successfully sheds light upon this issue in the bean bug *Riptortus pedestris*, a major pest of grain legumes in Africa and Asia. RNAi verified in this species that the circadian clock genes *period* (*per)* and *mammalian-type cryptochrome* (*cry-m*) function as negative regulators, and *cycle* (*cyc*) and *Clock* (*Clk*) function as positive regulators, in the circadian clock. RNAi directed against negative and positive regulators disrupted circadian clock, and interestingly produced distinct phenotypes in the circadian rhythmicity. Simultaneously, *per* and *cry-m* RNAi caused the insect to avert diapause under a diapause-inducing photoperiod whereas *cyc* and *Clk* RNAi induced diapause under a diapause-averting photoperiod. Thus, RNAi directed against negative and positive regulators had opposite effects not only in the circadian rhythm but also in the photoperiodic response. This study revealed that the circadian clock operated by *per*, *cry-m*, *cyc* and *Clk* governs seasonal timing as well as the daily rhythms. "Clock genes" would be the new candidates for the point of action to regulate diapause.

Annex 5: Glossary of terms related to dormancy and cold tolerance

Acclimation Change in physiological traits in response to environmental cues, typically to a single cue under strictly controlled laboratory conditions.

Acclimatization Change in physiological traits in nature in response to numerous environmental cues acting simultaneously.

Acute cold exposure Brief exposure to cold, usually over a period of seconds to hours. (Also cold shock)

Adaptation Change in population gene/genotype frequencies in response to selection pressure.

Antifreeze protein Protein that binds to ice crystals, preventing its growth in supercooled fluid (or preventing recrystallization in a frozen solution). Also known as Thermal Hysteresis Protein.

Chill coma The reversible inability to move (paralysis) at low temperatures.

Chill coma onset The point of entry into chill coma. (Also see critical thermal minimum, CT_{min}).

Chill coma recovery The process of recovery of movement when rewarmed from chill coma. Usually measured as a function of time (chill coma recovery time, CCRT).

Chill susceptible Insects that are killed at mild low temperatures (typically above or close to zero, far above their SCP).

Chill tolerant Insects that are not killed by mild low temperatures but die at relatively low temperatures (typically below zero but still well above their SCP).

Chronic cold exposure Prolonged exposure to cold, usually over a period of days to weeks (in an ecological sense: longer than a single night).

Critical thermal minimum (CT_{min}) The temperature at which an insect enters chill coma. (Also see chill coma onset).

Cryopreservation Storage of biological material at temperatures below cryogenic temperature of -140°C (theoretically for unlimited period of time).

Cryoprotectant Any molecule that protects cells, membranes and (macro) molecules from cold injury.

Cryoprotective dehydration A cold tolerance strategy that relies on considerable dehydration (almost complete loss of water) caused by the presence of ice in the environment (typically occurs in small and water-permeable soil animals).

Delayed mortality Mortality that occurs anytime later (not immediately) after the treatment (or environmental insult) is over (inability to complete the whole life cycle).

Developmental plasticity Environmentally-induced variations within a single genotype that occur during development encompassing more than one life stage.

Diapause Endogenously- and centrally-mediated interruption of ontogeny that routes the developmental programme away from morphogenesis or sexual maturation into an alternative diapause programme. The start of diapause usually precedes the advent of adverse conditions and the end of diapause need not coincide with the end of adversity. In some species, diapause is an obligate portion of the lifecycle and in some species diapause is facultative in response to environmental cues.

Diapause depth A term that typically refers to the level of metabolic suppression during diapause. Typically quantified as the difference between active and diapause metabolic rate.

Diapause Development Unknown physiological process(es) lead to more or less gradual decrease of diapause intensity and increase of sensitivity to diapause terminating conditions.

Diapause induction Occurs during specific ontogenetic stage (sensitive period) when cues from the environment are perceived and transduced into switching the ontogenetic pathway from direct development to diapause when the token stimuli reach some critical level (the response may be modified by other environmental factors).

Diapause intensity Describes the duration of refractoriness of an individual to resume development when it is transferred from diapause promoting/maintaining conditions to diapause averting/terminating conditions (typically measured as time needed to reach overt signs of development). Diapause intensity is most often viewed in the context of the ability to reinitiate normal development.

Diapause maintenance Endogenous developmental arrest that persists while the environmental conditions are favourable for direct development. Specific token stimuli may help to maintain diapause (prevent its termination). Metabolic rate is relatively low and constant.

Diapause programme Encapsulates the entire process of diapause from the beginning of the induction phase until the end of diapause and resumption of development.

Diapause termination Specific changes in environmental conditions or endogenous seasonal rhythms stimulate the decrease of diapause intensity to its minimum level and thus synchronize individuals within a population. By the end of the termination phase, a physiological state is reached in which direct development may overtly resume (if the conditions are permissive) or covert potentiality for direct development is restored but not realized (if the conditions are not permissive).

Direct chilling injury Injury arising from the cold shock. Underlying mechanisms include immediate effects of cold on cells or macromolecules (in the absence of ice formation) such as membrane phase transition, solute leakage, protein dissociation or denaturation.

Direct development When used in the context of diapause, a developmental program that does not include any diapause stage. In the broader developmental literature, direct development refers to situations where morphology is relatively invariant across ontogeny, i.e., that juvenile stages closely resemble mature adult stages.

Dormancy A generic term covering any state of suppressed development (developmental arrest) or suspended activity.

Fluctuating temperature (FT) A generic term covering a broad range of temporally dynamic thermal regimes, as opposed to unchanging, constant temperature (CT).

Freeze avoidant A cold tolerance strategy whereby insects survive low temperatures by depressing the supercooling point. Freeze-avoidant insects will survive low temperatures, but are killed if ice formation occurs (at SCP).

Freeze tolerant	A cold tolerance strategy that relies on the survival of internal ice formation.
Freezing injury	Injury linked to formation of ice crystals. Underlying mechanisms include mechanical effects of growing ice crystals, freeze dehydration and solute concentration.
***FTR - Fluctuating temperature regimes* (FTR)**	A storage protocol in which the prolonged exposures to cold (typically days) are interrupted by repeated short bouts (typically hours) at permissive temperature that allows recovery from chilling injuries. From the perspective of ecological relevance, temperatures normally fluctuate on several timescales, including daily and this may also be reflected in thermal stress experiments.
Glass transition	The phase transition from liquid to solid phase without formation of crystalline structure. This yields an amorphous, glass-like state. (Also called vitrification).
Ice nucleating agent	Any entity that can initiate ice formation in a supercooled fluid. Includes ice crystals, dust, microbes, some proteins.
Indirect chilling injury	Injury arising from chronic exposure to cold. Underlying mechanisms include cumulative effects of cold on whole organism such as disturbance of homeostatic processes, accumulation of toxic products, depletion of energy reserves.
Inoculative freezing	Freezing initiated by nucleators external to the insect, such as ice crystals in the environment.
Low temperature storage	Storage of biological material at temperatures below thresholds for optimal physiological activity, growth or development.
Phenology	The seasonal timing of life-history events.
Phenotypic plasticity	Different phenotypes expressed by a single genotype in response to different environmental conditions. Includes acclimation, acclimatization, etc.
Photoperiod	The duration of an organism's daily exposure to light - It includes the alternation of scotophase (dark part of the cycle) and photophase (light part of the cycle).
Physiological conditioning	Any treatment (pretreatment) that modifies the physiology of an organism. In the context of stress biology, conditioning is often associated with later alterations in a stress response.

Post-diapause quiescence	Exogenously imposed inhibition of development and metabolism, which follows the termination of diapause when conditions are not favourable for resumption of direct development.
Quiescence	Immediate response (without central regulation) to a decline of any limiting environmental factor below the physiological thresholds with immediate resumption of the processes if the factor rises above them.
Rapid cold-hardening	Improvement of cold tolerance by a short pre-exposure to a milder cold stress.
Recrystallization	The process of ice crystal growth in frozen solutions.
Sublethal effects	Decrease in fitness, performance and competitiveness of survivors (including ideally also their offspring). Sublethal effects typically result from indirect chilling injury.
Supercooling	Phenomenon by which water and aqueous solutions remain unfrozen at temperatures below the equilibrium melting point.
Supercooling point	The temperature at which an insect is measured to freeze (usually by observing the exotherm linked to release of heat during ice crystallization).
Thermal hysteresis	A difference between the melting point and the freezing point of an aqueous solution. Often indicates the activity of antifreeze compounds.
Thermoperiod	Daily temperature cycles. It includes the alternation of thermophase (warm part of the cycle) and cryophase (cold part of the cycle).