New insights to improve the Integrated Pest Management of three tortricid moths

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TESI DOCTORAL

New insights to improve the Integrated Pest Management of three tortricid moths

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ABSTRACT

The Sustainable Development Goals promoted by United Nations mainly aim to balance human growth with environmental preservation. Pest management can help to achieve Goal 2 (zero hunger) and Goal 12 (responsible consumption and production) as it contributes to increase yield, but it can also have a big impact on the surrounding environment. In this document, it is intended to contribute to these goals through the acquisition of knowledge that can be used in Integrated Pest Management (IPM) programs. Monitoring pest populations is a key procedure in IPM; it permits the application of control methods when the most susceptible stage of the pest occurs. Besides reducing the impact of current pest control strategies, the development of novel control methods with low environmental impact is needed to replace the most harmful methods. Behaviour manipulation has been successfully used to design new control methods based on volatile semiochemicals, such as mating disruption for moths or mass trapping in fruit flies. Nevertheless, insects use a wide range of chemical and physical cues to discern the surrounding environment; therefore, there are other options to develop new control methods based on behavioural interference.

In this PhD, three key fruit pests have been studied, *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Dennis & Schifermüller), with a special focus on *G. molesta* biology. *G. molesta* is causing severe damage to apple trees in the Girona province (NE Spain). In addition, in a preliminary evaluation by the IRTA researchers, it was detected that the official phenology model of *G. molesta* did not adjust to the population dynamics registered in apple orchards in Girona throughout the year. To corroborate this observation, the population dynamic of *G. molesta* in Girona and Lleida provinces was compared, as was the biology of two populations, one coming from Girona and the other from Lleida. The performance of *G. molesta* larvae in apple fruits at field conditions and at fixed temperatures in the laboratory was studied. Besides improving the current monitoring system, we also aimed to introduce new behaviour manipulation methods to IPM. We developed and tested a multi-choice arena to explore oviposition behaviour of *C. pomonella*, *G. molesta* and *L. botrana* to non-volatile cues. Two sugars (fructose and sucrose), two salts (KCl and NaCl) and a plant extract (neem oil) were tested. To further understand the perception mechanism of non-volatile stimuli by the three species, electrophysiologic recordings in sensilla chaetica of the adults' moths were carried out. Moreover, the number and position of sensilla chaetica in labial palps were recorded.

The study of the population dynamics of *G. molesta* showed the phenology model officially used estimated with enough precision the first generation in both provinces, but the whole phenology of the pest was only accurate for the Lleida province. In the Girona province, the generation time of *G. molesta* was longer than predicted by the model; as a consequence, one less generation was observed in Girona. This study confirms the lack of fit of the phenology model

for the development of the species in apple in the Girona province and the need to develop a new model for the development of the species in apple. The comparison of the development of the two populations (Girona and Lleida) in apples showed no differences between them. An increase in the development on apple fruits at the field experiment compared with that recorded at constant temperatures in the lab was found, especially marked when the larvae fed on fruits still attached to the trees. Remarkably, the results pointed out that the connection between the fruit and the plant may be delaying the development of *G. molesta* in apples.

Results on the oviposition behaviour showed that neem oil had the strongest effect. It had a clear deterrent effect on *G. molesta* and *C. pomonella.* Although on *L. botrana*, neem oil was deterrent at the highest concentration tested (10% in volume), it was stimulant at the lowest concentration (0.1%). The sugars tested generally reduce oviposition at the highest dose (1M); nevertheless, significant differences were found between species and sugars, e.g., in *C. pomonella*, 100mM of sucrose stimulate oviposition. Salts also showed differences between species; in *C. pomonella*, they increased oviposition at 100mM for KCL and at 100mM and 1M for NaCl, while in *G. molesta* and *L. botrana*, they had no significant effect. In this PhD, it was confirmed, for the first time in Lepidoptera, the contact-chemoreceptive function of the sensilla present in the labial palp. Moreover, the labial palp sensilla showed a higher response to sugars than the sensilla in the antenna, confirming the relevance of labial palp sensilla in the studied species.

The knowledge obtained in this PhD will help improve the IPM strategies. The differences found in the phenology of *G. molesta* between the Lleida and Girona populations are a strong reminder of the need to validate the phenology models in the actual conditions under which they are going to be used. Further studies should explore the causes of the differences detected between fruits attached and detached from the tree in the development of *G. molesta.* Neem oil seems a good candidate to explore the introduction of oviposition interference as a control method. The knowledge acquired in the electrophysiologic studies will help to better understand the whole system and avoid unforeseen effects that could impede the introduction of non-volatiles in IPM strategies.

RESUM

Els Objectius de Desenvolupament Sostenible promoguts per les Nacions Unides aspiren a equilibrar el creixement humà amb la preservació del medi ambient. El control de plagues pot ajudar a assolir l'objectiu 2 (fam zero) i l'objectiu 12 (producció i consum responsables), ja que contribueix a augmentar el rendiment dels cultius, però també pot tenir un impacte negatiu en el medi ambient. En aquest document, es pretén contribuir a aquests objectius a través de l'adquisició de coneixements que puguin ser utilitzats en els programes de Control Integrat de Plagues (CIP). El monitoratge de les poblacions de plagues és un aspecte clau al CIP; permet l'aplicació de mètodes de control a l'estadi més susceptible de la plaga. A més de reduir l'impacte de les estratègies de control de plagues actuals, també cal el desenvolupament de nous mètodes de control amb baix impacte per reemplaçar els mètodes més perjudicials. La manipulació del comportament s'ha utilitzat amb èxit per dissenyar mètodes de control basats en semioquímics volàtils, per exemple la confusió sexual en papallones nocturnes o la captura massiva en mosques de la fruita. Tanmateix, els insectes utilitzen una àmplia gamma de senyals químics i físics per discernir l'entorn que els envolta; per tant, hi ha altres opcions per desenvolupar nous mètodes de control basats en la manipulació del comportament.

En aquesta tesi doctoral, s'han estudiat tres plagues clau en fruiters, *Cydia pomonella* (L.), *Grapholita molesta* (Busck) i *Lobesia botrana* (Dennis & Schifermüller), amb un enfocament especial a la biologia de *G. molesta. G. molesta* està causant greus danys a les pomeres a la província de Girona (NE d'Espanya). A més, en una avaluació preliminar dels investigadors de l'IRTA, es va detectar que el model fenològic oficial de *G. molesta* no s'ajustava a la dinàmica poblacional registrada a les pomeres de Girona al llarg de l'any. Per corroborar aquesta observació, es va comparar la dinàmica poblacional de *G. molesta* a les províncies de Girona i Lleida, així com la biologia de les dues poblacions. Es va estudiar desenvolupament de larves de *G. molesta* en pomes en condicions de camp i amb temperatures constants al laboratori. A més de millorar el sistema de monitorització actual, també aspirem a introduir nous mètodes de manipulació del comportament en el CIP. Hem desenvolupat i provat un test de selecció múltiple per explorar el comportament d'oviposició de *C. pomonella, G. molesta* i *L. botrana* amb estímuls no volàtils. Es van testar dos sucres (fructosa i sacarosa), dues sals (KCl i NaCl) i un extracte vegetal (oli de neem). Per comprendre millor el mecanisme de percepció aquests estímuls en les tres espècies, es van realitzar registres electrofisiològics en sensílies caètiques de les arnes. A més, es van registrar el nombre i la posició de les sensílies caètiques als palps labials.

L'estudi de la dinàmica poblacional de *G. molesta* va mostrar que el model fenològic utilitzat oficialment estimava amb força precisió la primera generació a les dues províncies, però la fenologia al llarg de l'any només era predita correctament a la província de Lleida. A la província de Girona, el temps de generació de *G. molesta* va ser més llarg del previst pel model; com a conseqüència, es va observar una generació menys a Girona. Aquest estudi confirma la manca d'ajust del model fenològic per al desenvolupament de l'espècie en pomera a la província de

Girona i la necessitat de desenvolupar un nou model per al desenvolupament de l'espècie en pomera. La comparació del desenvolupament de les dues poblacions (Girona i Lleida) en pomes no va mostrar diferències entre elles. Es va trobar un increment en el desenvolupament en fruits de poma a l'experiment de camp en comparació amb el registrat a temperatures constants al laboratori, especialment marcat quan les larves s'alimentaven de fruits encara units als arbres. Sorprenentment, els resultats apunten que la unió entre el fruit i la planta pot estar endarrerint el desenvolupament de *G. molesta*.

Els resultats sobre el comportament de l'oviposició van mostrar que l'oli de neem va tenir més efecte. Va tenir un clar efecte dissuasori sobre *G. molesta* i *C. pomonella*. Tot i que a *L. botrana*, l'oli de neem va ser dissuasori en la concentració més alta (10 % en volum), va ser estimulant en la concentració més baixa (0,1 %). Els sucres provats generalment redueixen l'oviposició a la dosi més alta (1M); no obstant això, es van trobar diferències significatives entre espècies i sucres, per exemple, a *C. pomonella*, 100 mM de sacarosa estimulen l'oviposició. Les sals també van mostrar diferències entre espècies; a *C. pomonella* van augmentar l'oviposició a 100 mM per a KCL i a 100 mM i 1 M per a NaCl, mentre que a *G. molesta* i *L. botrana* no van tenir cap efecte significatiu. En aquesta tesi doctoral es va corroborar, per primera vegada en lepidòpters, la funció quimioreceptora de les sensílies presents al palp labial. A més, les sensílies del palp labial van mostrar una resposta més gran als sucres que les sensílies de l'antena, cosa que confirma la rellevància de les sensílies del palp labial en les espècies estudiades.

El coneixement obtingut en aquest doctorat ajudarà a millorar les estratègies de CIP. Les diferències trobades a la fenologia de *G. molesta* entre les poblacions de Lleida i Girona són un fort recordatori de la necessitat de validar els models fenològics en les condicions reals en què s'utilitzaran. Estudis futurs haurien d'explorar les causes de les diferències detectades entre fruits adherits i despresos de l'arbre en el desenvolupament de *G. molesta*. L'oli de neem sembla un bon candidat per explorar la introducció de la manipulació de l'oviposició com a mètode de control. Els coneixements adquirits als estudis electrofisiològics ajudaran a comprendre millor tot el sistema i evitar efectes imprevistos que podrien impedir la introducció de no volàtils en les estratègies de CIP.

RESUMEN

Los Objetivos de Desarrollo Sostenible promovidos por Naciones Unidas aspiran a equilibrar el crecimiento humano con la preservación del medio ambiente. El control de plagas puede ayudar a lograr el Objetivo 2 (hambre cero) y el objetivo 12 (producción y consumo responsables) ya que contribuye a aumentar el rendimiento de los cultivos, pero también puede tener un impacto negativo en el medio ambiente. En este documento, se pretende contribuir a estos objetivos a través de la adquisición de conocimientos que puedan ser utilizados en los programas de Control Integrado de Plagas (CIP). El monitoreo de las poblaciones de plagas es un aspecto clave en el CIP; permite la aplicación de métodos de control en el estadio más susceptible de la plaga. Además de reducir el impacto de las estrategias de control de plagas actuales, también se necesita el desarrollo de nuevos métodos de control con bajo impacto para reemplazar los métodos más dañinos. La manipulación del comportamiento se ha utilizado con éxito para diseñar métodos de control basados en semioquímicos volátiles, por ejemplo la confusión sexual en mariposas nocturnas o la captura masiva en moscas de la fruta. Sin embargo, los insectos utilizan una amplia gama de señales químicas y físicas para discernir el entorno que los rodea; por lo tanto, existen otras opciones para desarrollar nuevos métodos de control basados en la manipulación del comportamiento.

En esta tesis doctoral, se han estudiado tres plagas clave en frutales, *Cydia pomonella* (L.), *Grapholita molesta* (Busck) y *Lobesia botrana* (Dennis & Schifermüller), con un enfoque especial en la biología de *G. molesta. G. molesta* está causando graves daños a los manzanos en la provincia de Girona (NE de España). Además, en una evaluación preliminar de los investigadores del IRTA, se detectó que el modelo fenológico oficial de *G. molesta* no se ajustaba a la dinámica poblacional registrada en los manzanos de Girona a lo largo del año. Para corroborar esta observación, se comparó la dinámica poblacional de *G. molesta* en las provincias de Girona y Lleida, así como la biología de ambas poblaciones. Se estudió el desarrollo de larvas de *G. molesta* en manzanas en condiciones de campo y con temperaturas constantes en el laboratorio. Además de mejorar el sistema de monitoreo actual, también aspiramos a introducir nuevos métodos de manipulación del comportamiento en el CIP. Desarrollamos y probamos un test de selección múltiple para explorar el comportamiento de oviposición de *C. pomonella, G. molesta* y *L. botrana* con estímulos no volátiles. Se probaron dos azúcares (fructosa y sacarosa), dos sales (KCl y NaCl) y un extracto vegetal (aceite de neem). Para comprender mejor el mecanismo de percepción estos estímulos en las tres especies, se realizaron registros electrofisiológicos en sensilas *chaeticas* de las polillas. Además, se registraron el número y la posición de la sensilas *chaeticas* en los palpos labiales.

El estudio de la dinámica poblacional de *G. molesta* mostró que el modelo fenológico utilizado oficialmente estimaba con bastante precisión la primera generación en ambas provincias, pero la fenología a lo largo del año solo era predicha correctamente en la provincia de Lleida. En la provincia de Girona, el tiempo de generación de *G. molesta* fue más largo de lo previsto por el

modelo; como consecuencia, se observó una generación menos en Girona. Este estudio confirma la falta de ajuste del modelo fenológico para el desarrollo de la especie en manzano en la provincia de Girona y la necesidad de desarrollar un nuevo modelo para el desarrollo de la especie en manzano. La comparación del desarrollo de las dos poblaciones (Girona y Lleida) en manzanas no mostró diferencias entre ellas. Se encontró un incremento en el desarrollo en frutos de manzana en el experimento de campo en comparación con el registrado a temperaturas constantes en el laboratorio, especialmente marcado cuando las larvas se alimentaban de frutos aún unidos a los árboles. Sorprendentemente, los resultados apuntan que la unión entre el fruto y la planta puede estar retrasando el desarrollo de *G. molesta*.

Los resultados sobre el comportamiento de la oviposición mostraron que el aceite de neem tuvo el mayor efecto. Tuvo un claro efecto disuasorio sobre *G. molesta* y *C. pomonella*. Aunque en *L. botrana*, el aceite de neem fue disuasorio en la concentración más alta (10 % en volumen), fue estimulante en la concentración más baja (0,1 %). Los azúcares probados generalmente reducen la oviposición en la dosis más alta (1M); sin embargo, se encontraron diferencias significativas entre especies y azúcares, por ejemplo, en *C. pomonella*, 100 mM de sacarosa estimulan la oviposición. Las sales también mostraron diferencias entre especies; en *C. pomonella* aumentaron la oviposición a 100 mM para KCL y a 100 mM y 1 M para NaCl, mientras que en *G. molesta* y *L. botrana* no tuvieron ningún efecto significativo. En esta tesis doctoral se comprobó, por primera vez en lepidópteros, la función quimiorreceptora de la sensilas presentes en el palpo labial. Además, las sensilas del palpo labial mostraron una mayor respuesta a los azúcares que las sensilas de la antena, lo que confirma la relevancia de las sensilas del palpo labial en las especies estudiadas.

El conocimiento obtenido en este doctorado ayudará a mejorar las estrategias de CIP. Las diferencias encontradas en la fenología de *G. molesta* entre las poblaciones de Lleida y Girona son un fuerte recordatorio de la necesidad de validar los modelos fenológicos en las condiciones reales en las que se van a utilizar. Futuros estudios deberían explorar las causas de las diferencias detectadas entre frutos adheridos y desprendidos del árbol en el desarrollo de *G. molesta*. El aceite de neem parece un buen candidato para explorar la introducción de la manipulación de la oviposición como método de control. Los conocimientos adquiridos en los estudios electrofisiológicos ayudarán a comprender mejor todo el sistema y evitar efectos imprevistos que podrían impedir la introducción de no volátiles en las estrategias de CIP.

GENERAL INTRODUCTION

Human development is increasingly impacting the planet's welfare, and, to limit this impact, Sustainable Development Goals (SDGs) were promoted by the United Nations in 2015 (UN 2023). The SDGs aim to balance equitable human growth with protection of the Earth's ecosystems. They contain 17 goals that focus on different aspects of human rights, sustainable development, and the ecosystem protection. Improving pest management in agriculture can help achieve these goals, especially goal 2 (zero hunger) and goal 12 (responsible consumption and production). Arthropod pests are estimated to have a potential of reducing 18-20% the crop production worldwide (Sharma *et al.* 2017), limiting the impact of pest related loses will help reduce hunger and achieve food security. Advancing in pest management can also help goal 12 by reducing the side effects of pest treatments. Traditional pest management has been based on chemical insecticides, solely in Spain, 9961 tonnes of insecticides were sold in 2021 (MAPA 2023a). Insecticides are highly effective in reducing insect populations; however, they also have undesired impacts on human health and the surrounding environment. In addition, the indiscriminate use of insecticides has caused the appearance of resistance in the insect populations reducing their effectiveness (Devine & Furlong 2007). In this context, Integrated Pest Management (IPM) was first formulated in the '50 and '60 of the 20 century (Pickett *et al*. 1958, Stern *et al*. 1959, Kuenen 1961). Since then, the IPM concept has been further developed with a focus on reducing the environmental impact of control methods and boosting the natural pest control (IOBC-WPRS 2018). To achieve this goal, IPM combine all the available techniques and knowledge to rationalize the pest management with special focus on reducing insecticide sprays (Avilla & Riedl 2003, Ioriatti *et al.* 2011, Damos *et al* 2015).

IPM in fruit crops

On the European continent, the West Palearctic Section of the International Organization for Biological Control (IOBC/WPRS) actively promoted the new IPM concept, creating the Integrated Control in Fruit Orchards group in 1959 (Boller *et al.* 2006). The term Integrated Pest Management (IPM) quickly became popular as the idea of integrated pest management was greatly broadened during the 1960s to encompass also disease and weed control. The Food and Agriculture Organization of the United Nations (FAO) sponsored a conference in Rome, Italy in 1965 that used the term "integrated pest control" expressly (FAO 1966).

Currently, IPM is widely applied in fruit crops, especially in Europe since the EU Directive 128/2009 came into effect and IPM in fruit orchards became mandatory (Barzman *et al.* 2015). In Catalonia, the main fruit crops are grapevines, pome fruits and stone fruits, occupying 33.71% of the total productive area dedicated to tree crops, which correspond to 74.87% of the total production in weight (DARP 2021). Specific IPM guidelines have been developed for these crops (i.e. Batllori *et al.* 2012, Cambrany *et al.* 2012, IOBC 2023, MAPA 2023b). Arthropod pests control are addressed in these guides, as they can cause severe reductions in production (Sexton *et al*.

2007). The importance of arthropod pests in these fruit crops in Catalonia is also reflected in the amount of insecticides used, accounting for 40 % of the total amount of pesticides (Insecticides, fungicides, herbicides) used in stone and pome fruits orchards in Spain (MAPA 2023a). Different insect and mite groups have species considered pest in these crops (e.g. aphids, trips, moths, flies, spider mites). Within moths, the *Tortricidae* family (Lepidoptera), with more than 700 species considered agricultural pest (Zhang 1994), has a special impact in fruit orchards. Some of the tortricid species that are harmful include fruit borers (Knight *et al.* 2019), which cause direct damage to the valued good as they feed directly inside the fruit. All the most important fruit crops in Catalonia have important tortricid pests: *Cydia pomonella* (L.)*, Grapholita molesta* (Busck) and *Lobesia botrana* (Dennis & Schifermüller), are key pest of pome fruits, stone fruits and grapevines, respectively (Ioriatti *et al.* 2011, Damos *et al.* 2015). Although, *G. molesta* is generally considered a primary pest of peach and stone fruits, it can also attack apples and pears and, in some concrete location, cause severe loses (Damos *et al.* 2015, Escudero-Colomar & Vila 2018).

IPM strategies offer a variety of tools to reduce pests-related losses, from cultural practices to chemical sprays (Abrol 2015, Chouinard *et al*. 2015). Various control techniques were developed throughout the latter half of the previous century and the early decades of the current one, such as attract-and-kill (Curkovic & Brunner 2003, Navarro-Llopis *et al.* 2013); mating disruption (Waldner 1997, Witzgall 2001); the sterile insect technique (SIT) (Hendrichs *et al*. 1993); and mass trapping (Steiner 1952, Escudero-Colomar *et al*. 2005) and pest management in wide areas (Bassanezi *et al*. 2013, Paiva *et al*. 2019). In addition, new biological control agents were added, such as nematodes and other microorganisms (Tanada 1964, Andermatt *et al.* 1988, Poinar 2001, Campos-Herrera 2019). This represents a significant advancement in biological control (BC), which is now used in a variety of BC systems (Eilenberg *et al*. 2001).

Monitoring the pest population is always a key element (Petri *et al.* 2015), since IPM aims to use control methods only when they are necessary. Furthermore, for the tortricid species, monitoring the adult flight is especially critical because they spend most of the larval stage inside plant tissue (Rothschild & Vickers 1991, Knight *et al*. 2019). This aspect of larval behaviour makes larvae very difficult to target with currently available control methods due to the short time they spend outside the fruit after hatching. Monitoring pest populations becomes even more important due to the reduced time to apply control methods.

Pest monitoring

Populations of *C. pomonella, G. molesta* and *L. botrana* are commonly monitored in commercial orchards using delta traps baited with olfactory attractants. Even though it is a very simple method to monitor, it has a major setback, it requires direct observation. The temporal resolution of the system depends on the frequency of the field visits by skilled staff. In the studied area, field visits are usually conducted once a week to reduce costs, limiting the temporal resolution to a week basis. Much effort has been put into developing automated trap to reduce the periodic visits to the fields (Parsons *et al*. 2019, Preti *et al*. 2020). Nevertheless, farmers seem reluctant to implement them on a large scale, probably due to their higher cost and technical complexity (i.e. automated identification of target species).

Phenology modelling is a further resource for concluding pest monitoring and assisting in pest management, (Damos & Savopoulou-Soultani 2012, Rebaudo & Rabhi 2018), moreover, phenology models can be used to study population dynamics because they facilitate the comparison between population from different regions and years that registered different temperatures. Organisms pass through various phases and stages during their life cycle, which are known as the phenological stages of the species. Phenology can include individual traits, like the development stage, but also population characteristics, for example, the number of generations in a year. The phenology of most organisms is driven by environmental factors; these relations can be modelled using mathematical equations to track the life cycle indirectly through environmental data (Damos & Savopoulou-Soultani 2012, Rebaudo & Rabhi 2018). Building a phenological model requires a deep grasp of insect development and the factors that affect it. A species' life cycle information and the equations used to forecast its phenological stage are the components of phenology models. Temperature has the biggest influence on an insect's life cycle out of all the factors (Damos & Savopoulou-Soultani 2012, Rebaudo & Rabhi 2018). However, as observed in G. molesta (Myers *et al*. 2007, Silva *et al*. 2010, Najar-Rodriguez *et al*. 2013, Yang *et al*. 2016, Sarker *et al.* 2021), the host species can also significantly influence development. In addition to these two, there are other factors that, depending on the situation, may also have a significant impact. For this reason, the models must be validated for each location and crop for which they are to be utilized.

Modelling insect development

It is assumed that the relationship between temperature and development is a consequence of the effect of temperature on enzyme activity. As temperatures increase, so does enzymatic activity and, therefore, the biological processes that drive development. Like enzymatic activity, at extreme temperatures, development slows down to a halt, (Damos & Savopoulou-Soultani 2012). Therefore, development is faster near the optimum temperature and slows down as temperatures move away (Fig. 1). To model the temperature effect on development, laboratory studies at constant temperatures are performed to estimate the development time at different temperatures. Traditionally, the inverse of development time is used to model the relationship between temperature and development (Fig. 1). This parameter is known as the development rate, and it denotes the “amount” of development accumulated in one day.

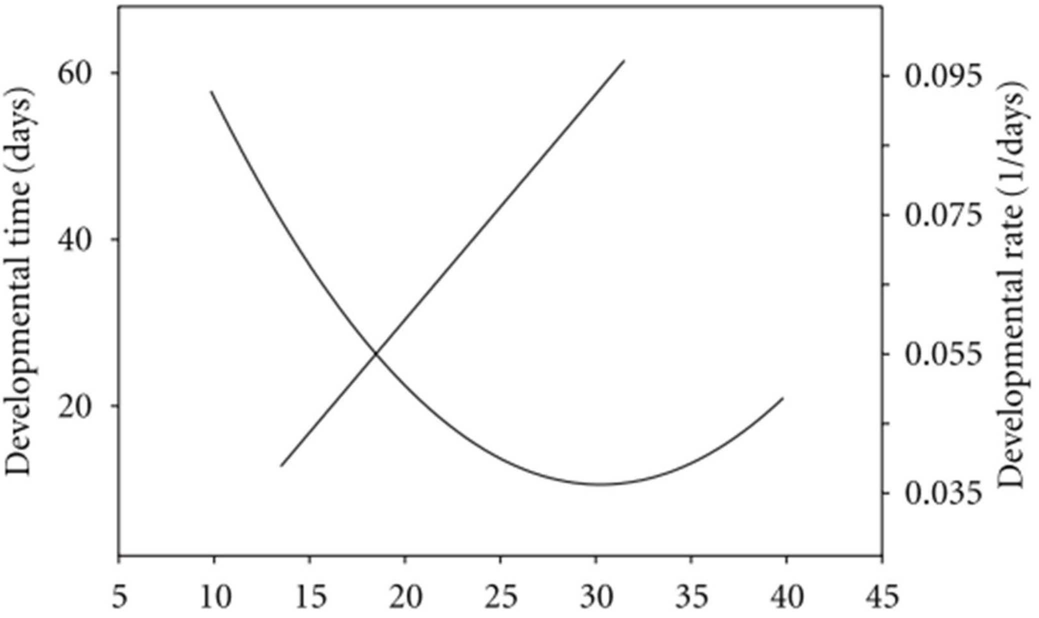

Figure 1. Typical response of the developmental time over temperatures and its relation with development rate. (Source: Damos & Savopoulou-Soultani 2012).

The development rate gradually increases from 0, corresponding to the Lower Development Threshold (LDT). As the temperature rises, the increase in development rate approaches linearity until it reaches the temperature for optimal development (Topt), after which it falls until it reaches the Upper Development Threshold (UDT), after which development ceases (Fig 2). The simplest models fit a linear function to the central temperatures; however, close to the LDT and UDT, the linear fit diverges from the linear function. Different equations have been proposed to model the development rate over the entire range of temperatures in order to improve the fit of the model in non-linear areas (Damos & Savopoulou-Soultani 2012, Rebaudo & Rabhi 2018). However, due to the simplicity of linear functions and the fact that the imbalance at high temperatures is already considered, most insect phenology models continue to use linear equations. To deal with the lack of linearity above Topt, a fixed “cutoff” is normally used. Depending on how the development rate is expected to evolve after UDT, three basic cutoff approaches have been proposed: horizontal, when the development rate is stable from Topt to UDT, intermediate; when it declines continuously until UDT, or vertical, when it ceases completely after Topt (UC-IPM 2023). The deviation from the linear equation at lower temperatures has little effect on the accumulated development and is usually disregarded.

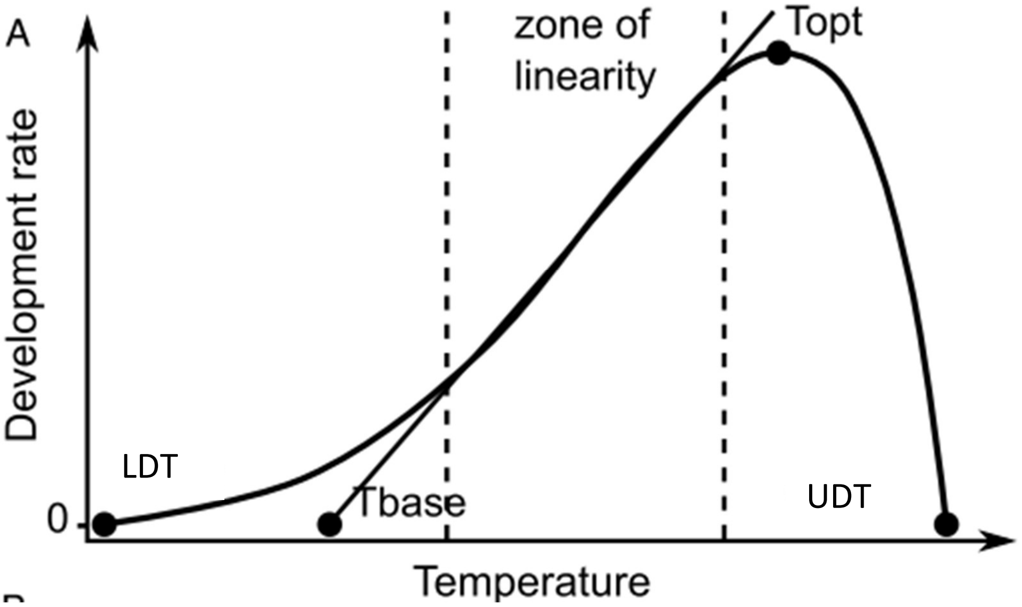

Figure 2. Theoretical development rate curve with respect to temperature with the most biologically relevant traits: Lower Development Temperature (LDT), Upper Development Temperature (UDT), optimum temperature (Topt). Lineal approximation made by lineal phenology models and the position of the base temperature used to calculate degree days (Source: Rebaudo & Rabhi 2018)**.**

Phenology models

Once the relationship between temperature and development is known, the phenology model can be built. Phenology models usually have an event that marks the start of the development for the target insect (e.g. a time of the year or a phenological event); this event is called a biofix (UC-IPM 2023). After the biofix has been reached, the model needs to track the phenological stage of the individual or population, or, in other words, the development that has been accumulated so far. Most phenology models use Degree Days (DD) to measure the accumulated development. The DDs are based on the observations of Blunck (1914), who found that at constant temperatures, the difference between the temperature experienced by the insects (Texp) and the Tbase of lineal models (Fig. 2) multiplied by the development time was always constant. This constant represents the amount of temperature (integral thermal) required to finish the development, and it is expressed in DD. One DD corresponds to 24 hours at one degree above the Tbase; it can be easily calculated at constant temperatures using the formula Texp-Tbase=DD. Under natural conditions, the temperature is not constant; therefore, different systems have been developed to calculate de DD (Zalom *et al*. 1983).

The first phenology models only considered temperature as a factor driving development, but with the improvement of computing systems and environmental data acquisition, the potential to increase phenology models complexity has increased considerably. For example, the introduction of stochastic models that take individual variability into account within populations helps to obtain a more realistic notion of the phenological stage of the whole population. In addition, other environmental factors, such as humidity, photoperiod, etc., can be added to the models to increase their accuracy. Phenology models can also be easily included now in web applications to facilitate knowledge transfer to farmers and advisors (Damos 2015). The improvement of the phenology models can help implement control methods that require precise timing, for example, punctual sprays of organic compounds that have a low active life. In summary, phenology models have great potential to improve pest monitoring and create decision support systems to improve IPM programs.

Pest control thought behaviour manipulation

New environmentally friendly control methods are also needed to reduce the impact of agriculture on the environment. In recent years some new strategies have appeared based on behaviour manipulation. Insects perceive their environment and react to external stimuli with a series of innate and learned behaviours (Agarwal & Sunil 2020). Pest management can take advantage of those stimuli induced responses to modify their behaviour. Giving the right stimuli at the proper moment can produce a behaviour from the pest that reduce the damage to the crops (Foster & Harris 1997). There are many type of stimuli (e.g. visual, chemical, physical) that insect can react to. All these stimuli are susceptible to be used to alter insect behaviour, however chemicals are the base of most of the current systems that apply behaviour manipulation (Agarwal & Sunil 2020), probably because the can be more easily applied.

Mating disruption is the most prominent technique based on behaviour manipulation. Many insects emit specific volatile blend to attract the opposite sex, in mating disruption, this blend (or some component) is released into the field at high quantities. In this environment saturated with sex pheromone males can not locate females, therefore the number of mated females is reduced and less females can lay fertilized eggs (Cardé & Minks 1995). Mating disruption have been successfully applied worldwide, especially to control tortricid pest. Mating disruption is estimated to cover 800,000 hectares of agriculture fields (Benelli *et al*. 2019). Volatile compounds are also used to attack insects to a selected place for different techniques (e.g. attract and kill, mass trapping, monitoring, push pull). Beside volatile chemicals, other types of stimuli can be used in pest management. Emitting specific sounds have been used successfully used to trap different groups of insect pests (Mankin 2012). Pest control application for substrate-borne vibrations are also underway (Polajnar *et al*. 2015). Visual cues are also exploited, alone in sticky traps (Shimoda 2018) or in combination with volatile stimuli (Abu-Ragheef 2020). Last but not least, non-volatile compounds have also a big potential for behaviour manipulation. Different strategies have been suggested so far, for example: adding food stimulants to insecticide mixtures to increase the consumptions of treated surfaces (Pszczolkowski 2004); also spraying oviposition deterrents on the host plants to reduce the number of eggs laid (Arnault *et al*. 2016). Oviposition deterrents are especially interesting for the species studied in this thesis because neonate larvae have little time to find a sustainable host, and females need to place eggs in the best place to ensure offspring survival (Thompson & Pellmyr 1991).

Chemical perception in insects

Volatile and non-volatile chemical perceptions has traditionally divided in two senses, olfaction and gustation (i.e. taste, contact chemoreception). However, both senses share a similar process. Unlike mammals, chemoreception is distributed along the insect body and usually combining other senses. For example, the antennae, which is the mainly olfactory organ, also contain gustation and temperature receptors on it (Chapman 1998). In insects, chemical compounds are detected in special structures found in the insect cuticle called sensilla. Sensilla are specialized cuticle structures innervated by one or more neurons (i.e. sensory cells). The neuron body is located at the base and the dendrites project to the tip of the sensillum. The neurons are supported by 3-4 accessory cells (i.e. sheath cells)(Fig. 3).

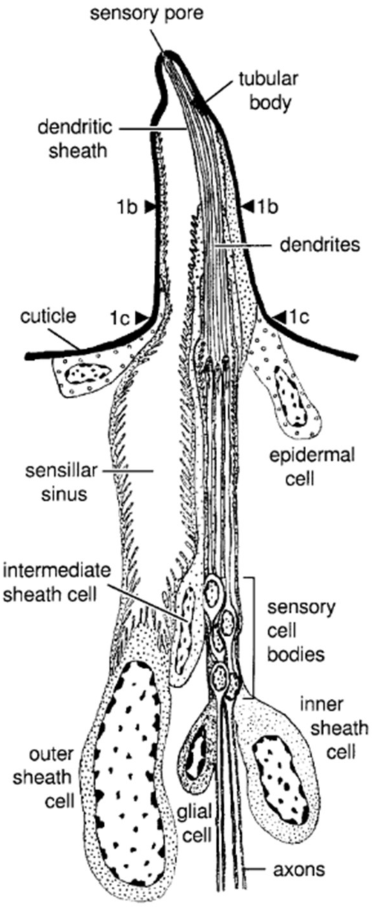

Figure 3. Structure of a typical gustatory sensillum with its principal element (Source: **Mitchell *et al.* 1999**).

Sensilla usually have an elongated (hair-like) shape, although other forms can also be found (Ryan 2002). Chemoreceptive sensilla have pores in the external cuticle, which allow the exchange of molecules with the surrounding environment. The number of pores present on a sensillum are indicative of their function. Multiple-pore sensilla usually have olfaction function; terminal uniporous sensilla have gustatory and tactile function; finally, aporous sensilla have tactile or thermo-hygro function (Hallberg & Hansson 1999). Another difference between olfactory and gustatory sensilla is the number of neurons present in each sensilla, in olfactory sensilla, especially in sensilla trichoidea, there is usually one Olfactory Receptor Neuron (ORN), while in gustatory sensilla they have between 1-8 Gustatory Receptor Neurons (GRNs) (Montell 2009). In most gustatory sensilla there is also a mechano-receptor neuron with a tubular body that projects into the (Marion-Poll 2020). Sensilla chaetica are common gustatory sensilla with hair-like shape that usually combine both functions, they are also called contact-chemosensory sensilla.

GRNs are usually classified by functional groups: sugar, salt, bitter and water depending on which substance activate their response. This classification is an adaption from the canonical gustation classes of mammals (Liman *et al*. 2014). However, recent research has shown that insect gustatory receptor proteins can also detect Ca^{2+}, CO^2, fats and hydrocarbons (Marion-Poll 2020). Molecular tools have given a new insight of the system of stimuli detection. Transmembrane receptors are present in the cellular membrane of the dendrites neurons that project to the sensilla tip. Four major receptor families have shown to have relation with gustation: gustatory receptors (GRs), ionotropic receptors (IRs) and Transient Response Potential (TRPs) (Scott 2018). In the present of the correct chemical stimuli, the receptor produce the depolarization of the membrane (receptor potential) which spreads towards the cell body of the neuron. If the receptor potential reaches a certain threshold, the cell membrane generates action potentials (commonly named spikes, Fig. 4) which are transmitted through the nervous system where all the information is integrated and a response if given if necessary (Dethier 1971, Ryan 2002).

Electrophysiology

The spikes can be recorded to observe the response of GRNs (also ORNs) to stimulations. Electrophysiological recording of single sensillum is a technique widely used for identifying the response of receptor neurons to different chemicals. This information can be used to understand the behaviour response to chemical stimulation. Hodgson *et al*. (1995) developed the tip recording to register gustatory sensilla responses. It consist in contacting the tip of the sensilla with a glass capillary containing a solution with the stimulus and a salt to allow for electric conductivity. The stimulus molecule penetrates though the terminal pore into the sensilla lymph and the neuron response producing a train of spikes (i.e. action potentials) if the right stimuli is used. The glass capillary function as an electrode (recording electrode) that allows the transmission the electric signal generated by the neurons. With a system of filters, amplifiers and a digitalizer, the spikes produced by the sensilla can be recorded by an electronic device (generally a computer). To obtain a recording with the minimum noise possible the insect is connected to the ground with another electrode (reference electrode) (Bretschneider & De Weille 2018).

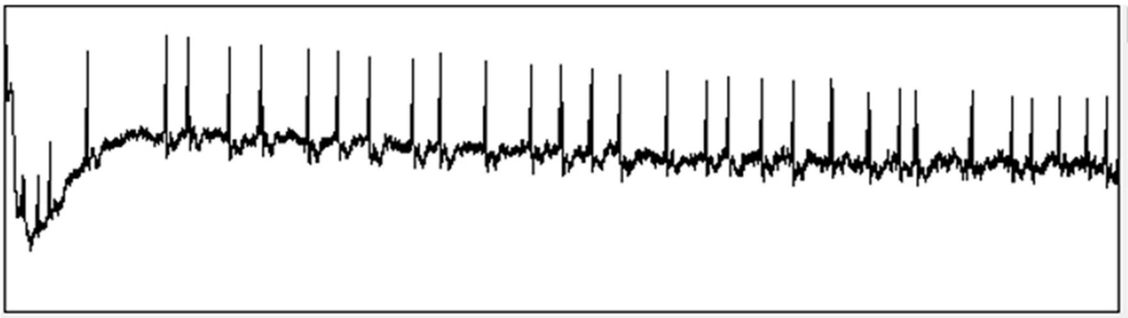

Figure 4. Recording from a sensillum chaetica obtained through the tip-recording technique showing a train of spikes in response to stimulation.

Upon stimulation, GRNs produce a train of spikes (Fig. 4). The frequency of spikes is and indicative of the amount of response from the GRN, it is used to compare between concentrations and stimuli. Sensilla chaetica usually contain more than one GRN, if more than one GRN response to the stimulation, the response of all of them is recorded. GRNs usually have different spike shape and amplitude, which allows for their differentiation. However, with tip-recording technique, spike shape can change during a stimulation or between stimulations, hindering its identification (Marion-Poll 2020). Also, interaction between GRNs of the same sensilla have been recently probed (Miriyala *et al*. 2018) with adds more complexity to the analysis of response of sensilla chaetica and its correlation with the stimulus applied.

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OBJECTIVES

The aim of the present thesis was to acquire new knowledge on the biology and behaviour of three insect pests with high economic in fruit trees and grapevines. I have explored different approaches to refine the IPM programs of the target species with the final goal of reducing environmental impact while reducing pest associated losses. The first part of the thesis focuses on improving the monitoring system of *G. molesta*¸ with special focus on Girona province (NE Spain), where it cause severe damage to apples, usually considered a secondary. To accomplish this purpose I studied the population dynamic of *G. molesta* in two regions with similar climate but in which it feeds on different hosts species. Also, the influence of apple can have in *G. molesta* development. The second part of the thesis is dedicated into unravelling the gustation system of the three tortricid moths by studying the effect on three phylogenetically related species, I hoped to obtain a new insights into the gustatory system that can be applied in pest control. I studied the effect of non-volatile stimuli in the oviposition behaviour of females, which the final goal to facilitate new controls method for IPM based on behavioural manipulation. Also the gustatory perception was analysed to better understand the whole gustatory system, which is bad represented in the literature.

Research objectives by chapters:

Chapter 1: Different population phenologies of *Grapholita molesta* (Busck) in two hosts and two nearby regions in the NE of Spain.

Study the population dynamic of *G. molesta* in Girona and Lleida provinces by analysing the data from the net of pheromone baited traps in commercial orchards routinely used to monitor *G. molesta* populations in both provinces. We also wanted to validate the phenology model used in the region to predict the pest phenology, which previous reports from IRTA researches showed lack of fit to Girona province populations.

Chapter 2: Host-Pest Interactions: Investigating *Grapholita molesta* (Busck) Larval Development and Survival in Apple Cultivars.

Evaluate some of the factors that can affect the development and survival of *G. molesta* larvae in apples and produce the change in the population dynamic detected in Chapter 1. Three main factors were evaluated: two insect population with different main host, three apple cultivars most planted in Girona and different temperature regime at constant and fluctuating temperatures.

Chapter 3: Oviposition by three tortricid moths on filter papers treated with different concentrations of sugars, salts or neem oil.

Develop a multi-choose arena to test the influence different concentration of non-volatile stimulus on the oviposition behaviour of three tortricid moths. Assess the response to three concentrations of biological relevant compounds (fructose, sucrose, NaCl, KCl) and a well know insect repellent (neem oil).

Chapter 4: Gustatory function of sensilla chaetica on the labial palps and antennae of three tortricid moths (Lepidoptera: Tortricidae).

Study the ability of the studied species to detect biological relevant compounds (fructose, sucrose, NaCl, KCl). Confirm the gustatory function of sensilla chaetica in labial palp by obtaining electrophysiologial responses to gustatory stimuli and compare the response of labial palp sensilla to antennal sensilla chaetica. Finally, assess the number and distribution of sensilla chaetica in the labial palps of adult moths

CHAPTER 1

Different population phenologies of *Grapholita molesta* (Busck) in two hosts and two nearby regions in the NE of Spain

Abstract

Grapholita molesta is an important pest of stone and pome fruits. In commercial orchards, integrated pest management programs use pheromone traps to monitor the population dynamics of *G. molesta* and adjust treatments. Phenology models can be used to forecast the population phenology of pests and to help optimise the time point at which to spray the orchards with insecticides. In the present study, the adult population phenologies of *G. molesta* in two provinces of north-east Spain were studied and their fit to the phenology model most used in both provinces. Weekly captures of adults in pheromone traps through the season were recorded over 5 y in a large number of commercial orchards, and these data were used to determine the number of generations of *G. molesta* in each province. Results showed significant differences between provinces in the generation time, being 97 Degree Days (DD) shorter in the Lleida province than in the Girona province. In Girona province, four generations were registered, while five were detected in Lleida. As a result of the differences found, the phenology model was not able to predict precisely the population dynamics in the Girona province.

Keywords: *Grapholita molesta*; population dynamics; phenology model; peach; apple; Spain, Catalonia

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Introduction

Grapholita molesta (Busck) (Lepidoptera: Tortricidae) is assumed to be a native of China, but it has spread over the world's temperate areas following the expansion of its main host, the cultivated peach (Chapman & Lienk 1971, Rothschild & Vickers 1991). This pest is also known to occasionally attack pome fruits, mainly after nearby peach orchards have been harvested (Roerich 1961, Rothschild & Vickers 1991, Balachowsky 1996). However, there are also reports of economic damage to apple orchards in Chile, Russia, Brazil, China, Italy, USA and Canada (González 1980, Zhao *et al.* 1989, Popovich 1992, Pollini *et al.* 1993, Reis *et al.* 1998, Felland & Hull 1999, Bellerose *et al.* 2007).

In Spain, *G. molesta* was first detected in 1974 in the Lleida province (Esteban 1974) where most peaches of the Catalonia Autonomous Community of Spain are cropped. In the early 2000s, *G. molesta* started to damage pome fruit orchards located in the Girona province (extreme NE of Spain) and also in Catalonia, where apples are the main crop fruit (Escudero-Colomar *et al.* 2012, 2019). *G. molesta* have a high potential impact on these two provinces because most of the fruit production of Catalonia is concentrated in them. The fruits crops cultivated in these provinces involve several hosts of *G. molesta* (e.g. peaches, apples and pears) (IDESCAT 2021). Several tools are available to control *G. molesta*: insecticide applications and mating disruption (MD) (Cardé *et al.* 1977, Cardé & Minks 1995). In the province of Girona, the attacks of *G. molesta* on apples made it necessary to install MD for *G. molesta* alongside MD for *Cydia pomonella* (L.), another key pest of apples from the moth family Tortricidae. In general, insecticide applications are used for both species to reinforce control with MD throughout the season, although control of *G. molesta* was not always enough to prevent damage (Escudero-Colomar *et al.* 2012, 2019).

G. molesta is a multivoltine species, presenting three to five generations depending on whether the environment is colder (e.g. Slovenia) or more temperate (e.g. California), respectively (Croft *et al.* 1980, Rice *et al.* 1982, Kanga *et al.* 2003, Tomše *et al.* 2004, Ahn *et al.* 2012). In mid-late autumn, when temperatures drop and the days shorten, the fifth instar larvae enter diapause until more favourable conditions occur at the end of winter (Rothschild & Vickers 1991). Population dynamics and the annual number of generations of a target species are usually measured in field conditions by recording adult flights with pheromone baited traps (Croft *et al.* 1980, Rice *et al.* 1984, Kanga *et al.* 2003, Kovanci & Walgenbach 2005, Damos *et al.* 2014). In the Girona and Lleida provinces, captures in traps and field observations indicate that *G. molesta* has at least four generations per year, but no studies have been conducted to confirm this.

Phenology forecast models are useful tools that predict the population dynamics of pests and can be used in IPM (Integrated Pest Management) programs to select the right moment to spray in order to control the target pest (Knight 2007, Damos *et al.* 2015). Phenology models can be tested through statistical and qualitative approaches, using in the latter, graphical comparisons between observed and predicted data (Bellocchi *et al.* 2010). Several phenology models were developed for *G. molesta* (e.g. Croft *et al.* 1980, Rice *et al.* 1984, Damos & Savopoulou-Soultani

2010). In the two provinces of the present study, the most used model to predict *G. molesta* progression in peach orchards is that of Croft *et al.* (1980), currently included in the web site of the Department of Agriculture of the Catalan Government (Ruralcat 2021), because it was originally validated in both peach and apple orchards in the USA (Croft *et al.* 1980). But, it was observed that in the Girona province, where apple is the main host of *G. molesta*, this phenology model is not precise enough to help in timing insecticides applications (Escudero-Colomar *et al.* 2012, 2019).

It is known that the development time for one species can vary between host species and even host varieties, as is the case in *G. molesta* (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang *et al.* 2016, Wang *et al.* 2018). The literature indicates that for *G. molesta*, on average, its development in apples requires between 20 and 60 more accumulated degree days (DD) than is required in peaches (Myers *et al.* 2007, Yang *et al.* 2016). Therefore, more knowledge on the population dynamics, the number of generations per year and the biology of the pest within the conditions of the two provinces is needed to enable better fitting of the forecasting model. Consequently, the main goals in the present study are:

a) to define the population dynamics of *G. molesta* in the Girona province where apple is the predominant fruit crop, as well as in the Lleida province where peach is the main fruit crop;

b) to determine the number of generations of *G. molesta* per year in each province;

c) to test the accuracy of the Croft *et al.* (1980) phenology model for both provinces, including the development delay on apples reported by Myers *et al.* (2007) and Yang *et al.* (2016).

Materials and Methods

2.1. Study area

Data used in this report come from commercial orchards of two provinces of the extreme north–east of Spain, Girona and Lleida, just 250 km apart (Fig. 1A). The province of Girona has two main fruit growing areas, in the north, the Alt Empordà and the south, the Baix Empordà (Fig. 1C). In both areas, apple is the main crop. Due to the fact that there are small climatic differences between both areas that translate into differences in the phenology of plants and pests, it was decided to keep both areas separated in studying the phenology of *G. molesta* and then fitting those data to the most commonly used forecasting phenology model in each case. In the province of Lleida, three areas were delimited based on the predominant tree cultivation: in the north an area composed of mixed orchards of pome and stone fruit trees (hereafter Mixed area), in the south an area with orchards mainly of stone fruit (hereafter Stone area) while in the east a third area with predominance of pome fruit orchards (hereafter Pome area) (Fig. 1B). The five areas studied share the same bioclimate and thermotype (related to a temperature range), but the two in the province of Girona differ from the three in the province of Lleida in that the ombrotype (related to the yearly rainfall), and also have slight differences in weather conditions (Table 1; Rivas-Martinez & Rivas-Saenz 2021).

2.2. G. molesta monitoring system

Data were collected from the monitoring white delta traps baited with Pherobank® sex pheromone (Wijk bij Duurstede, The Netherlands), the standard used in all the studied areas. They were placed in commercial orchards in Girona province (apple orchards) and in Lleida province (peach orchards). Moth captures in traps were checked weekly from the beginning of March until the end of October. Data were collected during a 5-year study period (2015-2019) from orchards without MD. The number of traps varied between years in the five areas studied according to the number of orchards and traps that conform to the study requirements: to use the monitoring material described above and not use MD to control *G. molesta* (Table 2). The remarkable decrease in the number of traps over time in the areas of Girona was due to the increasing number of apple orchards using MD to control the pest. Mean weekly captures and standard errors of those means were calculated for each area and year to draw the population dynamic curves.

The daily maximum and minimum temperature were obtained from the official weather stations belonging to the Meteorological Service of Catalonia (Fig. 1) (Ruralcat 2021). The selected weather stations correspond to the municipalities of Sant Pere Pescador for the Alt Empordà area, La Tallada d'Empordà for the Baix Empordà, Aitona for the Stone area, Albesa for the Mixed area and El Poal for Pome area (Fig. 1 and Table 1), because they were the most indicative of the weathers in each area studied. The model proposed by Croft *et al.* (1980) and Rice *et al.* (1984) predicts the first moth catch at 126 DD after January 1st and a generation time (from egg to egg) of 535 DD. The accumulated DD in each area was calculated from the daily temperatures using a single sine method of calculation described in Zalom *et al.* (1983) with a lower threshold of 7.2ºC, an upper threshold of 32.2ºC and a horizontal cut off. Moreover, 60 DD (the maximum reported development delay when *G. molesta* larvae feeds on apples compared to peaches (Myers *et al.* 2007, Yang *et al.* 2016) was added to the number of DD required for the generation time in Girona areas to check whether this delay also fits to the development of *G. molesta* populations in apples.

To assess the agreement between model predictions and the observed data at field, graphical plots were made. They were used to judge qualitatively the model ability to predict population dynamics (Bellocchi *et al.* 2010).

Table 1. Bioclimatic (based on the Worldwide Bioclimatic Classification System) and meteorological data for the five studied areas. Meteorological data were obtained from the official weather stations associated with each area from 2015-2019. The UTM (Universal Transverse Mercator) coordinates for each weather station are indicated Minimum and maximum temperatures for the period of diapause of *G. molesta* were calculated from October to February, and equivalent temperatures for the developing period from March to September.

Area	**Bioclimate**		**Bioclimate belt**		**Weather data**					
	Type	**Subtype**	**Thermotype**	**Ombrotype**	**Station location**	**Min. diapause temp.**	**Max. diapause temp.**	**Min. devel. temp.**	**Max. devel. temp.**	**Yearly rainfall**
Alt Empordà, (Girona)	mediterranean pluviseasonal-oceanic		meso-mediterranean	subhumid	507995X 4669451Y (31T)	4.80	16.85	12.38	24.49	546.98
Baix Empordà (Girona)	mediterranean pluviseasonal-oceanic		meso-mediterranean	subhumid	505127X 4655771Y (31T)	4.51	16.84	12.48	25.28	541.84
Stone area, (Lleida)	mediterranean pluviseasonal-oceanic	steppic	meso-mediterranean	dry	288002X 4595926Y (31T)	3.30	15.47	11.41	28.09	315.46
Mixed area, (Lleida)	mediterranean pluviseasonal-oceanic	step-pic	meso-mediterranean	dry	306325X 4625793Y (31T)	3.47	13.77	11.85	26.52	382.68
Pome area, (Lleida)	mediterranean pluviseasonal-oceanic		meso-mediterranean	dry	323310X 4615624Y (31T)	2.37	13.96	10.95	26.68	350.36

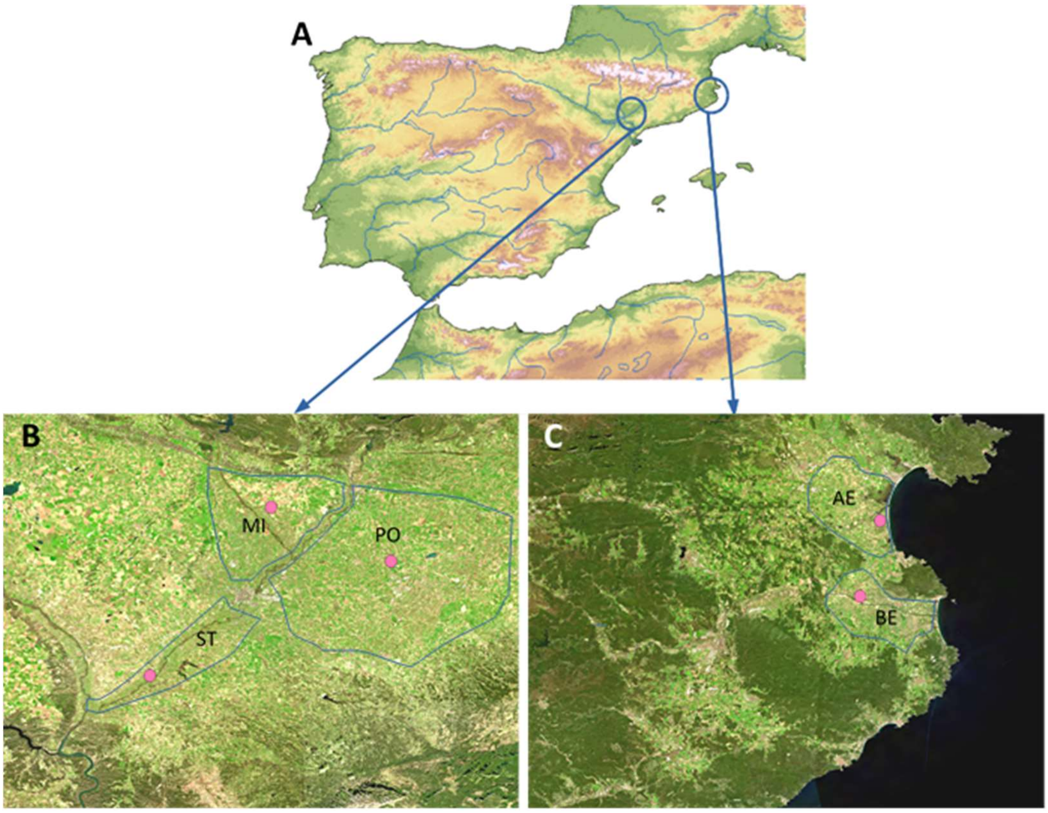

Figure 1. (A) Map of Spain showing the location of the two provinces of north-east (B) Lleida, (C) Girona, hosting the five fruit growing areas studied: Girona: AE: Alt Empordà (pome fruit growing area), BE: Baix Empordà (pome fruit growing area), and Lleida: ST: Stone fruit growing area, MI: Mixed stone and pome fruit growing area, and PO: pome fruit growing area. The locations of the official weather stations are indicated with pink dots. 2.3. Weather recordings, *G. molesta* phenology model fit and degree-days calculation

2.4. Determination of the beginning and the end of each generation

Average weekly moth catches for each province and area were plotted for each year to determine the length of adult flight periods. The shift between two flight periods was determined from the minima between two flight period peaks. When no clear minima could be observed in any of the studied years due to overlaps between flights, this period was compared with the same period in the other years for the same area to find the most probable time point when the shift occurs (for example, see the year transition between the third and fourth flight in year 2017; Fig. 2a). The start of the first flight and the end of the last flight were only recorded in those years in which the data had a complete coverage of these specific periods. The duration of the flight is assumed to be the generation time from adult to adult and it is compared to the

Croft *et al.* (1980) model egg to egg generation time along with the corrected value when the larvae feed on apples. The accumulated DD was also used to follow and define each of the flights.

Table 2. Number of traps fulfilling the study requirements in each area and year. AE: Alt Empordà, BE: Baix Empordà, ST: Stone area, MI: Mixed area, PO: Pome area.

Province	Area	Number of traps				
		2015	2016	2017	2018	2019
Girona	AE	104	109	111	15	40
	BE	167	78	53	23	25
Lleida	ST	17	37	13	52	30
	MI	47	32	25	13	23
	PO	32	52	14	21	16

2.5. Statistical analysis

Statistical analyses were performed with R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). A multiple factor ANOVA (aov function) was conducted to determine the factors influencing the generation time expressed in DD. The factors analysed were: “province”, “area”, “observation year” and “seasonal flight order” (e.g. second flight). Stepwise model selection was used to remove those factors and interactions that were non-significant ($p<0.05$). Normality was checked using the shapiro.test function and homoscedasticity with the bartlett.test. Tukey’s multiple comparisons test was calculated with the function HSD.test (Mendiburu 2002) for the factors “seasonal flight order” in each “province” separately. The Standard error of the mean for the generation time was calculated using the function jackknife (Kostyshak 2019).

Results

3.1. Population dynamics

Fig. 2 shows the population dynamics of *G. molesta* throughout the season in the two fruit growing areas of Girona province and in the three of Lleida province. The mean captures per week were lower in the Girona areas than in the Lleida areas. The maximum average number of captures in the Girona province (10 captures/trap/week) was registered in the Alt Empordà area fruit growing area in 2017, while in the Lleida province the maximum reached was 50 captures/trap/week in the Pome fruit area in 2018. The pattern of the population dynamics shows several differences between the provinces e.g.: i) *First moth capture*: In the two areas of Girona, the first moth was captured in mid-late March, while in the three Lleida areas, the first moth captured occurred in the second week of March, ii) *First generation peak*: In the Girona province, the first generation peak was in mid-April and in the Lleida province it occurred in mid-March. iii) *Generation identification*: In both provinces the separation between the first and second generation was very clear, with 2-3 weeks of low captures, but from the second generation onward, in the Lleida areas, the separation between generations drastically decreased and some overlap appeared. Bi-modal peaks were also present, which makes the separation between generations more complex, highlighting the value of a phenology model to identify each generation. In the two areas of the Girona province, it was easier to identify the different generations from the second generation onward. Nonetheless, to properly identify them, the phenology model was also applied. iv) *Nº of generation/season*: In Girona province, four generations were present in a year while in Lleida province five generations occurred. Similarities between the provinces were also observed: i) *Relative level of captures in each generation*: In general the second generation had a lower number of catches than the first, and, from the third generation onward, the number of catches increased, the last generation being associated with the most catches; ii) *Timing of the final captures of the year*: Final captures were recorded at the end of September to the beginning of October in all the studied areas.

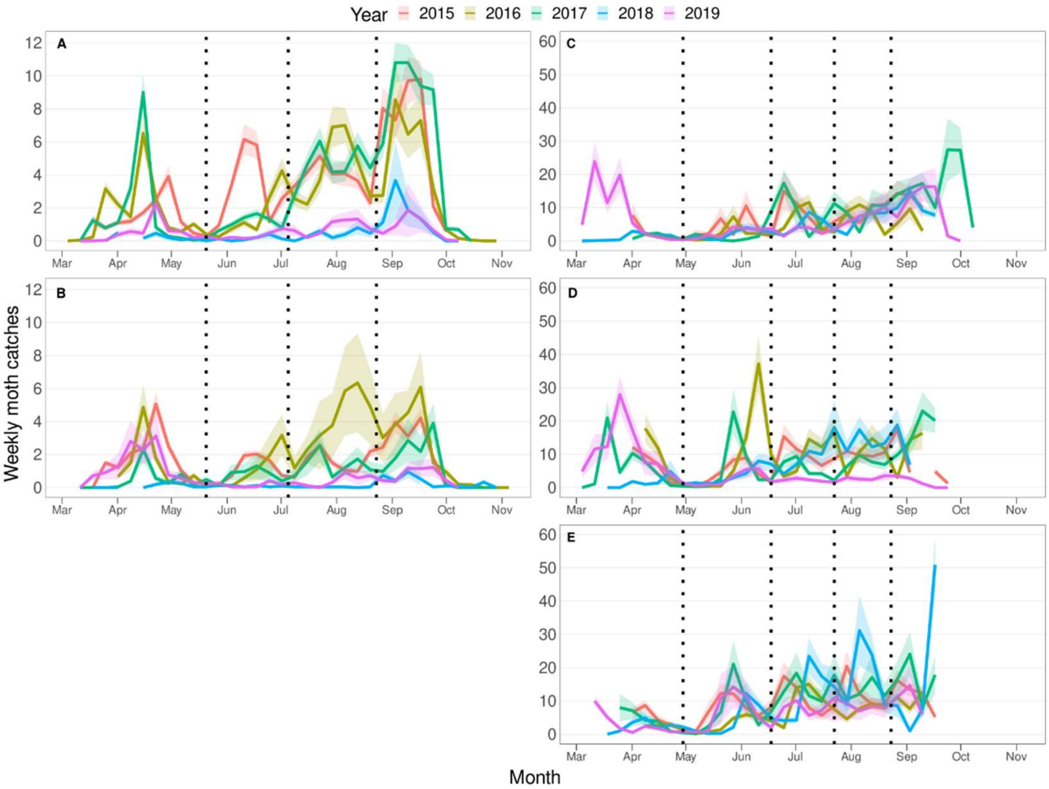

Figure 2. Weekly moth catches (mean±SE) for each year (2015-19) and for each area. A: Alt Empordà, B: Baix Empordà, both of Girona province; C: Stone area; D: Mixed area; E: Pome area, the three of Lleida province. Dotted vertical lines indicate the approximate minima between flights. Shades of each line indicates the SE. Note: the y-axis scale is different for AB than for CDE.

3.2. Phenology model

The phenology model described by Croft *et al.* (1980), applied to the weather data collected for each of the five areas, predicted five generations during the period when moths were flying in both the provinces studied. In the Girona province, after the second generation there was only partial correspondence between the predicted generations and those observed (Fig. 3 A, B, Table S1 of supplementary material), even after applying the maximum delay of 60 DD reported for *G. molesta* developing on apples (Myers *et al.* 2007, Yang *et al.* 2016) (Fig. 4 A, B, Table S1 of supplementary material). In the Lleida province, the phenology model accurately predicted all the generations in the Stone and Mixed areas (Fig. 3 C, D, Table S1 of supplementary material), but in the Pome area the generation peaks occur approximately 100 DD earlier than predicted (Fig. 3 E, Table S1 of supplementary material). Moreover, the first moth catch seems to be delayed in Girona while in Lleida it is close to the 160 DD predicted by the model developed by Croft *et al.* (1980), although there are few data on this period in both provinces. The use of DD to represent the generations reduced the differences in flight synchronization between years within areas (Fig. 3), and together with the representation of the phenology model helps to determine more accurately and easily each generation (compare Fig. 2 and Fig. 3 and Table S1 of Supplementary material).

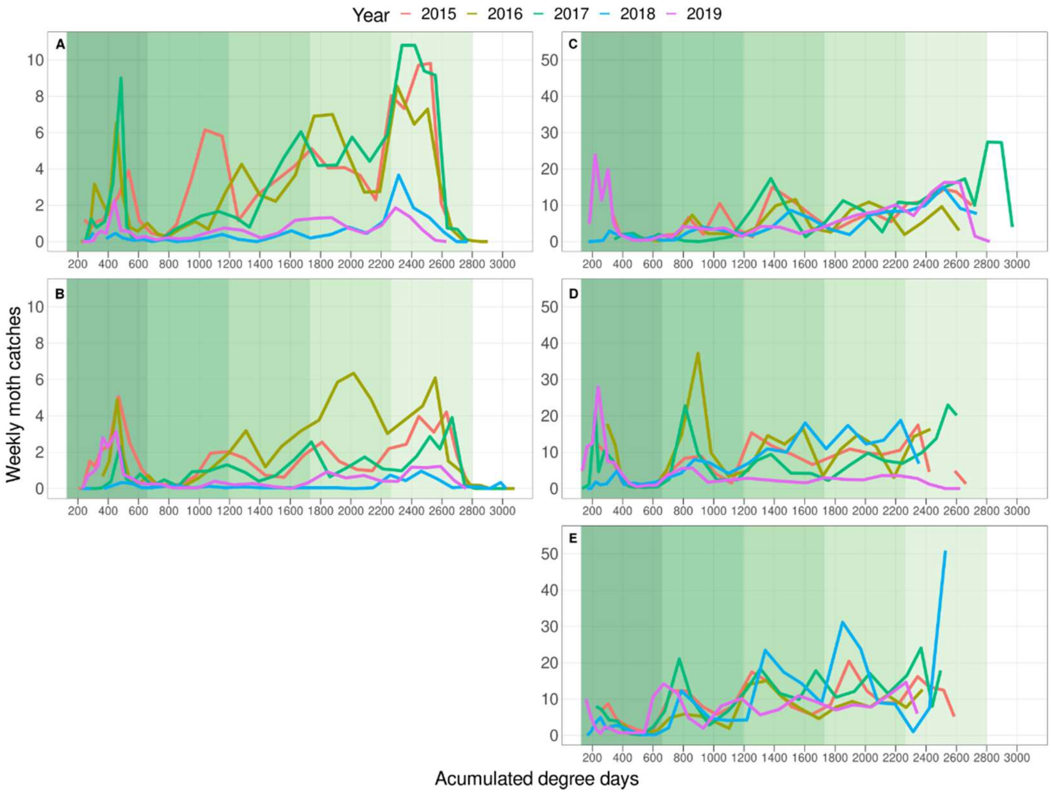

Figure 3. Mean weekly moth catches plotted against accumulated DD for each year (2015-19) and for each area. A: Alt Empordà, B: Baix Empordà, both of Girona province; C: Stone area; D: Mixed area; E: Pome area, the three of the Lleida province. Background colours indicate the predicted generation range of the phenology model (535 DD) Croft *et al.* (1980). Note: the y-axis scale is different for AB than for CDE.

All data analysed met the assumptions of normality and homoscedasticity ($p>0.05$). There was a significant effect of the factors “province” ($F_{1,96}=10.17$, $p<0.0001$) and “seasonal flight order” ($F_{1,96}=38.7$, $p<0.0001$) on generation time. No differences were detected between “area” ($F_{3,92}=0.36$, $p=0.784$) and “year” ($F_{1,92}=0.57$, $p=0.451$) and no interactions among factors were detected. Regarding the generation time in each province, the results showed that average generation time was higher in the Girona province (625.6±2 DD) than in the Lleida one (528.1±14.2 DD). The relative length of generations follows the same pattern in both provinces but there are differences between the absolute generation length in each province. In both provinces the first generation was the shortest and the third and fifth (a fifth generation only occurred in Lleida) were the longest (Fig. 5). In the Girona province, the first generation was significantly shorter than all the others (see Table S2 in supplementary material). The Lleida first generation was also shorter than the third and the fifth, but it was not statistically different from the second and the fourth (see Table S2 in supplementary material).

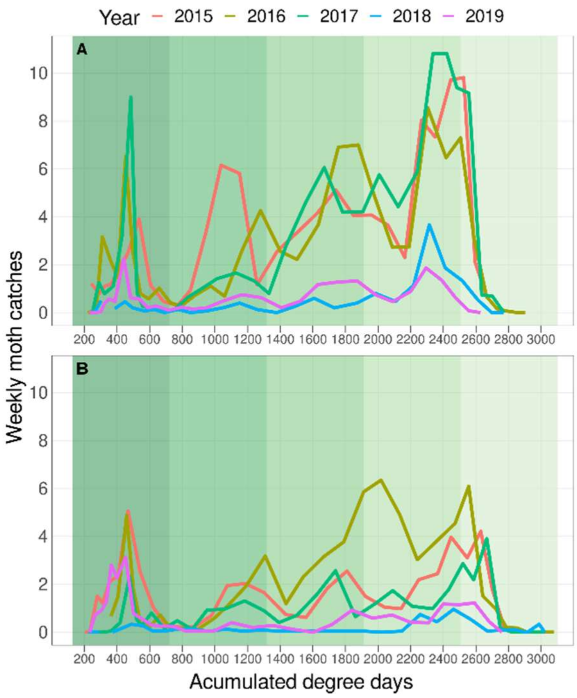

Figure 4. Mean weekly moth catches in the Girona province plotted against DD for each area and year. A: Alt Empordà, B: Baix Empordà, both of Girona province. Background colours indicate the predicted generation range of the phenology model (Croft *et al.* 1980) plus 60 DD for feeding on apples (595 DD in total) as reported by Myers *et al.* (2007) and Yang *et al.* (2016).

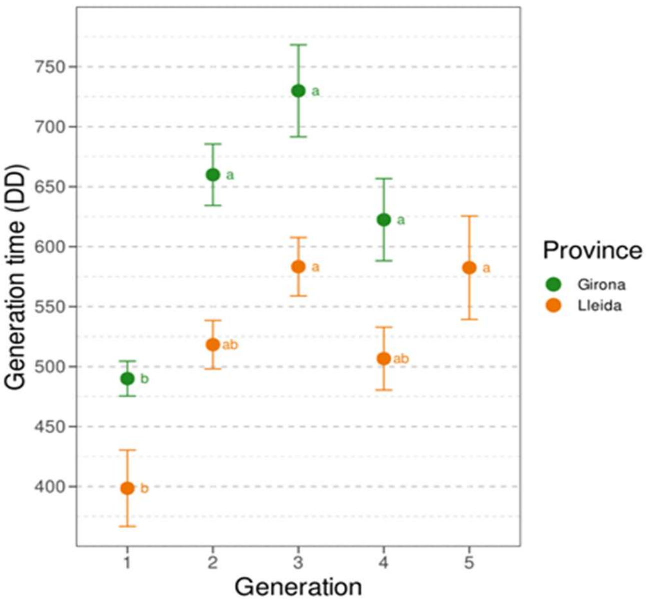

Figure 5. Duration of each generation in DD (mean±SE) in each province. Different letters indicate significant differences between generations within a province ($p<0.05$, Tukey).

Discussion

In this study, two main differences were found between the Girona and Lleida provinces, the first regarding the population dynamics of *G. molesta* and the second in the fit to the phenology model currently used to forecast population progression over a season. The main difference is that in the Girona province, four generations were observed while in the Lleida province there were five despite moths being captured for a similar time period in both regions. The population dynamics observed in both provinces follows the same pattern already reported in other studies based on experimental or untreated orchards (Rice *et al.* 1982, Russell 1986, Kovanci & Walgenbach 2005, Damos & Savopoulou-Soultani 2010). Ellis (2006) suggested that the use of moth capture data from orchards treated with insecticide applications makes population dynamics analysis harder. In the present study, although data were obtained in commercial orchards without MD and insecticides were used to control pests, similar population dynamics curves were obtained to those previously reported for other areas of the world for untreated experimental orchards. Therefore, analysis of data coming from a high number of orchards may have reduced the influence of the use of insecticides on the population dynamics curves. Nevertheless, other authors have demonstrated that it is possible to validate a phenology model using data obtained in orchards with insecticides applications (Joshi *et al.* 2016).

The number of captures was generally lower in the Girona province than in the Lleida province. This difference may be due to a lower survival of *G. molesta* in apples (Myers *et al.* 2006), although specific studies for the populations of the study area are lacking. Other factors that can influence the lower moth capture rates in the Girona province are the high number of orchards using MD for *G. molesta* as a control method and the absence of stone fruit orchards. The effect of MD in reducing moth population levels when MD is applied in a large area is well known (Cardé & Minks 1995, Il'ichev *et al.* 2002, Knight 2008, McGhee *et al.* 2011). The highest number of captures was found in the Lleida province in the Pome area, at the end of the flying period. Even though apples predominate in this area, peaches are also grown. Only occasional economic damage has been reported on late-growing varieties of apples in this region, therefore MD against *G. molesta* is only applied to the isolated peach orchards cropped between the apple orchards, reducing the effect of MD and allowing migration of nearby mated females from untreated zones once most of the peach orchards have been harvested (Knight 2008, Rothschild & Minks 1974).

The first moth is captured later in the Girona province than in the Lleida province. This fact is not only reflected in the flight start date but also in the fact that in Girona *G. molesta* requires more accumulated DD for the flight start. However, the exact moment when the moths begin to fly is still unknown, since in some years, and especially in the three areas studied in the province of Lleida, the phenology model predicted the beginning of the first flight before the monitoring traps were placed in the area. In addition to this, in some years, the first portion of the moth catch curve is descending, suggesting that the first flight was already present when the first monitoring traps were set. Higher temperatures during diapause development can lead to a lower development rate (McGhee *et al.* 2011). Because first moth catches begin earlier in Lleida even though winter temperatures there were lower than in Girona (Table 1), our results suggest that the optimum temperature for diapause development in *G. molesta* seems close to the temperatures registered in Lleida. However, diapause termination is controlled by many factors in *G. molesta* and some of them are still unknown (Russell 1986, Rothschild & Vickers 1991, Sausen *et al.* 2011). Therefore, more studies on the diapause of this pest in both provinces will help in better understanding the differences observed between areas and in turn improving the precision of the phenology model, with regards the first generation. Another factor that can have an influence in the timing of the first moth catch is the crepuscular temperature. The flight of male *G. molesta* is halted when temperatures are below 15ºC, and in early spring moths get caught in pheromone traps between 4 and 1 h before sunset (Rothschild & Minks 1974, Kim *et al.* 2011). During the period when the first moths appear in both provinces (mid-February to mid-April), the average daily temperature of the five areas over the five years of the study was below the reported threshold or slightly above (considering the standard deviation) (Alt Empordà:12.7±2.7ºC, Baix Empordà:13.0±2.8ºC, Stone area:14.7±3.8ºC, Mixed area:13.1±3.6ºC, Pome area:13.2±3.6ºC; Ruralcat 2021). This may therefore influence the timing of the first moth catches.

In contrast, the final captures in the season happen at a similar time of the year in both provinces (early October). Photoperiod is a key factor regulating the start of diapause in *G. molesta* (Russell 1986, Rothschild & Vickers 1991, Sausen *et al.* 2011) and, in the present study, both provinces have a very similar photoperiod (maximum 3-min difference between provinces; Manatech 2021).

Differences in the generation time of *G. molesta* in successive generations were reported by Damos and Savapolou-Soultani (2010), and we report similar findings in the present study. One of the factors that can influence the development of the pest is the photoperiod, especially in the last generations when the photophase is shorter than that of the generations developed during summer (Rothschild & Minks 1974). Bimodal peaks and generation overlaps have also been reported for the species (Russell 1986, Damos & Savapolou-Soultani 2010). These two factors make difficult in this species the mathematical validation of a phenology model. For this reason, and to check whether or not the model is useful in predicting the phenology of the insect, the approach proposed in this study was adopted. Both characteristics appear more conspicuously in the populations in the Lleida province than in Girona. Damos and Savapoulou-Soultani (2010) suggested that extreme high temperatures might explain the overlapping of generations. Given that in Lleida the daily thermal amplitude during the flying period of *G. molesta* is higher than in Girona (Table 1), our results support this. In addition, there is a greater mixture of host crops in the Lleida province, which may contribute to there being more overlapping of generations due to different development rates of moths in different hosts and varieties (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang *et al.* 2016, Wang *et al.* 2018). The use of DD to represent the population dynamics instead of date, reduces the effect of temperature over the years and helps to identify generations, despite overlaps, as demonstrated in the present study (As an example, refer to year 2016, third and fourth generations, in Figs 2C and 3C).

Several studies recommend evaluating phenology models in the local conditions of the area of interest due to possible adaptations of the pest (Pitcairn *et al.* 1992, Kean & Kumarasinghe 2007, Schaub *et al.* 2017, Damos *et al.* 2018). Even though the model described in Croft *et al.* (1980) was validated in the USA (California and Michigan), on peach and apple orchards (generation time: 535DD), the results obtained in the present study confirm the need to evaluate phenology models in local conditions. While the Lleida population have a similar generation time to the USA (528.1±14.2 DD), in Girona the development time is much higher (625.6±2 DD) despite the fact that the two provinces are separated by only 250 km. The difference between the generation time predicted by the model and the real generation time registered in the province of Girona, correspond to the DD accumulated in 2 or 3 days (depending on the temperature registered during the development period of each generation). These 2 or 3 more days are a small error in the model when predicting the phenology of the pest, but it accumulates with each generation (Myers *et al.* 2007), resulting in an error large enough to reduce efficacy of most insecticide applications due to lack of timing between the phenology of insects and the insecticide application. Due to the fact that the second, third and fourth generations are longer in Girona province than that predicted by the phenology model, the error in the prediction of these generations would be around of 12-15 days. The delay in the development of the pest on apples (Myers *et al.* 2007, Yang *et al.* 2016) could partially explain the increase in the DD needed in the Girona areas, although the DD increase found in the present study corresponds to 2.3

times more than if the DD was the maximum of 60 reported in the literature. Differences in development time within apple varieties has also been observed (Myers *et al.* 2007, Silva *et al.* 2010, Wang *et al.* 2018), so more information about the time of development of the species in the varieties cultivated in the studied areas should help to better understand the results obtained and to improve the phenology model.

On the other hand, in the province of Girona, the delay in the start of the first generation added to the increase in the duration of the following generations, suggest an adaptation to local conditions (Damos *et al.* 2014). This adaptation could also be a result of an increase in the lower development threshold temperature in populations of Girona as it was reported in other areas of the world (Ahn *et al.* 2014). Although the two provinces share the same bioclimate, it seems that the small differences in some climatic conditions (e.g. those corresponding to the ombrotype) as well as the different hosts present in the two provinces, could have induced/facilitated the development of local adaptations in the populations. This hypothesis requires study to develop a phenology model that effectively forecasts the development of the *G. molesta* population throughout the year when the species develops in apples in the Girona province.

Most papers on phenology model validation use data from a few orchards (e.g. Croft *et al.* 1980, Rice *et al.* 1982, Knight 2007, Damos & Savopoulou-Soultani 2010, Ahn *et al.* 2014, Damos *et al.* 2014, Joshi *et al.* 2016, Schaub *et al.* 2017, Damos *et al.* 2018). This document uses a large data set from two provinces and for a period of 5 years for phenology model validation. In Lleida province, a good fit of the predicted phenology by the model to the population dynamics of *G. molesta* was found, while in the Girona the model did not properly predict the development of the pest. These results are practical and useful because they explain the difficulties found at the field level in predicting the development of the pest based on the model and, therefore, in deciding the appropriate time to spray. These results also show the need for an adjustment of the model used, or to develop a new one, in order to facilitate decision-making at the field level.

Conclusions

In our study we found a different population phenology of *G. molesta* in two closely located fruit producing provinces growing different crops. These differences result in four generations of the moth a year in the Girona province and five in the Lleida province. The phenology model commonly used in both provinces, developed by Croft *et al.* (1980), does not predict accurately the population dynamics of the species in the Girona province where apples are the main crop, but it has an acceptable fit to the data for the Lleida province, where peaches are the main crop, including in the areas with a mix of peach and pome fruits.

Developing phenology models for a multivoltine species over large areas requires in-depth knowledge and understanding of the biology of the species in the environments and crops in which it grows. Thus, it seems clear that studies on the development of *G. molesta* on apple and peach are necessary, to better fit a model for each of the provinces considered in this document. This will allow to identify the differences in biology of *G. molesta* that explain the different population dynamics found between the provinces, and will open the way to adjust a model to the development of the species in each one.

Finally, the methodology used in the present document shows to be practical for making faster decisions regarding the proper fit of a phenology model in a new area or the need to adjust it.

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Supplementary material

Table S1. DD predicted by the phenology model (Croft *et al.* 1980), applying the correction of 60 DD delay (Myers *et al.* 2007 and Yang *et al.*2016) and the registered in each area in each of the years studied.

Moth catches	Predicted by phenology model (535DD)	Predicted by phenology model (595DD)	DD registered up to the event																								
			Girona										Lleida														
			Alt Empordà					Baix Empordà					Stone area					Mixed area					Pome area				
			2015	2016	2017	2018	2019	2015	2016	2017	2018	2019	2015	2016	2017	2018	2019	2015	2016	2017	2018	2019	2015	2016	2017	2018	2019
First moth catch in the season	126	126	250	250	250	250	300	250	350	350	350	250	*	*	300	200	150	*	*	150	200	100	*	*	*	160	*
Minima in between 1st and 2nd generation	661	721	750	800	700	750	750	800	800	800	800	800	600	625	550	700	550	550	550	550	550	550	575	550	500	600	500
Minima in between 2nd and 3rd generation	1196	1361	1250	1500	1350	1400	1400	1550	1450	1400	1450	1600	1250	1150	900	1250	1175	1125	1100	1050	1100	1000	1025	1100	975	1150	925
Minima in between 3rd and 4th generation	1731	1911	2175	2150	2125	2100	2100	2150	2250	2300	2150	2150	1800	1775	1600	1900	1575	1650	1725	1750	1750	1600	1650	1700	1550	1700	1300
Minima in between 4th and 5th generation	2266	2506	2725	2800	2775	2700	2550	2850	2900	2825	3000	2750	2175	2250	2100	2275	2325	2100	2150	2250	2350	2000	2150	2250	2150	2300	1800
Final 5th generation	2801	3101	-	-	-	-	-	-	-	-	-	-	2850	2650	3000	2850	2850	2700	*	2750	*	2550	2650	*	*	*	2400

(-) = No flight produced; (*) No enough data for the event determination

Table S2. Mean pairwise comparison of the difference generation times of *G. molesta* (in DD) in two generations of the same year, for each province. p-values were calculated with a Tukey test.

Province	Generations compared	Generation time difference (DD)	p-value	
Girona	1-2	-170.00	0.0014	**
	1-3	-240.00	0.0000	***
	1-4	-132.50	0.0160	*
	2-3	-70.00	0.3534	
	2-4	37.50	0.8073	
	3-4	107.50	0.0665	.
Lleida	1-2	-119.76	0.0744	.
	1-3	-184.76	0.0012	**
	1-4	-108.10	0.1320	
	1-5	-183.93	0.0033	**
	2-3	-65.00	0.3813	
	2-4	11.67	0.9976	
	2-5	-64.17	0.5080	
	3-4	76.67	0.2226	
	3-5	0.83	1.0000	
	4-5	-75.83	0.3383	

CHAPTER 2

Host-Pest Interactions: Investigating *Grapholita molesta* (Busck) Larval Development and Survival in Apple Cultivars

Abstract

Grapholita molesta (Busck) is a major pest affecting many Rosaceae family species. In certain regions, *G. molesta* causes significant damage to apples (*Malus domestica* (Borkh.)), which are generally considered secondary hosts. To better understand changes in the performance of *G. molesta* in apples, this study recorded the survival and development time of larvae in three different apple cultivars. Two *G. molesta* populations were examined: one that mostly attacks peaches (from the Lleida province of Spain) and another that attacks apples (from the Girona province). Tests were conducted at constant temperatures in the laboratory on fruits detached from the tree, as well as under field conditions on fruits still attached to the tree and on fruits detached from it. The cultivar influenced development time in both the laboratory and field conditions. The cultivar also affected survival, but only in the fruits still attached to the tree under field conditions. These results suggest that excising the apples from the tree affects the fitness of *G. molesta*. Survival was higher in the Girona population under laboratory conditions, but no other differences were detected between the populations.

Keywords: attached fruit; fitness; Integrated Pest Management; phenology model; Tortricidae; tree factor

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Introduction

Grapholita molesta (Busck) (Lepidoptera: Tortricidae) is a pest found worldwide, with larvae that feed inside fruits (Rothschild & Vickers 1991), causing direct damage to the fruit, but it can also feed inside green shoots (Myers *et al.* 2006, 2007) and apple burrknots (Bisognin *et al.* 2012). Peach is generally considered its primary host, although it can infest many fruit trees in the Rosaceae family. The fitness of *G. molesta* is influenced by many factors; in general, it is higher on peach than on other hosts such as plum or apple (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang *et al.* 2016, Sarker *et al.*, 2021). However, the level of fruit maturation affects the development and survival of *G. molesta* (Du *et al.* 2015, Sarker *et al.* 2021), and the fitness of *G. molesta* is inverted in immature fruits, with higher fitness observed in apples than in peaches (Sarker *et al.* 2021). In addition, differences in the development time of *G. molesta* larvae reared on different cultivars of the same plant species have also been reported (Myers *et al.* 2007, Yang *et al.* 2016, Wang *et al.* 2018).

Grapholita molesta spends most of its larval stage inside host tissues (Rothschild & Vickers 1991). During larval development, the options to manage the pest are greatly reduced. Therefore, precisely monitoring this pest is especially important to target the most accessible stages (Knight *et al.* 2019). Phenology models can predict the stage of a given species, and for this reason, they have been widely used in Decision Support Systems to predict the phenology of pests in commercial orchards and help farmers control them (Welch *et al.* 1978, Bange *et al.* 2004, Höln *et al.* 2007, Rebaudo & Rabhi 2018). In poikilothermic organisms, such as insects, temperature plays a major role in their development (Rebaudo & Rabhi 2018). Many insect phenology models use development data from constant temperatures to model the relationship between temperature and development (Howe 1967, Wagner 1984, Rebaudo & Rabhi 2018).

To study the effect of temperature on *G. molesta*, experiments are usually conducted under controlled environmental conditions with fruits detached from trees (Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Du *et al.* 2015, Yang *et al.* 2016, Wang *et al.* 2018, Sarker *et al.* 2021) for practical reasons. However, many fruits show an increased maturation process while detached from the tree (Paul *et al.* 2012), and in some pear cultivars, fruits attached to trees cannot complete their maturation (Lindo-Garcia *et al.* 2019). In apples, different maturation rates have also been reported between attached and detached fruits (Lin & Walsh 2008, Fernández-Cancelo *et al.* 2022). This change in maturation has been shown to affect the survival rate of *Cryptophlebia illepida* (Butler) larvae (Follett *et al.* 2007) and could influence the results of other similar studies, as suggested by Myers *et al.* (2007). However, information from studies at constant temperatures is critical for modeling the relationship between development rate and temperature (Rebaudo & Rabhi 2018). Therefore, this possible effect of fruit detachment needs to be taken into account to use this data to model insect development. Additionally, some fruits attacked by *G. molesta* in natural conditions fall prematurely from the tree (Dustan 1960). The information obtained from detached fruits could also be used to include larvae that developed in fallen fruits into the phenology model.

Grapholita molesta can have three to five generations depending on the region (Croft *et al.* 1980, Rice *et al.* 1982, Kanga *et al.* 2003, Tomše *et al.* 2004). During the adult stage, *G. molesta* can colonize other areas in search of better conditions, such as a host that is in a more optimal phenology stage for the new generation, or it can move from a harvested orchard to another where fruits are still present (Rothschild & Minks 1974, Sciarretta & Trematerra 2006). Therefore, the time required for *G. molesta* to complete its development can change during the season, creating difficulties in predicting its phenology. In Catalonia, a region in Northeast Spain, *G. molesta* is present in the two major fruit production regions: Girona in the Northeast, close to the Mediterranean Sea, and Lleida in the interior, far from the Mediterranean Sea. However, it behaves differently in these regions. In the Girona province, apple is the most common host for *G. molesta*, and alternative hosts are scarce. In this province, *G. molesta* has been causing serious losses in apples since the last decade (Escudero-Colomar *et al.* 2012). On the contrary, in the province of Lleida, there is a mixture of host species, but in some areas, apple and pear are dominant. However, no significant losses in apples caused by *G. molesta* have been reported so far, even though significant numbers of *G. molesta* adults are also detected in apple orchards (Amat *et al.* 2021). Further differences are detected in the number of generations; *G. molesta* has four generations in Girona and five in Lleida, although both provinces share similar temperature and climatic conditions (Amat *et al.* 2021).

Different statistical methodologies can be used to analyze development and mortality data in entomology (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Du *et al.* 2015, Yang *et al.* 2016, Wang *et al.* 2018, Sarker *et al.* 2021). "Survival" analysis is common for mortality data (e.g., Chakroun *et al.* 2007, Bareil *et al.* 2018, Martínez *et al.* 2021). However, survival analysis can also be used for many other "time-to-event" data, such as development times (Ma 2021). Survival analysis has some characteristics that make it useful for studying insect development. For example, it can incorporate censored (incomplete) data into the analysis (Bressers *et al.* 1991, Ma 2021).

Additionally, it compares the whole follow-up period of the data (Bland & Altman 2004), not just the mean, as in most usual methods (e.g., ANOVA). Survival analysis was first designed for human health studies, in which the exact time of the event may not be observed due to difficulties in sampling. Censoring was developed within survival analysis to include the information provided by this incomplete data in the analysis. There are different types of censoring depending on when the event occurs in relation to the sampling period. Right censoring applies when the studied event has not occurred before the experiment ends, for example, insects that have not finished their development in the period studied (Ma 2021). These individuals are usually omitted or considered to have finished their development in most development studies. However, more information can be extracted by including them as censored data in survival analysis, leading to more accurate conclusions (Bressers *et al.* 1991).

This study aims to investigate the development time and survival rate of two Spanish populations of *G. molesta* (Girona and Lleida) on three apple cultivars ('Gala', 'Golden', and 'Fuji') under different conditions, including laboratory versus field conditions and fruits attached to the tree versus fruits detached from it.

Materials and methods

Insects

Two populations of *G. molesta* from Northeast Spain were captured, one from orchards around the village of Ventalló in the province of Girona (hereafter referred to as the Girona population) and the other from Menàrguens village in the province of Lleida (hereafter referred to as the Lleida population). Adults and larvae were captured in Girona in 2018 and Lleida during 2017–2018. Adult insects were captured using Ajar-like traps baited with terpinyl acetate, while larvae were collected from infested fruits and shoots. The Girona insects were mainly collected in apple orchards, while the Lleida insects were collected in peach orchards. Adult insects were kept in an environmental chamber at 25 ± 1ºC under a 16:8 h light:dark photoperiod, and larvae were reared in a semi-artificial diet at the same temperature and photoperiod (Ivaldi-Sender 1974). Fresh eggs (0-24h), collected from a wax paper that was constantly available for the females to lay eggs, were used to infest the fruits.

Plant material

The orchards involved in the study were located at the IRTA Mas Badia research center (42.054271 N, 3.062348 E, La Tallada d'Empordà, Girona, Spain). The experiments were conducted on trees managed with a special Integrated Pest Management (IPM) system called "Fruit.net," which aims to obtain fruits without residues at harvest (Batllori *et al.* 2012). Mating disruption was used for codling moth (*Cydia pomonella* (L.)), mass trapping for medfly (*Ceratitis capitata* Wied.), and Phytoseiidae for controlling European red mite (*Panonychus ulmi* Koch). No insecticide treatment was used since the flowering of the apple trees. Three apple cultivars, 'Gala', 'Golden', and 'Fuji', were used to test the development of *G. molesta*, which are usually harvested in August, September, and October, respectively, in Girona province. To avoid the effect of the fruit maturation stage on the development of *G. molesta* (Sarker *et al.* 2021), experiments on each cultivar started when the fruits reached 75% of maturation for the corresponding cultivar. The period considered in this study as fruit maturation started at "full bloom" (phenology stage BBCH 65) and ended on the expected harvest day. The dates at which these stages of apple tree phenology were reached were provided by the personnel responsible for the experimental orchards, who are specialists in apple tree physiology.

Development time and survival of two populations of G. molesta at laboratory conditions

Four different environmental chambers were used to test five temperatures (16, 18, 22, 26, and 30ºC) in the laboratory. The chamber temperature was monitored throughout the experimental periods using data loggers (HOBO ext temp/RH pro v2 or HOBO pendant, Onset Computer Corporation, MA, USA) (Supplementary Table S2).

At 75% fruit maturation for each cultivar, apples were harvested for laboratory experiments and stored at 4ºC in fruit conservation chambers for up to 4 months. One day before the start of each temperature experiment, the apples were placed at the corresponding temperature. To infest the apples with *G. molesta* eggs, a piece of wax paper containing one egg less than 24 h old was placed in the calyx of an apple, and the calyx was covered with parafilm. The infested apples were kept in individual plastic containers with corrugated cardboard inside to allow larval pupation and to facilitate the detection of mature larvae. The containers were closed with a mesh and an elastic band to prevent the larvae from escaping. One hundred eggs of each population were placed on fruits of each cultivar (‘Gala’, ‘Golden’, and ‘Fuji’). Each temperature was tested in two different chambers at different dates (50 eggs each time) to reduce any possible effect of chamber and maturation during storage in the conservation chambers.

Daily records were kept of egg hatching and mature larval emergence from the fruit to calculate the larval development time. The phenology model described by Croft *et al.* (1980) was used to determine the end of the experiment at each temperature. This model is currently available on the Ruralcat website (https://ruralcat.gencat.cat/), which offers services to advisers and producers in the study area on the phenology state of pests during fruit growth. The hourly temperature recorded by the data loggers was used to calculate the number of degree-days (DD) at each temperature. DD was calculated using the thresholds described in Croft *et al.* (1980) (7.2ºC and 32.2ºC) with a horizontal cut-off. When the DD accumulated for each temperature exceeded the development time for egg and larval development described in the phenology model (~300 DD) plus 150 DD, the apples in which the larva had not emerged were checked to detect the presence of living larva that had not finished their development.

Development time and survival of two populations of G. molesta at field conditions

In the field experiments, apples were infested with eggs following the same methodology used in the laboratory experiments unless specified otherwise. Infestation occurred when each cultivar reached 75% maturation. The time at which the larva emerged from fruits was recorded daily. This time corresponds to the egg plus larval development time since the time of egg hatching was not recorded in field conditions. Three experiments were conducted in the field: i) fruits attached to trees, ii) fruits detached from trees but kept in the same field, and iii) diet control. Throughout the experimental period, air temperature in the experimental fields was monitored using two data loggers (HOBO ext temp/RH pro v2, Onset Computer Corporation, MA, USA) (Supplementary Table S2 for details).

The procedure for each experiment was:

i) Fruits attached to the tree: Apples infested with *G. molesta* eggs were naturally left attached to the trees and covered with mesh sleeves to prevent the entrance of predators and parasitoids, as well as the escape of *G. molesta* larvae. This treatment was carried out for three consecutive years. In 2018 and 2019, 300 eggs from each population were placed on the same number of apples over two consecutive days (150 each day) at 75% maturation of each of the three apple cultivars. In 2020, 150 eggs from each insect population were placed on the 'Gala' cultivar and 100 on 'Golden' and 'Fuji' on the corresponding dates. The phenology model described in Croft *et al.* (1980) and the temperatures recorded *in situ* with the data loggers were used to predict the end of larval development. When the larvae were near the end of their development, corrugated cardboard was placed inside the sleeves to allow larval pupation and facilitate the detection of mature larvae. The emergence of larvae from apples was checked daily until a further ~175 DD after the first mature larva emerged to ensure that enough larvae were detected. At the end of the experiment, all infested apples were collected and examined to detect the presence of living larvae inside the fruit or any indication of larval feeding. Since egg hatching was challenging to record under field conditions, detecting larval feeding in infested apples was considered evidence of egg hatching, and the number of apples with larval feeding was treated as the number of hatched eggs.

ii) Fruits detached from trees: In 2020, apples of the same age as those used in the previous experiments were separated from the trees but kept in the same orchard under field conditions and were infested with *G. molesta* eggs following the same procedure described earlier. One day after the field experiment with attached fruits started for each cultivar, 150 eggs of each population were individually placed in detached 'Gala' cultivar fruits, and 100 of each population were placed in apples of 'Golden' and 'Fuji' cultivars. Infested apples were placed inside two field cages under the same trees used for the attached fruit experiment. The phenology model (Croft *et al.* 1980) and the temperatures recorded with data loggers placed inside the cages were used to determine when the larvae were nearing the end of their development. Larval emergence from apples was checked daily until 175 DD after the first larva emergence was detected. When the experiment ended, all apples were examined to detect living larvae or indications of larval feeding, as in the attached fruit experiment. Larval feeding was also used as an indicator of egg hatching.

iii) Field control: In 2019, *G. molesta* development was monitored in the corresponding period of each cultivar when field experiments were conducted using a standard artificial larval diet. Three hundred eggs from each population were placed in a semi-artificial diet (the same as used for larval growth in insect breeding) in cages protected from direct sunlight in the same research station (in an old meteorological field cage station) near the experimental orchards. These controls were placed on the same day that the experiment with attached apples was infested in each cultivar. The proportion of hatched eggs was recorded, and the emergence of mature larvae was checked daily until no more mature larvae were detected.

Statistical analysis

All data analyses were performed using R 4.1.1 (R Core Team 2022). Larval survival under laboratory conditions was calculated as the proportion of mature larvae that emerged plus the remaining living larvae inside the fruit with respect to the number of hatched eggs. In field conditions, egg hatching was not recorded; therefore, larval survival was calculated as the proportion of mature larvae that emerged plus living larvae with respect to the number of apples with evidence of larval feeding. Survival data were analyzed using a generalized linear model with a Poisson function for error distribution. Model selection started from the simplest model containing no main effects. Then, each main factor was sequentially added, and finally, interactions between significant factors were tested. For model comparison, the likelihood ratio test (LRT) and the Akaike information criterion (AIC) were used, and models with lower AIC values and significantly different LRT were selected. Means of survival for each significant factor were compared using a Tukey's test for multiple pairwise comparisons. For laboratory conditions, the effect of temperature, population, and cultivar were analyzed. For the field-attached fruit experiment, population, cultivar, and year were analyzed. For the field detached fruit experiment, population and cultivar were analyzed. For the field control experiment, population and cultivar were analyzed. Data from the different experiments (laboratory conditions, attached fruits, detached fruits, and field controls) were analyzed independently.

Development time analyses were conducted using the Kaplan-Meier method (Kaplan & Meier 1958). Larvae still inside the apple at the end of the experiments were added as right-censored individuals (i.e., the event did not occur at the end of the experiment). The log-rank test was used to evaluate the effect of individual factors on the cumulative proportion of larvae that completed development as a function of development time expressed in DD. Multiple pairwise comparisons were made to compare the levels of significant factors using a log-rank test and a Benjamini-Hochberg correction to avoid false positives. In laboratory experiments, larval development time was analyzed. In field experiments, egg plus larval development was analyzed, as well as in comparisons between field and laboratory conditions.

Results

Survival

The statistical analysis of laboratory experiments conducted at constant temperatures revealed that "temperature" was the most significant factor in explaining larval survival (Deviance [Dev.] = 628.29, df = 2, $p < 0.001$). The highest survival rate was observed at 22°C and 26°C (87% and 83%, respectively), while the lowest survival rate was recorded at 14°C (23%) (Fig. 1). Additionally, the insect population origin had a significant effect on the model (Dev. = 10.97, df = 1, $p < 0.001$), with the survival rate in the Girona population being 7% higher than that of the Lleida population (72% and 66%, respectively). No significant differences were detected among cultivars (Dev. = 4.83, df

= 2, p = 0.09), and the interaction between temperature and insect population was not significant (Dev. = 8.03, df = 4, p = 0.09).

Experiments conducted under field conditions revealed that: i) in *fruits attached* to the tree, larval survival was primarily influenced by the factors "cultivar" (Dev. = 88.23, df = 2, p < 0.001) and "year" (Dev. = 62.01, df = 1, p < 0.001), with no interaction between the two factors detected (Dev. = 0.46, df = 2, p = 0.79). The insect population did not affect larval survival in attached fruits under field conditions (Dev. = 0.02, df = 1, p = 0.88). The 'Golden' cultivar had the highest survival rate, while 'Gala' had the lowest (Fig. 1). Larval survival was 1.6 times higher in 2018 (43%) than in 2019 (26%). In 2020, larval survival was similar to previous years (30%). However, due to high mortality in eggs (84%), the final number of emerged larvae was very low (1–8 depending on the cultivar and population), so data from attached fruits in 2020 were not used for statistical analysis. ii) In *detached fruits* (in 2020), larval survival was not affected by either cultivar (Dev. = 0.68, df = 2, p = 0.71, Fig. 1) or population (Dev. = 0.09, df = 1, p = 0.76). iii) In the *field control* (in 2019, with an artificial diet), differences were found in larval survival between the cultivar periods (Dev. = 103.45, df = 2, p < 0.001, Fig. 1) but not between population origins (Dev. = 0.15, df = 1, p = 0.69).

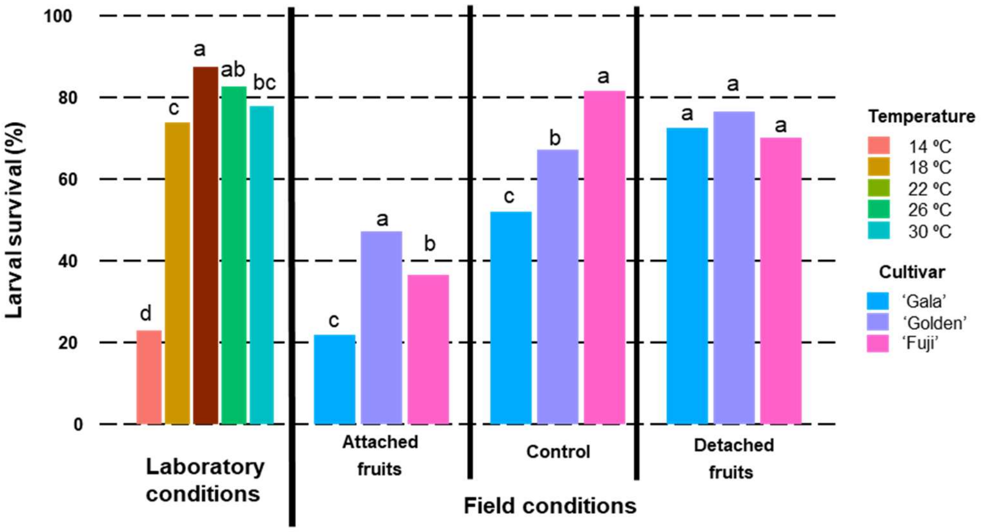

Figure 1. Effect of temperature on larval survival at laboratory conditions and effect of cultivar on larval survival at field conditions. Values within the same experimental conditions (vertical lines) with different letters indicate significant differences (Tuckey test: p<0.05). Note: in field control, "cultivar" correspond to the period of the year in which the field experiments were conducted for each cultivar; insect population and year of field experiment have been combined in this plot to facilitate representation.

Development time

Under laboratory conditions, the factors "cultivar" (χ^2 = 97.77, df = 2, p < 0.0001, Fig. 2A) and "temperature" (χ^2 = 268.15, df = 4, p < 0.0001, Fig. 2B) had a significant effect on larval development time, with no significant differences observed between populations (χ^2 = 0.17, df = 1, p = 0.59). The development time was significantly shorter in 'Gala' compared to the other two cultivars ('Golden' and 'Fuji'), and, in turn, the development time in 'Golden' was significantly shorter than in 'Fuji' (Fig. 2A). At 14°C, larval development time was equal in all cultivars; at 18°C and 26°C, *G. molesta* larvae had significantly shorter development time in 'Gala' and 'Golden' cultivars compared to 'Fuji'; at 22°C and 30°C, differences in larval development time were found among all three cultivars studied (Supplementary Fig. S1). In general, the shortest larval development time was recorded at 26°C and the longest at 14°C. The development time of larvae increased at 22°C, 18°C/30°C, and 14°C, respectively (Fig. 2B). The effect of temperature on development time was similar across all cultivars (Supplementary Fig. S2).

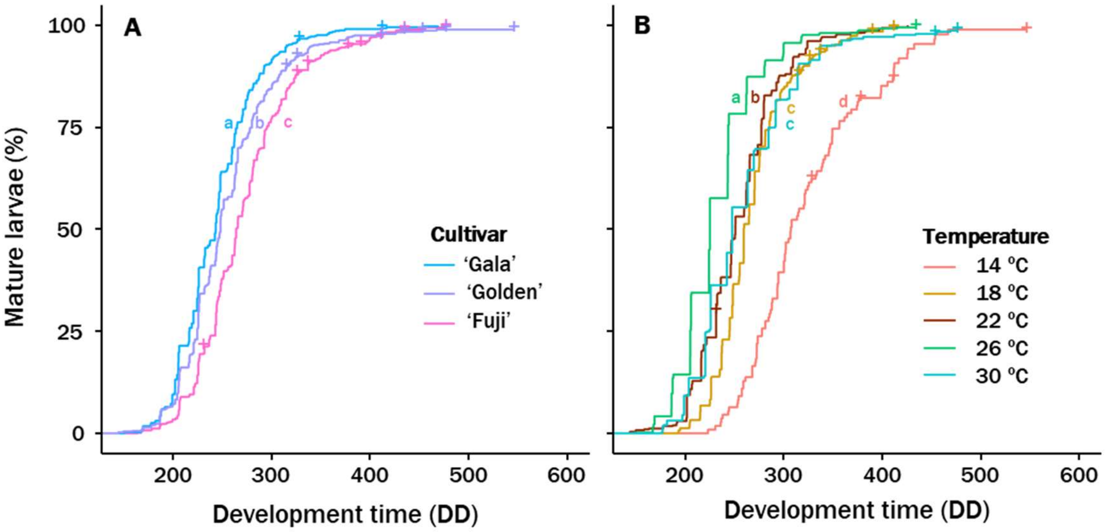

Figure 2. Cultivar (A) and temperature (B) effect on cumulative proportion of larvae that completed development as a function of development time since egg hatching (larval development time expressed in Degree-Days, DD) at laboratory conditions. Different letters indicate significant differences between curves (Log-Rank test p<0.05). Crosses marks times when censored data occurred.

Under field conditions: i) in the *attached fruits* during 2018, the irrigation system in the 'Golden' cultivar orchard failed, and the plants suffered from water deficit during the experiment. In these attached apples, the median development time (50% of mature larvae) was approximately 100 DD shorter than that recorded in the same orchard in 2019 and 2020 (Supplementary Fig. S3). Water deficit clearly affected the fruit aspect and probably the performance of the larvae, *Spodoptera littoralis* (Boisduval) showed preference for drought stressed apple leaves (**Gutbrodt *et al.* 2012**), suggesting a benefit from feeding on stressed plants. No other factor was observed to explain this difference in development time. For this reason, data from 'Golden' 2018 were excluded from the statistical analysis. In general, the cultivar had a significant effect on egg plus larval development time in attached fruits (χ^2 = 53.13, df = 2, p < 0.0001). In 2018, no differences were detected between 'Gala' and 'Fuji'. Egg plus larval development time in 'Golden' cultivar was shorter than that in 'Fuji' in 2019, but no differences were detected between 'Gala' and 'Golden' or between 'Gala' and 'Fuji' (Fig. 3A). Significant differences in development time between the years 2018 and 2019 were also found (χ^2 = 74.29, df = 1, p < 0.0001); the development time in 2019 at 50% of larval emergence was around 40 DD shorter than that in 2018 (Fig. 3A). Data from 2020 was not used due to the low number of mature larvae recovered. ii) Insect population did not affect development time in fruits attached to the tree ($\chi^2 = 0.25$, df = 1, p = 0.61).

ii) In *detached fruits*, egg plus larval development time was significantly affected by the cultivar ($\chi^2 = 23.28$, df = 2, $p < 0.0001$), with 'Golden' cultivar allowing for a shorter development time (Fig. 3B). In this experiment, population origin was also not significant ($\chi^2 = 1.51$, df = 1, p = 0.22).

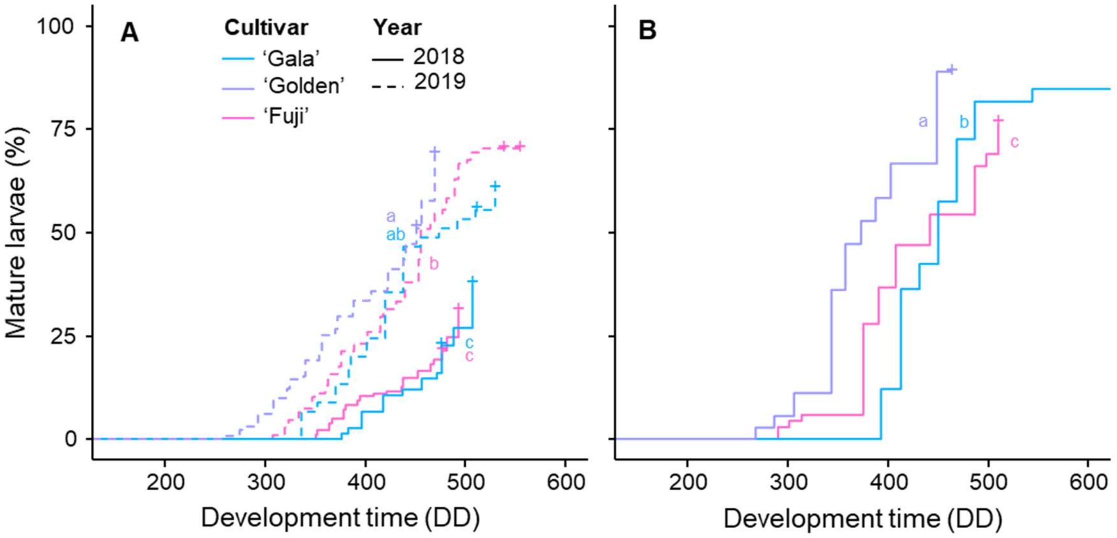

Figure 3. Cumulative proportion of larvae that completed development as a function of development time since egg laying (egg plus larval development time expressed in Degree-Days, DD) in field conditions, (A) fruit attached to the trees, (B) fruit detached from the trees. Crosses mark times when censored data occurred. Different letters indicate significant differences between curves (Log-Rank test p<0.05).

iii) The *field control* in 2019 showed differences in egg plus larval development time between the periods in which the attached fruit experiment was conducted for each cultivar ($\chi^2 = 346.43$, df = 2, $p < 0.0001$). The development time was shorter for the 'Gala' period, followed by the 'Golden' period, while the 'Fuji' period showed the longest development time (Table 1).

Laboratory vs. *field comparisons*: Differences were observed in egg plus larval development time within constant temperatures in the laboratory (14, 18, 22, 26, and 30ºC) and between them and field-attached fruits and detached fruits (χ^2 = 268.15, df = 4, p < 0.0001). Constant temperatures had a similar effect on egg plus larval development as on larval development alone; development time was shortest at 26ºC and increased at 22, 18, 30, and 14ºC. Development in all temperatures tested in the laboratory was shorter than in all field conditions. Within field conditions, detached fruits had a shorter development time than attached fruits (Supplementary Table S1). Also, during 2020, development time seemed shorter in attached fruits (Supplementary Fig. S3); however, this difference could not be confirmed due to the low number of mature larvae recovered. Analyzing each cultivar independently, differences between conditions (laboratory, field-attached, and detached) were also observed ('Gala' χ^2 = 131.89, df = 4, p < 0.0001, 'Golden' χ^2 = 105.35, df = 4, p < 0.0001, 'Fuji' χ^2 = 73.42, df = 4, p < 0.0001). The differences between conditions in the 'Gala' and 'Fuji' cultivars were similar to those observed when data from all varieties were analyzed together. In 'Golden', the development time was shorter in both field conditions (compared to the other cultivars) and could not be statistically differentiated from 14ºC in laboratory conditions. However, the difference within field conditions was similar to the 'Gala' and 'Fuji' cultivars. Detached fruits had a shorter development time than attached fruits (Fig. 4).

Table 1. Egg plus larval development time (expressed in Degree-Days, DD) for 25, 50, and 75% of the total larvae recovered in the field control experiment with semi-artificial diet. Different letters indicate significant differences between curves (Log-rank test p<0.05).

Cultivar period	n	25%	50%	75%	
'Gala'	249	237.04	255.91	255.91	a
'Golden'	362	249.55	264.37	280.45	b
'Fuji'	440	269.44	282.64	294.88	c

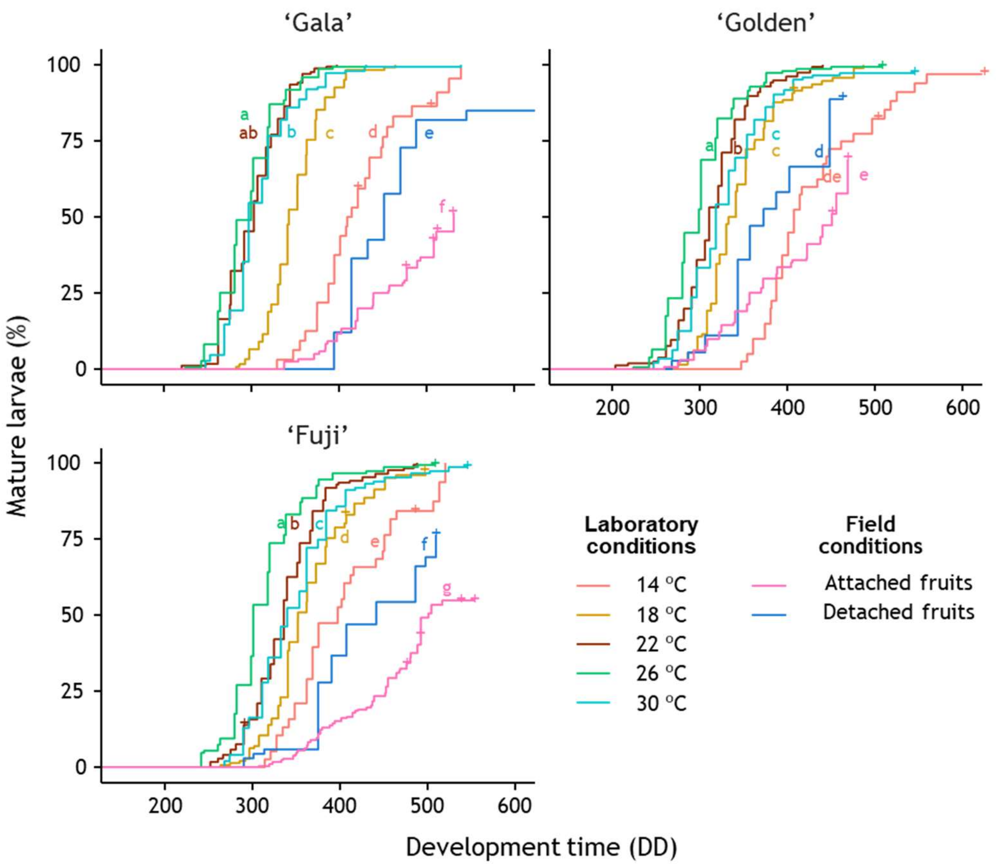

Figure 4. Cumulative proportion of larvae that completed development as a function of development time since egg laying (egg plus larval development time expressed in Degree-Days, DD). Crosses mark times when censored data occurs. Different letters indicate significant differences between curves (Log-Rank test $p<0.05$). Note: only data from year 2019 was used to calculate 'Golden' curve in attached fruits.

Discussion

Insect phenology models can be used to compare development time between different periods under field conditions (e.g. Myers *et al*, 2007). In this study, the phenology model was also used to compare the results from field trials and laboratory experiments at a constant temperature. In general, the larval development time recorded under laboratory conditions was longer than that predicted by the model (215 DD, Croft *et al.* 1980). Nevertheless, this delay in development (~43 DD) falls within the range detected by other studies that have tested *G. molesta* development in apples (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang *et al.* 2016).

Although phenology models account for the effect of temperature on development time, differences among temperatures were detected in this study. The phenology model used (Croft *et al.* 1980) assumes a linear relationship between temperature and development (Welch *et al.* 1978). However, the relationship between temperature and development is almost linear near the optimum temperature, but at temperatures near the development thresholds, this linear relation is lost (Damos & Savopoulou-Soultani 2012 Mironidis 2014, Rebaudo & Rabhi 2018). The results presented in this study confirm this observation. The shortest development time (in DD) recorded in this study was at 26ºC, which is closest to the optimum temperature (25ºC) found in another study (Chen *et al.* 2019). The development time increased as temperatures deviated from the optimum until it reached the longest development time at 14ºC.

Phenology models are typically developed using data obtained under constant temperature conditions in the laboratory to determine insect thermal requirements (Rebaudo & Rabhi 2018). However, temperature fluctuations can increase the development rate of *G. molesta* compared to similar constant temperatures (Chen *et al.* 2019). In other species, such as *Helicoverpa armigera* (Hübner), development was found to be shorter under fluctuating temperatures below 25ºC compared to constant temperatures with the same means (Mironidis 2014).

Contrary to these findings, our results showed a longer development time for *G. molesta* under field conditions (both attached and detached fruits) than under laboratory conditions at temperatures closest to the mean temperatures recorded during field experiments. This suggests that factors other than temperature were influencing *G. molesta* development. It is possible that the phenology model did not perform well under the particular climatic conditions of the region, as it was developed for a much colder region (Croft *et al.* 1980). In fact, in the field control with the same food source, the DD required to complete egg and larval development were lower in the period of the 'Gala' cultivar than in the period of the 'Fuji' cultivar, indicating the inaccuracy of the model.

It is also important to note that the actual temperatures experienced by larvae inside apples may differ from external temperatures. Moreover, high-temperature variability has been reported inside apples depending on their position in the canopy (Saudreau *et al.* 2009). Thus, the temperature recorded in the field experiments may not be perfectly correlated with the actual temperature experienced by the larvae inside the fruits. However, air temperature is generally used when applying phenology models, and therefore it seems to be the best approximation for this study.

Important differences were observed among the experiments conducted. The development time at 26ºC in laboratory conditions was more than 200 DD shorter than that for the attached fruits in the 'Gala' and 'Fuji' cultivars. The development time was also shorter for detached fruits under field conditions in all cultivars. The effect of using detached fruits to study insect development, even if acknowledged in some cases (e.g. Myers *et al.* 2007), has generally been overlooked. In the present study, this effect was observed. Survival was also much higher in detached apples than in attached ones, reaffirming the effect of excising fruits on larval fitness.

Part of the observed differences were due to the limitations of the phenology model detected in the field control. However, this effect could not explain all the differences detected. For example, survival during the 'Fuji' field experiment was highest in the field control, but in the attached fruits, it was higher in the 'Golden' cultivar. Therefore, another factor must have modified larval fitness in attached fruits. The "tree factor" modulating apple maturation (Lin & Walsh 2008, Paul *et al.* 2012, Fernández-Cancelo *el al*. 2022) was probably affecting the development of the larvae, as in other species (Follett *et al.* 2003). Additionally, direct and indirect plant defense responses triggered by herbivore feeding (Mithöfer & Boland 2012, Mauch-Mani *et al.* 2017) could have interfered with larval development and survival.

The presence of secondary metabolites as well as the activation of plant defenses, could have been responsible for the longer development time and lower survival rate. For instance, *Choristoneura fumiferana* (Clemens) was found to increase the presence of a secondary metabolite (maltol) when feeding on its plant hosts (Leclair *et al.* 2015). The presence of maltol in plant tissue has been associated with a decrease in the survival of *C. fumiferana* and an increase in its development time (Williams *et al.* 2019). In another tortricid moth, *C. illepida*, larvae fed on detached fruits showed three times the survival rate of those fed on attached fruits (Follett *et al.* 2003). Therefore, the activation of plant defense mechanisms could have reduced the fitness of *G. molesta* larvae in attached apples, as detected in this study.

Insects can evolve to overcome plant defense systems. In some tortricid moths, specific host races have been identified (Cisneros & Barnes 1974, Goyer *et al.* 1995). Host races perform better in certain plant species within the total species host's range. In *G. molesta*, adaptation to different host cultivars has been previously reported (Monteiro & Niederheitmann 2022). *G. molesta* exhibits worldwide genetic variation (Kirk *et al.* 2013) and also varies among close populations (Torriani *et al.* 2010, Damos *et al.* 2014). Some of the genetic differences may be due to specific host races. However, it does not seem that the *G. molesta* population in the Girona province has experienced considerable adaptation to feed on apples. No differences in development and survival rates were detected under field conditions, and only a small but significant increase in survival was detected in Girona populations when developing in apple fruits under laboratory conditions. The reduced difference in larval fitness among populations with different crop origins indicates the pest's ability to easily adapt to different food sources and its potential capacity to attack multiple hosts in mixed crop areas.

In the present study, differences in development time and survival rates of *G. molesta* among apple cultivars were observed, consistent with previous reports (Myers *et al.* 2007, Yang *et al.* 2016, Wang *et al.* 2018). However, differences among cultivars were not constant between treatments. This lack of congruence could result from the phenology model used to compare treatments. Additionally, the maturation process in attached and detached apples can vary among cultivars. The maturation process of 'Gala' and 'Golden' is highly increased after fruits are harvested (Lin & Walsh 2008, Fernández-Cancelo *et al.* 2022), while the effect of fruit detachment on 'Fuji' is lower (Lin & Walsh 2008). Furthermore, the plant defense mechanism may differ between cultivars, adding more complexity to comparing them.

In conclusion, despite developing in different hosts, no remarkable differences in development and survival were found between Girona and Lleida populations of *G. molesta*. The development times recorded under laboratory conditions were similar to those from previous studies and the effect of cultivars. The differences found between experiments at constant temperature and those in the field indicate the need to adjust the currently used phenology model to the natural conditions experienced in the field. Moreover, the difference between fruits still attached and those detached from the trees should be further evaluated. All this new knowledge will offer a clear improvement in the forecast of pest development and, consequently, in the usefulness of phenology models in IPM in the studied areas and other similar regions.

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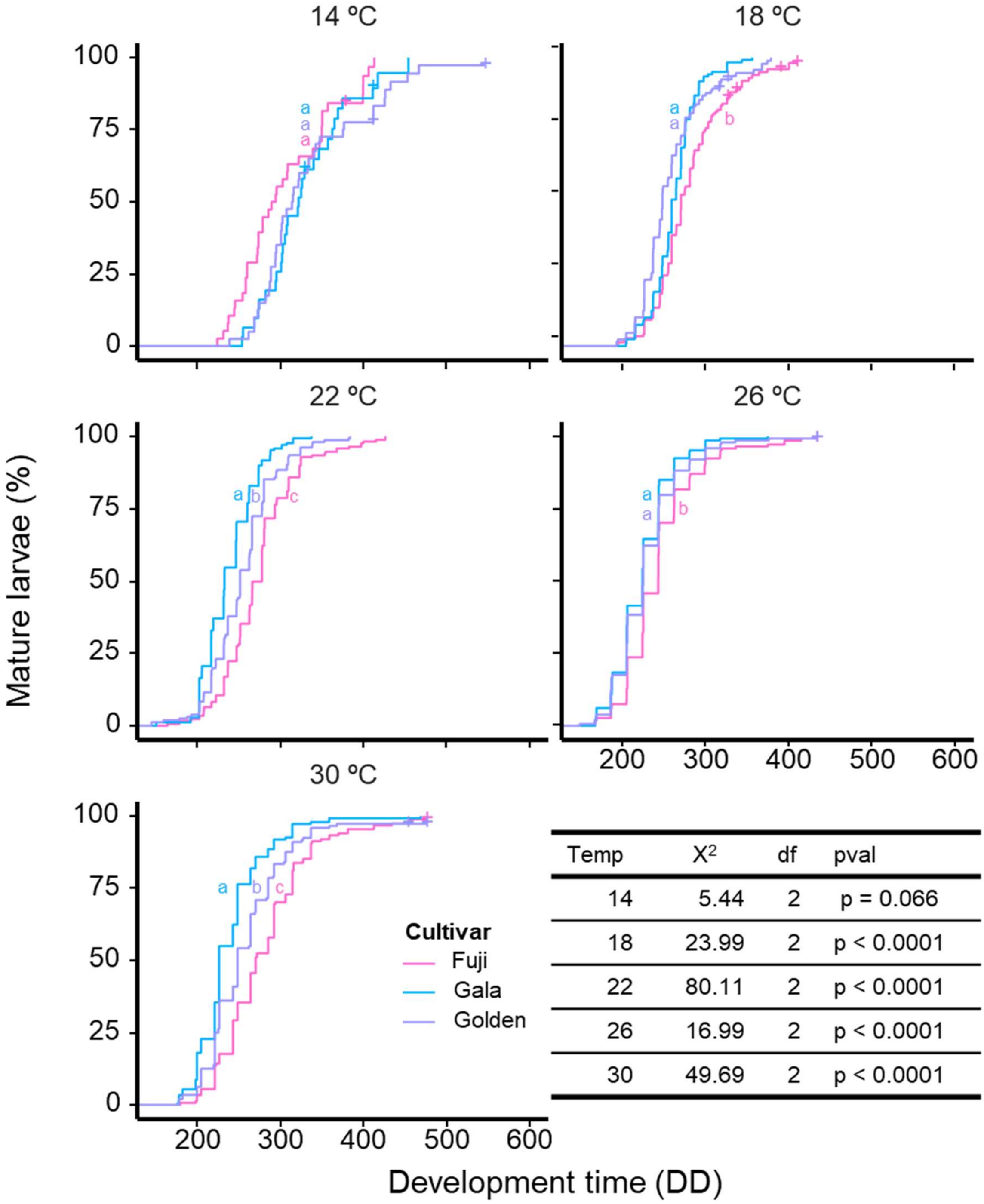
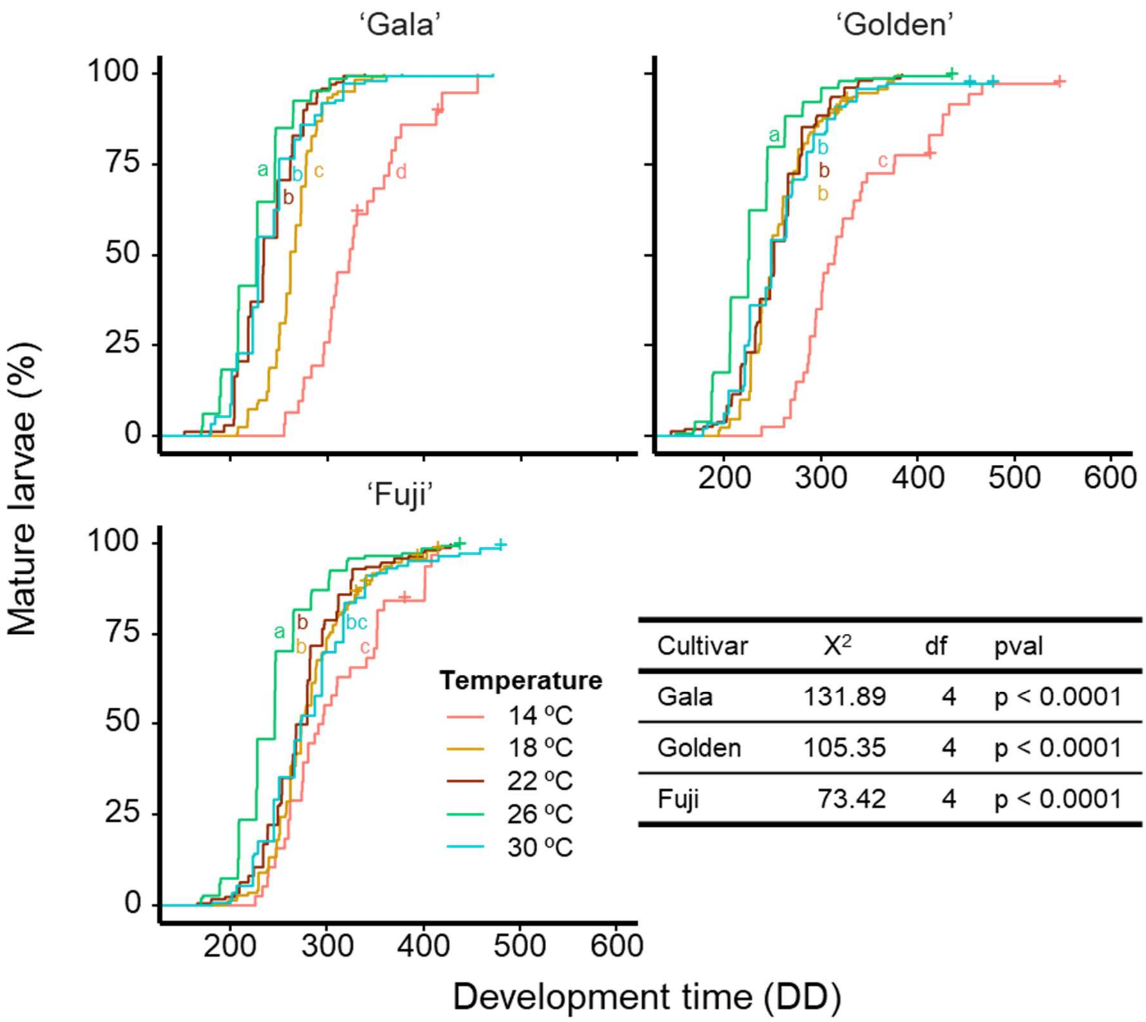
Supplementary material



Temp	X²	df	pval
14	5.44	2	p = 0.066
18	23.99	2	p < 0.0001
22	80.11	2	p < 0.0001
26	16.99	2	p < 0.0001
30	49.69	2	p < 0.0001

Figure S1. Cumulative proportion of larvae that completed development as a function of development time since egg hatching (larval development time expressed in Degree-Days, DD) at laboratory conditions. Different letters indicate significant differences between curves (Log-Rank test p<0.05), each temperatures was analyzed independently. Crosses marks times when censored data occurred. The table shows the results of the log-rank test within each temperature.



Cultivar	X^2	df	pval
Gala	131.89	4	p < 0.0001
Golden	105.35	4	p < 0.0001
Fuji	73.42	4	p < 0.0001

Figure S2. Cumulative proportion of larvae that completed development as a function of development time since egg hatching (larval development time expressed in Degree-Days, DD) at laboratory conditions. Different letters indicate significant differences between curves (Log-Rank test p<0.05), each cultivar was analyzed independently. Crosses marks times when censored data occurs. The table shows the results of the global log-rank test within each cultivar.

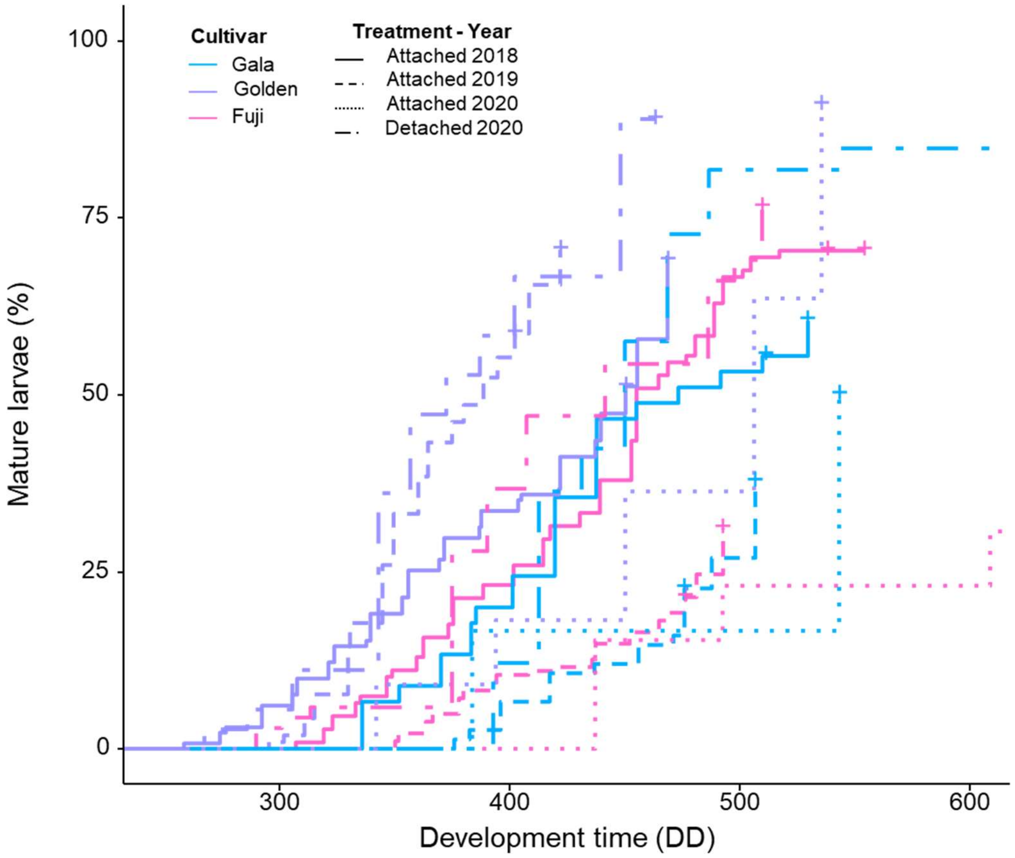


Figure S3. Cumulative proportion of larvae that completed development as a function of development time since egg laying (egg plus larval development time expressed in Degree-Days, DD) for all conditions and years tested at field conditions. Crosses marks times when censored data occurred.

Table S1. Egg plus larval development time (expressed in Degree-Days, DD) for 25, 50 and 75 % of the total larvae in laboratory and field conditions. Different letters indicate significant differences among curves within each treatment (Log-rank test $p<0.05$). Note: for attached fruits, data from year 2018 of Golden cultivar was not used.

Treatment	n	25%	Median dev. time	75%	
26 ºC	449	279.62	300.96	319.59	a
22 ºC	498	290.29	315.17	339.69	b
30 ºC	441	296.09	317.97	362.03	c
18 ºC	395	329.36	341.83	372.22	d
14 ºC	110	373.90	406.06	453.99	e
Field detached fruits	137	375.17	431.50	486.76	f
Field attached fruits	541	435.86	501.39	NA	g

Table S2. Summary of the temperatures recorded at laboratory conditions (a) and at field conditions (b).

a

Temperature	n	Chamber	Mean	SEM
14	4036	3	13.96	0.010
	3648	4	14.14	0.008
18	1164	1	17.80	0.025
	898	4	18.20	0.032
22	783	1	20.93	0.012
	819	2	21.88	0.016
	950	4	22.07	0.012
26	683	2	26.05	0.053
	541	4	25.98	0.016
30	662	1	29.75	0.064
	627	2	29.08	0.040

b

Year	Treatment	Cultivar	n	Mean	SEM	Min	Max
2018	Attached	Gala	751	25.64	0.21	15.66	39.21
2019	Attached	Gala	728	25.03	0.20	14.91	44.27
2019	Control	Gala	481	25.01	0.20	15.89	34.18
2020	Attached	Gala	734	25.05	0.21	13.57	40.66
2020	Detached	Gala	807	28.42	0.18	14.77	45.12
2018	Attached	Golden	650	23.52	0.18	14.19	33.77
2019	Attached	Golden	706	24.17	0.21	12.45	38.50
2019	Control	Golden	457	24.56	0.20	15.34	35.02
2020	Attached	Golden	817	23.09	0.21	10.52	38.06
2020	Detached	Golden	649	25.20	0.29	10.74	44.47
2018	Attached	Fuji	823	21.72	0.16	12.79	33.48
2019	Attached	Fuji	1009	21.11	0.15	11.30	33.04
2019	Control	Fuji	649	22.15	0.19	12.27	33.16
2020	Attached	Fuji	1466	18.96	0.16	2.88	34.20
2020	Detached	Fuji	1443	22.80	0.14	11.66	47.45

CHAPTER 3

Oviposition by three tortricid moths on filter papers treated with different concentrations of sugars, salts or neem oil

Abstract

Oviposition behaviour is an essential parameter in the life history of insects. The manipulation of oviposition behaviour with contact chemical stimuli can be employed in pest control. Relatively few oviposition chemical stimuli have been described for tortricid moths. A laboratory arena was developed to measure the effect of gustatory stimuli on the number of eggs laid by the tortricid moths *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Dennis & Schifermüller), major pests of apples, peaches and grapes worldwide. NaCl, KCl, sucrose and fructose (which have demonstrated behavioural and/or physiological effects on the moth species) and neem oil (a strong insect deterrent) were tested. Mated females were released in 1.48 L choice-test arenas containing 3 doses of a given test stimulus (0.1, 1 and 10% v/v in ethanol for neem oil, or 10^1, 10^2 and 10^3 mM in water for salts and sugars) or solvent control, and the number of eggs laid on each of the four different filter paper pieces was recorded 24h later. The configuration of the oviposition arenas had to be adapted to each species in order to maximize oviposition on the filter paper. Neem oil dramatically reduced the number of eggs laid as the dose increased, but the lowest neem oil dose (0.1% v/v) unexpectedly increased *L. botrana* oviposition relative to solvent control. In general, salts increased oviposition at the mid dose (10^2 M) and sugars reduced it at the highest dose (10^3 mM), with a higher number of significant effects in *C. pomonella* than in *G. molesta*, and in *G. molesta* than in *L. botrana*. Our study shows that ubiquitous plant chemicals may be used by tortricid moths to asses oviposition substrate suitability and that neem oil is a strong oviposition deterrent of leafrollers. Our oviposition setup is a convenient tool to test the effect of tastants on the oviposition behaviour of tortricid moths.

Keywords: oviposition deterrent; oviposition stimulant; behavior manipulation; sucrose; fructose; NaCl; KCL; Tortricidae

Chapter not submitted: Amat C, Prasad R & Gemeno C (2023) Oviposition by three tortricid moths on filter papers treated with different concentrations of sugars, salts or neem oil.

Introduction

Oviposition behaviour is a fundamental parameter in the life history of oviparous animals (Refsnider & Janzen 2010). In phytophagous insects, female oviposition choices have a profound impact on larval fitness because they are often restricted to live on the plant that their mothers chose to lay them as eggs (Thompson & Pellmyr 1991). If the mother makes a bad choice the larvae will be restricted to a suboptimal diet and may experience decreased fitness or die (Benda *et al.* 2011). Egg location also determines susceptibility to natural enemies and environmental stressors such as insolation and desiccation. In addition, the number of larvae per plant or plant part could affect larval fitness due to competition, so females may also may evaluate previous oviposition in the host in order to reduce competition and increase larval fitness (Mayhew 2001).

Lepidopteran oviposition behaviour has been reviewed (Thompson & Pellmyr 1991, Renwick & Chew 1994). Female moths and butterflies asses host suitability using different sensory modalities. Olfaction and vision play a main role before landing on the plants, but once landed females have access to additional information, mainly chemical (non-volatile chemicals sensed through gustation, also referred to as taste or contact chemoreception), and mechanical (through mechanosensation) (Ramaswamy 1988, Honda 1995, Städler 2002). Most of the on-plant chemo- and mechano-sensory information is located on the cuticle, which is coated with a variety of chemical cues as well as numerous microscopic structures of varied functions (Müller & Riederer, 2005, Barthlott *et al.* 2017). In addition, internal chemical cues may become available to the insect where the cuticle breaches.

Gustatory receptor neurons (GRNs) are typically housed inside microscopic cuticular projections (i.e., sensilla) which in the Lepidoptera, as in the majority of insects, are on the legs, cephalic appendages, the anterior sections of the digestive system and the ovipositor (King & Gunathunga 2023a). Just by walking on the plant females gather chemical information passively through their legs. In addition, female butterflies reportedly drum on the plant substrate with their forelegs to gather additional chemical information from the plant, and female moths and butterflies drag their ovipositor over the plant substrate, potentially to sample its chemical composition (Ramaswamy 1988, Cury *et al.* 2019). Tactile and gustatory information normally go together because mechanosensory neurons are present in most of the same appendages that bear GRNs, either in separate specialized mechano sensilla or paired with gustatory neurons in mechano-taste sensilla (Marion-Poll 2020, King & Gunathunga 2023a).

Gustatory stimuli may arouse or reduce behaviour, or have no effect, and these actions are described with specific terminology for different types of behaviour (feeding, ovipositing, etc) (Wallingford *et al.* 2017). Oviposition stimulants and deterrents can be employed in pest management in different ways. In push-pull strategies, deterrents can push the pest away from the crop while stimulants can be used to attract them to alternative sinks (Pickett *et al.* 1997, Eigenbrode *et al.* 2016). Oviposition marking pheromones, those that reduce larval competition, have been used successfully to reduce Tephritid fly populations (Benelli *et al.* 2014). Oviposition is also relevant in the control of insects of medical importance, such as mosquitoes (Mwingira *et al.* 2020). Synthetic chemicals, such as modern insecticides, can also change oviposition behaviour (Gould 1991). Oviposition cards to monitor eggs in species which eggs are hard to find could provide a very useful IPM tool (Markheiser *et al.* 2018).

Leafroller moths (Lepidoptera: Tortricidae) are economically important fruit pests (van der Geest & Evenhuis 1991). Females lay eggs on the plant surface and the larvae feed on diverse plant tissues (i.e., fruit, twigs, etc.). There are, however, relatively few oviposition taste stimuli described for tortricids. Foster and Howard (1998) found that the organic-solvent extracts of the host plants stimulated oviposition in *Epiphyas postvittan*a (Walker), and Grant *et al.* (2000) tested the oviposition response of *Choristoneura fumiferana* (Clemens) to a series of aliphatic carboxylic acids. Rid *et al.* (2018) show that oleanoic acid, the most abundant compounds on the skin of grapes, stimulated oviposition of *Lobesia botrana* (Dennis & Schiffermüller). As the most economically significant tortricid pests for apple, peach, and grapevine in the world, respectively, we have described the response of GRNs from the antennae and labial palps of adult males and females of *Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *L. botrana* (Knight *et al.* 2019, Benelli *et al.* 2023). Salts (KCl, NaCL) and sugars (glucose and fructose) elicited a response from the GRNs (Amat *et al.* 2022).

In the present study we wanted to determine if the compounds that stimulate the GRNs can modify the behaviour of the moths. We chose oviposition behaviour because it has a great potential in IPM. In addition, sugars affect the oviposition behaviour of *C. pomonella* (Lombarkia & Derridj 2008, Arnault *et al.* 2016), and *L. botrana* (Maher *et al.* 2006). We also included neem oil, an essential oil derived from the tissues of the neem tree, *Azadirachta indica* (A. Juss.) which is a strong insect repellent (Campos *et al.* 2016). In order to perform multiple-choice oviposition tests under laboratory conditions a convenient oviposition arena was developed.

Material and Methods

Insects

Larvae were reared on a semi-artificial diet modified from Ivaldi-Sender (1974) at 25^{o}C under a 16:8 light:dark photoperiod. *G. molesta* and *L. botrana* pupae were sexed and kept in 1-L containers provided with 10% sucrose in water in separate male and female environmental chambers and the adults were collected every 1-3 days. *C. pomonella* adults emerged in the larval cages and were collected daily. 1 to-3-day-old males and females were mixed in a ratio 1.5:1, respectively, starting at least 5 hours before the onset of the schotophase. On the next day, the now 2 to-4-day-old females were placed in the oviposition arenas. No food or drink was provided in the oviposition arenas. Mating status was checked after the test as indicated below (most females were mated).

Chemical stimuli

Water soluble stimuli [NaCl (CAS: 7647-14-5, ref. S7653), KCl (CAS: 7447-40-7, ref. P3911), sucrose (CAS: 57-50-1, ref. S9378) and fructose (CAS: 50-48-7, ref. F0127), Sigma-Aldrich, Madrid, Spain] were diluted in deionized water. Three concentrations of each stimulus (10, 100 and 1000 mM) were prepared in 4-ml aliquots and kept at -20ºC. The same water stock was kept at -20ºC for the control. Neem oil from cold pressed seeds (Batch no 9344732, Gonaturals, Vivere Gmbh, Hamburg, Germany) was diluted in absolute ethanol to obtain three 0.1, 1 and 10 % v/v dilutions. Neem oil dilutions and the ethanol solvent were kept at -20ºC in 4-ml aliquots.

Oviposition arenas

The oviposition arenas (Fig. 1) were adapted from the mating boxes used for insect rearing. A 138-mm-high x 117-mm-internal diameter (i.e., 1.48 L) section of solid gray PVC pipe was stopped in one end with a polymethyl methacrylate (PMMC) clear plate glued into it. The other end of the cylinder was covered with a lid made with a 40-mm section of a 125-mm inner diameter solid gray PVC pipe with grey nylon mosquito screen (mesh size = 1 mm) glued on it. The walls were lined with 5-mm thick green kitchen scourer, protruding 5-mm outside the cylinder to ensure sealing with the mosquito screen lid. The PMMC was lined with either green kitchen scourer (*C. pomonella* and *G. molesta*) or with 2-mm thick black felt (*L. botrana*). The oviposition substrate consisted of filter paper pieces (Whatman No.1, Global Life Science Solutions Operations UK Ltd, Buckinghamshire, UK). In the *C. pomonella* and *G. molesta* arenas the four 50-mm x 75-mm (37.5cm^2) filter-paper pieces were pinned long-side vertical onto the scourer lining the walls, spaced 30 mm from each other, 10-mm below the mosquito screen lid and 53-mm from the other cage end. In the *L. botrana* arenas the four triangular filter paper pieces were cut from a 110-mm radius disc (19.63 cm^2 each), and were stapled to the black felt lining the PMMC, leaving 10-mm distance between them. *C. pomonella* and *G. molesta* arenas stood upside up, with the mosquito screen lid on top, while *L. botrana* arenas stood upside down, with the nylon screen lid on the floor, and the filter papers on the ceiling. This difference in arena orientation maximized the number of eggs lays on the filter papers relative to the other arena surfaces (i.e., kitchen scrub, nylon screen or felt), as will be discussed later.

Experimental procedure

4 mL aliquots of the taste stimuli were defrosted on demand and frozen again until finished. Filter paper pieces with the treatment name lightly written with pencil were laid on a plastic screen so as to facility evaporation. Test solutions were applied using micropipetes with disposable plastic tips, wetting the paper completely and as evenly as possible. The *C. pomonella* and *G. molesta* filter papers received 0.5 ml test solution, and the *L. botrana* papers 0.26 ml, resulting in the same stimulus quantity per unit area (Table S1). A different pipette tip was used for each compound, the control treatment was loaded first and then the test stimuli, from the lowest to the highest concentration using the same pipette tip for a given compound. Latex gloves were exchanged when handling filter papers of different stimuli. Filter papers were left

at room temperature until they were completely dry and then were kept inside plastic bags at 4ºC until used, but for no longer than 15 d.

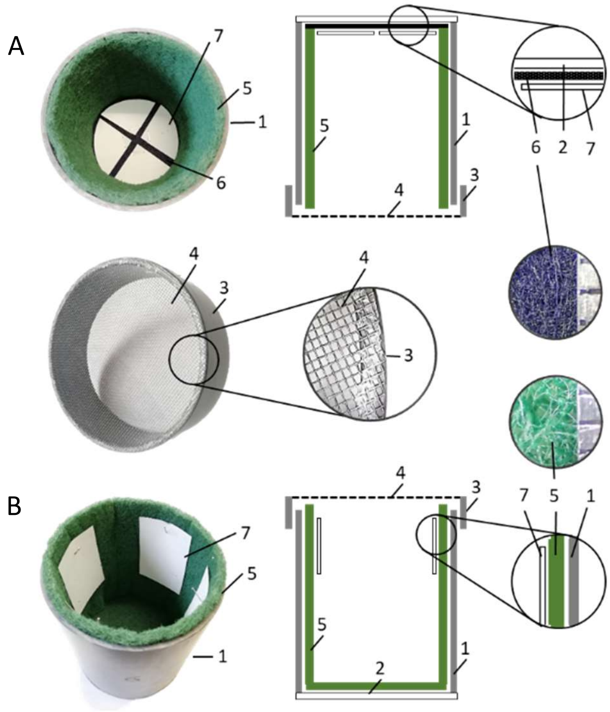


Figure 1. Configuration of the oviposition arenas for *L. botrana* (A), *C. pomonella* and *G. molesta* (B). The distribution of all elements is showed in a real image and a scheme with details of the most important sections. 1: PVC pipe; 2: PMMC stop; 3: PVC pipe lid; 4: mosquito screen; 5: kitchen scourer; 6: black felt; 7: oviposition substrate (filter paper).

Females were introduced in the test arenas at least 5 h before the onset of the schotophase. The arenas were placed in the same environmental chamber where the colony is kept, at 24.5 ± 1^{o}C under a 16:8 light:dark photoperiod. The number of eggs laid on the filter papers was recorded 24 h after females were released in the oviposition arenas. Females were placed in ethanol for later determination of mating status by means of spermatophore dissection (N = 50/species). For cleaning, scourer and felt were submerged in 60^{o}C tap water for 20 min and then were rubbed thoroughly and let dry before reused. The mosquito screen was cleaned with a paper towel soaked in 75% ethanol, and the cages were rinsed in water.

Statistics

Data curation, statistical analyses and plots were done in R 4.2.2 software (R core team, 2022). The number of eggs per female was estimated by dividing the number of eggs in a filter paper by the number or females placed in the arena (4 to 5). Generalized lineal model (GLM) with Poisson error distribution was used to analyse the effect of species, stimulus and concentration. Model selection started from the simplest model containing no main effects, then main factors and interactions were added sequentially. Models were compared to each other with likelihood ratio test (LRT) and selected according to the Akaike information criterion (AIC). The model with the best fit was used to conduct pairwise comparison between relevant groups of significant factors. Pairwise comparisons used the Tukeys´s test in the package "emmeans()".

Results

For each species and stimulus combination between 72 and 80 females were placed in between 15 to 16 arenas. *C. pomonella* laid between 11 and 70 eggs per female (36.82 ± 0.75, mean ± SEM), followed by the *G. molesta* with between 12 and 70 eggs (34.05 ± 0.72, mean ± SEM) and the *L. botrana* with between 2 and 62 eggs (24.82 ± 0.75, mean ± SEM).

The percentage of mated (i.e. carrying a spermatophore) females of *L. botrana*, *G. molesta* and *C. pomonella* females was 94, 90 and 96%, respectively (N=50). A small proportion of females carried two spermatophores. Dead or morbid females at the end of the test occurred in 17 out of 229 arenas (Table S3) with 6.24 ± 2.74%, 0.53 ± 0.32%, 0.54 ± 0.33 for *G. molesta*, *L. botrana* and *C. pomonella* (mean ± SEM), respectively. The highest morbidity/mortality occurred in the sucrose *G. molesta* arenas (15%). The number of eggs laid by females did not correlate negatively with female morbidity or death (Fig. S1)

The statistical model that best explained oviposition contained all main factors (stimulus, concentration and species) and 2nd- and 3rd-order interactions (Table S4). The factor with the highest deviance (35%) was the interaction between stimulus and concentration, followed by species (23%), and by the interaction between species and stimulus (15%) (Table 1).

Table 1. ANOVA output of the selected GLM model that analyses the number of eggs laid by individual females as a function of species ("sp": *C. pomonella*, *G. molesta, L. botrana*), stimulus ("stim": glucose, fructose, NaCl and KCl), and stimulus concentration ("con": solvent control and 3 concentrations). Treatments are shown in decreasing order of deviance.

	Df	Deviance	Resid.Df	Resid.Dev	Pr(>Chisq)	% Dev.	Cum. Dev.
NULL			915	2872			
stim:con	12	344.55	880	1998	<0.001	34.73	34.73
sp	2	225.53	913	2647	<0.001	22.73	57.47
sp:stim	8	146.67	898	2358	<0.001	14.79	72.25
sp:stim:con	24	118.11	856	1880	<0.001	11.91	84.16
con	3	117.29	906	2504	<0.001	11.82	95.98
stim	4	24.94	909	2622	<0.001	2.51	98.50
sp:con	6	14.91	892	2343	0.021	1.50	100.00

Pairwise comparisons of parameter estimates using the best-fit model revealed that *L. botrana* laid fewer total eggs than the other two species, that fructose and KCl received more eggs than the other three stimuli, and that the highest concentration had the fewest number of eggs (Table S5). Pairwise comparisons of different parameter interactions are shown in Figs. 2 and 3.

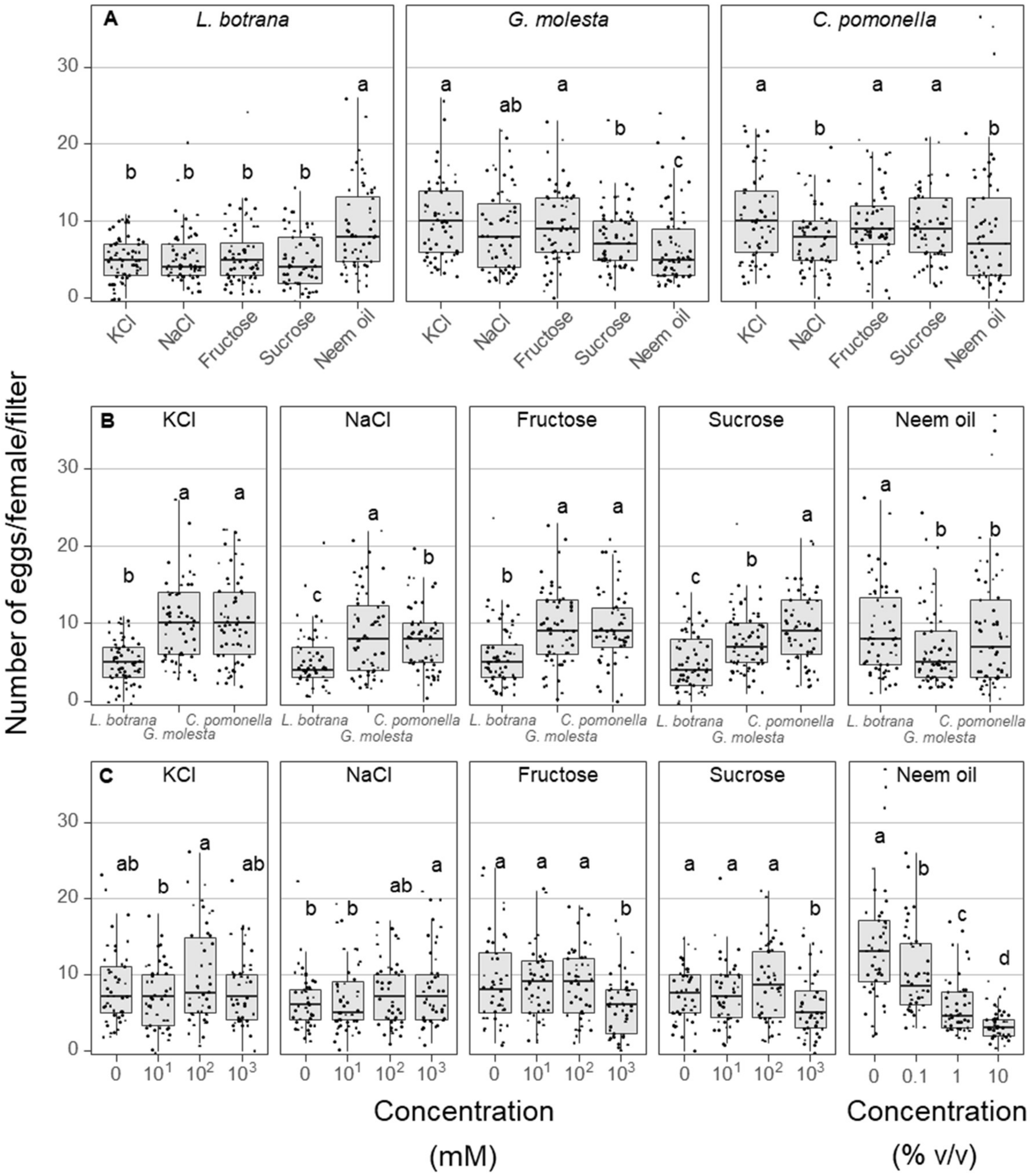


Figure 2. Effect of species*stimulus (A), stimulus*species (B) and stimulus*concentration (C) on the number of eggs laid by female *L. botrana, G. molesta* and *C. pomonella* on filter papers loaded with different concentrations of salts, sugars or neem oil. Within each plot, a different letter among treatments indicates a significant difference (Tukey´s test, $P < 0.05$). Box plots show median (horizontal line), first and third quartile (box) and 1.5*inter-quartile range (vertical bars).

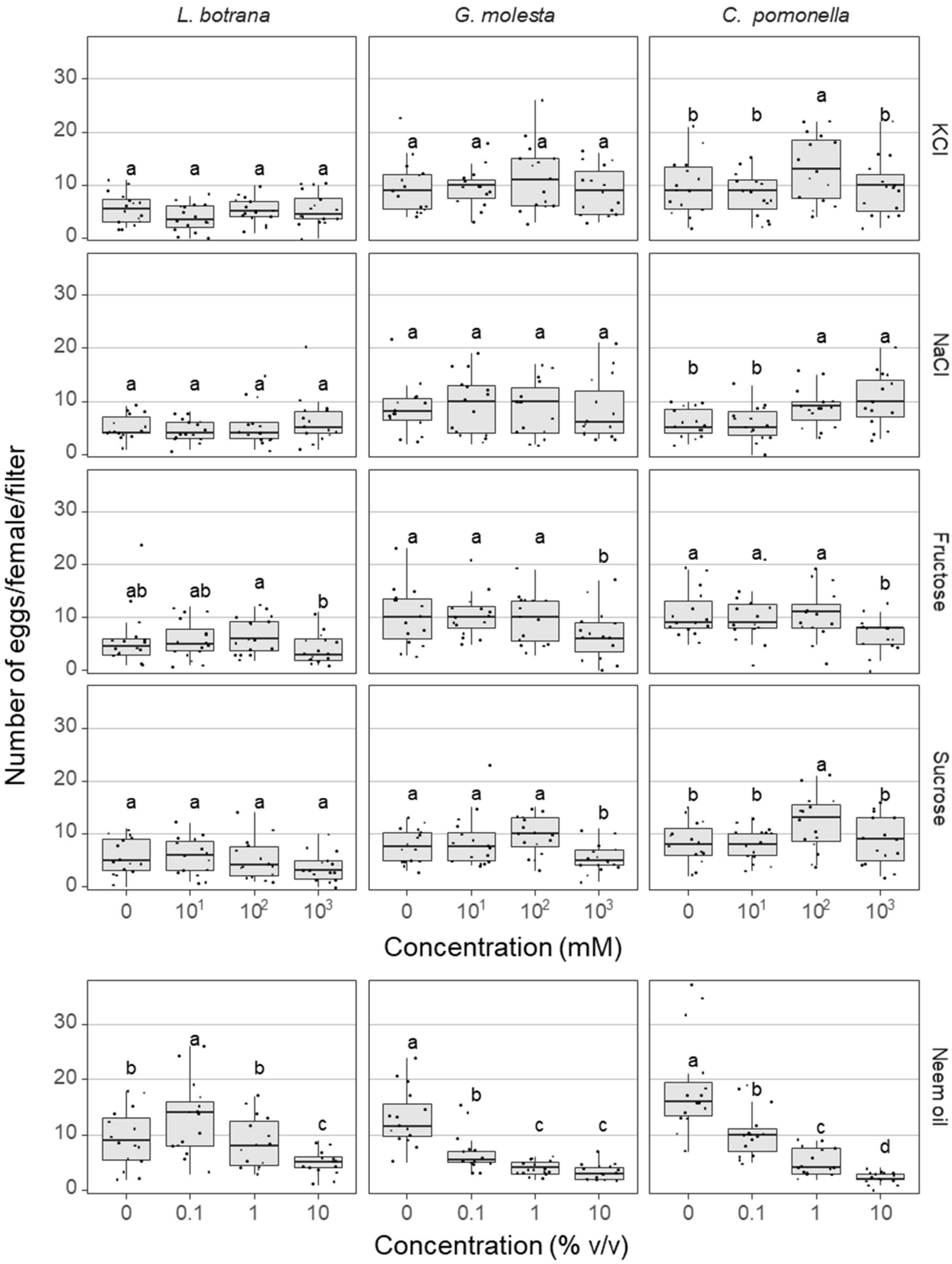


Figure 3. Effect of species*stimulus*concentration on the number of eggs laid by female *L. botrana, G. molesta* and *C. pomonella* on filter papers loaded with different concentrations of salts, sugars or neem oil. Within each plot, a different letter among treatments indicates a significant difference (Tukey´s test, P < 0.05). Box plots show median (horizontal line), first and third quartile (box) and 1.5*inter-quartile range (vertical bars).

Neem oil received the lowest total number of eggs relative to the other 4 stimuli in *C. pomonella* and *G. molesta* (except for NaCl in *C. pomonella*), while the opposite was true in the *L. botrana* (it laid more eggs in neem oil arenas than in the other treatment arenas) (Fig. 2A and 2B, Table S6). Neem oil had the strongest dose-effect (deterrent), whereas salts and sugars only affected oviposition at the highest dose (sugars deterring and NaCl stimulating, relative to the control) (Fig. 2C, Table S6). These general patterns depended on the species. For example, sucrose and fructose deterred the oviposition of *C. pomonella* and *G. molesta* but not *L. botrana*, and NaCl stimulated the oviposition of *C. pomonella* but not of the other two species (Fig. 3, Table S7). Finally, despite being the strongest deterent in all three species, the lowest neem oil dose stimulated *L. botrana* oviposition (Fig. 3, Table S7), which caused the higher oviposition in neem oil arenas of *L. botrana* over *C. pomonella* and *G. molesta* described above (Fig. 2B, Table S7).

Discussion

Oviposition arenas

Several oviposition arena arrangements were tried before settling on a definitive one. It is pertinent to describe this process as it reveals peculiarities about the oviposition behaviour of each species. The two aspects that had to be considered in the design of the oviposition arenas were: 1) choosing a suitable oviposition substrate material, and 2) discouraging oviposition on the remaining areas of the arena. Moths tend to oviposit on smooth surfaces (Thompson & Pellmyr 1991, Markheiser *et al.* 2018) so two types of materials are commonly used in oviposition tests: impermeable materials made of plastic, glass or metal (e.g. Maher & Thiery 2004), or smooth but porous materials such as paper (e.g. Grant *et al.* 2000). The plant surface is covered by waxes, so waxed kitchen paper (the oviposition substrate in our rearing facility) was our first choice. Stimulus was applied with a domestic atomizer, but upon drying on the waxed paper we observed that the highest sugar concentrations formed unevenly distributed solid droplets of uneven sizes, much smaller than the insects, but changed the texture of the substrate. Furthermore, the droplets were hygroscopic so their consistency varied from solid to sticky with ambient humidity. Because insects sense microscopic variations in surface texture (Al Bitar *et al.* 2010, Sanchez-Alcañiz *et al.* 2017), we opted for a porous material that did not change its mechanical properties with the stimulus. Preliminary observations indicated that females laid as many eggs on untreated filter paper as they did on waxed paper, so filter paper was chosen as the oviposition substrate. The number of eggs that females laid on the filter paper at the end of the study was similar to what has been reported using other studies (Rid *et al.* 2018, Da Silva *et al.* 2011, Weissling *et al.* 1996), confirming the suitability of filter paper as oviposition substrate for these species. Porous substrates such as filter paper have the advantage, over non-porous ones, that the test stimulus diffuses evenly. Yet, a disadvantage of filter paper over impermeable substrates is that the stimulus moves inside and outside, so the actual concentration of stimulus available to the insect sensory organs per surface area can only be guessed. In this regard, impermeable substrates provide a closer estimation of the stimulus available to the insect.

The second aspect to take into account is that to maximize oviposition on the target substrate the remaining of the arena (which is usually made of plastic or other smooth materials) needs to be made of a material that discourages oviposition (e.g. Curtis *et al.* 1990, Grant *et al.* 2000, Maher *et al.* 2004). To this end, in our first setup the oviposition cage walls were lined with black felt. *L. botrana* and *C. pomonella* laid most of the eggs on the filter paper (which was placed over the felt), but *G. molesta* laid a significant number of eggs (11%, N = 11) on the felt. In order to reduce oviposition outside the target substrate, felt was replaced by kitchen scourer, which has a significantly more intricate surface and reduced significantly the number of eggs laid by *G. molesta* outside the target substrate. Yet, with this new setup, both *G. molesta* and *L. botrana* laid a significant number of eggs on the lid (30% and 11%, N = 10 and 9, respectively), which at this initial stage was covered with polyester organza. The organza was replaced with nylon mosquito screen and now *G. molesta* and *C. pomonella* laid most of the eggs on the filter paper (97% and 99.9%, N = 10 and 8, respectively), but *L. botrana* laid a sizable number of eggs on the mosquito screen (18%, N = 10). Because we had observed that *L. botrana* had a propensity to lay eggs on the ceiling of the cages, regardless of the material, we inverted the vertical position of the *L. botrana* arenas and placed the filter paper on the ceiling while the lid sat at the bottom, and with this last modification *L. botrana* laid 88% of eggs on the target substrate (N = 4). As a final improvement, we lined the ceiling of the *L. botrana* arenas with black felt in order to detect any eggs that they laid just outside the filter paper.

Rid *et al.* (2018) found that *L. botrana* laid 72% of the eggs on the ceiling of small (5-cm diameter) glass plates, but because the surface was treated with stimuli it cannot be concluded that *L. botrana* had a tendency to lay eggs on the ceiling. However, Markeiser *et al.* (2018, Figure 5) made an interesting observation regarding the location and shape of the oviposition substrate on *L. botrana* and *Eupoecilia ambiguella* (Hübner) (Lepidotera: Tortricidae, another grape pest) oviposition. Both species preferred to lay eggs on a convex-shaped (over a concave-shaped) surface if it is placed on the ceiling of the arena, while it is avoided if on the floor, except that when it is shaded *E. ambiguella* (nor *L. botrana*) prefers it. Differences in oviposition behaviour among species under artificial conditions, such as laboratory arenas, probably reflect specific host adaptations by insects, such as the plant smoothness, location of different organs and spatial structure. A better understanding of oviposition behaviour may help design better oviposition test arenas. Using artificial plant models, Foster and Howard (1998) showed that *E. postvittana* females prefer to lay eggs on the leaves that have a simulated midrib vein made out of wax. They also showed that females lay more eggs on the dummy plants that have a denser leaf array. Physical cues, such as stem diameter and distance between leaves and stem affect oviposition choices in the noctuid *Busseola fusca* (Fuller) (Calatayud *et al.* 2008). Our test species are adapted to lay eggs on specific plant parts that vary seasonally. *L. botrana* lays eggs on the bracts of flowering grape bunches in the first generation, but on following generations they lay eggs on the fruit (Benelli *et al.* 2023). *G. molesta* lays eggs mainly on leafs (Russell 1986) and *C. pomonella* oviposit mainly on leafs and less on branches or fruits (Curtis 1990, Wearing 2016). Female moths walk on the substrate until they find a suitable place to lay eggs, and in the process they gather information about the size, shape, texture and spatial structure of the host, as well as its odour, colour and taste. It has been suggested that the good attachment of *C. pomonella* to very smooth surfaces is an adaptation for choosing most favourable substrates for oviposition (Al Bitar *et al.* 2010). Oviposition arenas provide an oversimplified environment where many of these natural cues are either absent or greatly modified. Yet, these arenas offer a convenient tool to screen many gustatory compounds under controlled conditions and provide information for further tests under more natural conditions, in a similar way as the wind tunnel and olfactometer serve to screen volatile compounds. In addition, our simplified oviposition arenas allow for multiple choice tests and compare several stimuli simultaneously, or to build dose-response curves which are a lot more informative than single-dose tests, as shown by the response of *L. botrana* to neem oil.

Ecological relevance of taste stimuli

Sugars and salts are present on all living tissues, and together with bitter and water sensation constitute the four fundamental taste categories in most insect gustatory sensilla (Marion-Poll 2020). Insects sense other compounds with gustatory sensilla that are difficult to fit in these 4 categories, such as carboxylic and nucleic acids and cuticular hydrocarbons (King & Gunathunga 2023b). Tastant molecules are not sensed in isolation but as mixtures, and this combination is what elicits behavioural responses under natural conditions (Marion-Poll 2020). In our tests, single sugar and salt stimuli altered oviposition behaviour, which is relevant given their commonality and that they were tested in isolation. Future test should explore the effect of compound mixtures or of these compounds on natural substrate.

Salts are a limiting resource to most organisms, including phytophagous insects, and adults of some lepidopteran species actively seek salts on minerals or even faeces, urine or tears (Mitchell *et al.* 2019). Ingesting salts may serve the nutritional requirements of adults (Ravenscraft & Boggs 2016), but whether females use salts to choose hosts larval hosts is practically unexplored. Maher *et al.* (2006) showed that KCl deterred oviposition in *L. botrana*, while we found the opposite effect. Laboratory and field tests with Na-enriched host plants showed no oviposition effects in two butterfly species, even when the concentration was potentially toxic to the larvae (Mitchell *et al.* 2019). Certainly, when salt levels are toxic females may choose not to oviposit, and this may be the reason why salts deter mosquito oviposition (Day 2016).

Sugar content on fruit and leaf surface is little explored, on apples sugars were detected on water surface washing of fruits and leaves (Lombarkia & Derridj 2002) Sugars could guide oviposition-site selection for these species in the different host phenology stages. Whereas *C. pomonella* larvae feed only on the fruit of their host plants (Barnes 1991), *G. molesta* larvae feed on new shoots on the first generation (when fruits are still absent), and on fruits on subsequent generations (Rothschild & Vickers 1991). *L. botrana* larvae feed on flowers, green berries or ripe berries of grapevines as they become available on the first, second and third moth generations, respectively (Benelli *et al.* 2023). Thus, sugar content on plant surface could help females to identify the best oviposition substrate in each generation. Tarsal contact-chemoreceptor sensilla of *L. botrana* are sensitive to fructose and sucrose, and females lay more eggs on substrates treated with fructose and glucose (Maher *et al.* 2006). Derridj *et al.* (1986) showed that *Ostrinia nubilalis* (Hübner) females prefer to lay eggs on *Zea mays* (L.) with a higher content in free sugars (glucose, sucrose, fructose) induced by maleic hydrazide. Since this increase happened on the internal plant tissues it is not clear how the females had access to this information. However, because insect gustatory sensilla typically house four cells that detect water and sugars, salts, and bitter substances, detecting compounds belonging to these taste valences may be unavoidable, and thus these stimuli may eventually influence behaviour under laboratory condition, even if they are not particularly relevant under natural conditions where other gustatory stimuli are present. Anyway, host-plant sugars alter the oviposition behaviour of *C. pomonella* (Lombarkia & Derridj 2008), and altering their normal distribution may be used on its control (Arnault *et al.* 2016).

Neem oil contains at least 100 biologically active compounds and affects diverse aspects of insect behaviour and physiology, including oviposition (Mordue & Blackwell 1993, Campos *et al.* 2016, Kumari & Kaushik 2016). Seljåsen and Meadow (2006) showed a 50% reduction in the number of eggs laid by *Mamestra brassicae* (L.) on cabbage leaves treated with 0.5% neem oil. Bruce *et al.* (2004) report dramatic reductions in the number of eggs batches and eggs laid by a noctuid and a pyralid moth, already from the lowest dose on tested on the plant. These tests, as ours, and many other that test the effect of neem oil on egg laying, involve contact between females and neem oil. There is physiological evidence that neem oils acts by contact. since neem oil, or related terpenoids, appear to inhibit the electrophysiological response of gustatory sensilla in the labial palps of termites (Ohmura *et al.* 2006) and the tarsal sensilla of *Plutella xyllostella* (L.)(Qiu *et al.* 1998). However, some neem oil components are volatile and it is possible that the effect we observed on oviposition could be mediated by olfaction instead of taste. Several insect clades, including leafhoppers (Saxena & Basit 1982), mosquitoes (Su & Mulla 1999) and lepidoptera (Gajmer *et al.* 2002), respond to neem oil volatiles. We antennoctomized females to test the effect of neem oil volatiles, but they did not outlive the 24 oviposition test period required.

The increased oviposition of *L. botrana* at the lowest neem oil dose (0.1%) was unexpected. Preliminary observations indicate that an even lower concentration (0.01%) did not increase oviposition further. Grant *et al.* (2000) report a very interesting observation, somewhat related to ours, where nonanoic acid (a 9-carbon carboxylic acid) loaded on filter paper in a petri dish strongly stimulated oviposition by *C. fumiferana* at the low of two doses tested (78 nmol/cm2) whereas it strongly deterred it at a 10-times higher dose (780 nmol/cm2). They observed the opposite effect of low and high dose (although not so pronounced) with decanoic acid too, but it did not occur with the other carboxylic acids tested (from C-4 to C-28 carbon length) (Grant *et al.* 2000). This highlights the importance of testing mode than one dose of test compounds. Many plant-derived behaviourally-active insect gustatory stimuli, such as neem oil, are non polar. For example, *L. botrana* oviposition behaviour is deterred by the lipids presents on its own eggs and the skin of grapes (Gabel & Thiéry 1996, Rid *et al.* 2018). *Nicotiana tabacum* (L.) methanol extracts deter *G. molesta* oviposition when applied to peach twigs (Sarker & Lim 2018). GRNs on the legs of L. botrana respond to sweets (Maher *et al.* 2006), and we have examined the responses of antennal and labial palp GRNs to the two salts and sugars (Amat *et a*l. 2022). It would be interesting to determine the response of GRNs to neem oil and its constituents too. However, unlike olfaction, recording the electrophysiological responses of GRNs to non-polar compounds is challenging because dissolving non-polar molecules in the electrophysiological saline solution requires the use of non-polar solvents which may alter the normal functioning of the neurons (Marion-Poll 2020).

Mechanism

The oviposition tests reveal that females detect the test compounds, but not which body part bears the gustatory sensilla involved in the detection. Gustatory sensilla occur on head appendages, mouthparts, legs and ovipositor, so any of these organs could be involved (Mbata *et al.* 1997). Female butterflies reportedly drum on the plant substrate with their forelegs to gather chemical information from the plant, and some moths do that too (Wallace *et al.* 2004). Female lepidoptera such as *O. nubilalis* or *Papilio* have more taste sensilla on the prothorathic legs possibly allowing them to analyse the surface of the leaves on which they will lay eggs (Marion-Poll 2020). Female moths and butterflies drag their ovipositor over the plant substrate to sample its chemical composition (Ramaswamy 1988, Cury *et al.*, 2019). Taste sensilla have been characterized in a number of moth species (e.g. Waladde 1983, Maher *et al.* 2006, Seada *et al.* 2016). Moths have been seen tapping the substrate with their antennae while ovipositing (Hattori 1986). Few accounts (e.g. Myers *et al.* 1981) discuss the employment of mouth parts or labial palpi in the context of moth egg laying.

Conclusions

Our study increases the list of oviposition-modifying gustatory compounds described for tortricid moths, and it shows that neem oil is a potent oviposition deterrent. Making a suitable oviposition arena is essential in order to test different compounds simultaneously on several moth species, and testing several doses was fundamental to reveal subtle differences, such as the unexpected increase in oviposition with the lowest neem oil dose by *L. botrana*. Carrying out tests under more natural conditions is needed to demonstrate the potential of these compounds in pest control, as has been shown for sugars in *C. pomonella* by Lombarkia and colleagues (reviewed in Knight *et al.* 2019). More research is needed to identify a larger number of compounds with potential gustatory effects, and also to determine the mechanism of sensory detection, specifically for non-polar compounds.

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Supplementary material

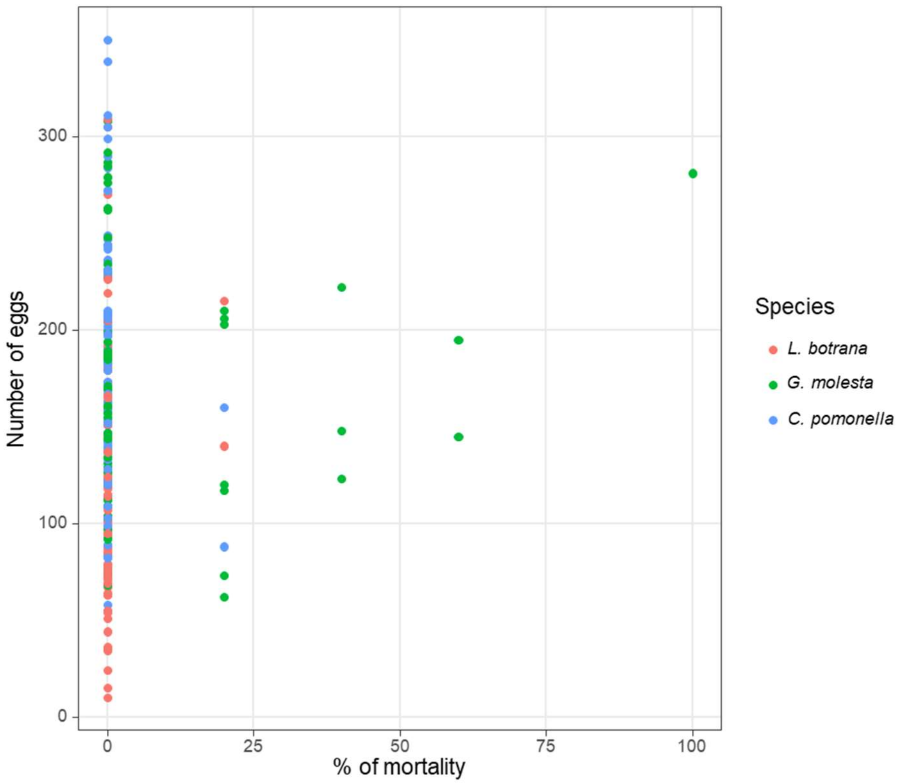

Figure S1. Relation between the number of eggs registered in each arena in function of the dead and potentially moribund females.

Table S1. Stimuli source and final concentration per surface area of filter paper.

Stimulus	CAS	Sigma	Concentration mM (sugars and salts), v/v (neem oil)	Amount in filter paper µg/cm^2 (sugars and salts), µl/cm^2 (neem oil)
KCl	7447-40-7	P3911-500G-M	10	9.94
			100	99.37
			1000	993.75
NaCl	7647-14-5	S7653-1KG	10	7.79
			100	77.90
			1000	779.01
Fructose	50-48-7	F0127-1KG	10	24.02
			100	240.15
			1000	2401.53
Sucrose	57-50-1	S9378-1KG	10	45.63
			100	456.29
			1000	4562.86
Neem oil	-		0,1	1.33
			1	13.33
			10	133.30

Table S2. Shapiro test for the of normality of the distribution of the number of eggs (P < 0.05 = not normally distributed)

Species	Stimulus	Concentration	p-value
L. botrana	KCl	0	0.26
L. botrana	KCl	10	0.39
L. botrana	KCl	100	0.81
L. botrana	KCl	1000	0.13
L. botrana	NaCl	0	0.07
L. botrana	NaCl	10	0.60
L. botrana	NaCl	100	0.01
L. botrana	NaCl	1000	0.01
L. botrana	Fructose	0	0.00
L. botrana	Fructose	10	0.13
L. botrana	Fructose	100	0.10
L. botrana	Fructose	1000	0.06
L. botrana	Sucrose	0	0.20
L. botrana	Sucrose	10	0.47
L. botrana	Sucrose	100	0.06
L. botrana	Sucrose	1000	0.24
L. botrana	Neem oil	0	0.51
L. botrana	Neem oil	0.1	0.51
L. botrana	Neem oil	1	0.12
L. botrana	Neem oil	10	0.91
G. molesta	KCl	0	0.07
G. molesta	KCl	10	0.81
G. molesta	KCl	100	0.29
G. molesta	KCl	1000	0.11
G. molesta	NaCl	0	0.06
G. molesta	NaCl	10	0.18
G. molesta	NaCl	100	0.17
G. molesta	NaCl	1000	0.01
G. molesta	Fructose	0	0.42
G. molesta	Fructose	10	0.15
G. molesta	Fructose	100	0.25
G. molesta	Fructose	1000	0.40
G. molesta	Sucrose	0	0.34
G. molesta	Sucrose	10	0.01
G. molesta	Sucrose	100	0.62
G. molesta	Sucrose	1000	0.53
G. molesta	Neem oil	0	0.28
G. molesta	Neem oil	0.1	0.00
G. molesta	Neem oil	1	0.10
G. molesta	Neem oil	10	0.02
C. pomonella	KCl	0	0.70
C. pomonella	KCl	10	0.60
C. pomonella	KCl	100	0.19
C. pomonella	KCl	1000	0.42
C. pomonella	NaCl	0	0.15
C. pomonella	NaCl	10	0.88
C. pomonella	NaCl	100	0.38
C. pomonella	NaCl	1000	0.78
C. pomonella	Fructose	0	0.04
C. pomonella	Fructose	10	0.86
C. pomonella	Fructose	100	0.62
C. pomonella	Fructose	1000	0.79
C. pomonella	Sucrose	0	0.96
C. pomonella	Sucrose	10	0.52
C. pomonella	Sucrose	100	0.61
C. pomonella	Sucrose	1000	0.35
C. pomonella	Neem oil	0	0.01
C. pomonella	Neem oil	0.1	0.06
C. pomonella	Neem oil	1	0.03
C. pomonella	Neem oil	10	0.11

Table S3. Mortality in the oviposition arenas for *C. pomonella, G. molesta* and *L. botrana.*

Species	Stimulus	Number of females	Number morbid/ dead	% morbid/ dead	Number of arenas with morbid/dead
L. botrana	KCl	80	0	-	0
L. botrana	NaCl	75	1	1.33	1
L. botrana	Fructose	80	0	-	0
L. botrana	Sucrose	75	1	1.33	1
L. botrana	Neem oil	75	0	-	0
G. molesta	KCl	75	1	1.33	1
G. molesta	NaCl	74	7	9.46	5
G. molesta	Fructose	74	4	5.41	2
G. molesta	Sucrose	80	12	15.00	5
G. molesta	Neem oil	80	0	-	0
C. pomonella	KCl	75	1	1.33	1
C. pomonella	NaCl	72	0	-	0
C. pomonella	Fructose	73	0	-	0
C. pomonella	Sucrose	72	1	1.39	1
C. pomonella	Neem oil	75	0	-	0

Table S4. Model selection for the number of eggs laid showing Akaine information criterion (AIC) and P-value of pairwise model comparisons. Factors: Species, stimulus and concentration.

Model type	AIC	DF model	DF residual deviance	Residual deviance	LRT P-value
Null	6260	0	915	2872	-
Main effects	5910	9	906	2504	<0.001
Main effects and 2nd-order interactions	5456	35	880	1998	<0.001
Main effects, 2nd- and 3rd-order interactions	5386	59	856	1880	<0.001

Table S5. Observed and estimated mean number of eggs per species and stimulus. Corresponds with Figure 2 in the main text.

	mean SEM				
Species	Observed		Estimated		
L. botrana	6.00	0.24	5.74	0.14	b
G. molesta	8.51	0.28	8.15	0.17	a
C. pomonella	9.17	0.32	8.51	0.18	a
Stimulus					
KCl	8.34	0.38	7.94	0.21	a
NaCl	7.28	0.34	7.04	0.20	b
Fructose	8.13	0.35	7.84	0.21	a
Sucrose	7.40	0.32	7.04	0.20	b
Neem oil	8.25	0.47	6.99	0.21	b
Concentration					
Control	8.99	0.38	8.45	0.20	a
Low	7.99	0.31	7.62	0.19	b
Medium	8.26	0.33	7.80	0.19	ab
High	6.29	0.29	5.83	0.17	c

Table S6. Observed and predicted number of eggs laid by females for several treatment combinations: (A) species within stimulus, (B) stimulus within species, and (C) concentration within stimulus.

A

		Observed		Estimated		
Species	Stimulus	mean	SEM	mean	SEM	
L. botrana	KCl	5.05	0.34	4.99	0.28	b
L. botrana	NaCl	5.38	0.43	5.35	0.30	b
L. botrana	Fructose	5.67	0.50	5.58	0.30	b
L. botrana	Sucrose	5.03	0.44	4.96	0.29	b
L. botrana	Neem oil	8.97	0.73	8.46	0.39	a
G. molesta	KCl	9.97	0.65	9.92	0.41	a
G. molesta	NaCl	8.75	0.68	8.74	0.38	ab
G. molesta	Fructose	9.35	0.63	9.22	0.40	a
G. molesta	Sucrose	7.84	0.49	7.67	0.35	b
G. molesta	Neem oil	6.78	0.62	5.87	0.32	c
C. pomonella	KCl	10.23	0.72	10.08	0.41	a
C. pomonella	NaCl	7.70	0.53	7.48	0.36	b
C. pomonella	Fructose	9.53	0.59	9.37	0.40	a
C. pomonella	Sucrose	9.30	0.58	9.19	0.39	b
C. pomonella	Neem oil	9.10	1.04	6.88	0.39	a

B

		Observed		Estimated		
Stimulus	Species	mean	SEM	mean	SEM	
KCl	*L. botrana*	5.05	0.34	4.99	0.28	b
KCl	*G. molesta*	9.97	0.65	9.92	0.41	a
KCl	*C. pomonella*	10.23	0.72	10.08	0.41	a
NaCl	*L. botrana*	5.38	0.43	5.35	0.30	c
NaCl	*G. molesta*	8.75	0.68	8.74	0.38	a
NaCl	*C. pomonella*	7.70	0.53	7.48	0.36	b
Fructose	*L. botrana*	5.67	0.50	5.58	0.30	b
Fructose	*G. molesta*	9.35	0.63	9.22	0.40	a
Fructose	*C. pomonella*	9.53	0.59	9.37	0.40	a
Sucrose	*L. botrana*	5.03	0.44	4.96	0.29	c
Sucrose	*G. molesta*	7.84	0.49	7.67	0.35	b
Sucrose	*C. pomonella*	9.30	0.58	9.19	0.39	a
Neem oil	*L. botrana*	8.97	0.73	8.46	0.39	a
Neem oil	*G. molesta*	6.78	0.62	5.87	0.32	b
Neem oil	*C. pomonella*	9.10	1.04	6.88	0.39	b

C

		mean SEM				
Stimulus	Concentration	Observed		Estimated		
KCl	0	8.35	0.73	8.14	0.43	ab
KCl	10	7.20	0.62	6.77	0.40	b
KCl	100	9.87	0.93	9.24	0.47	a
KCl	1000	7.96	0.70	7.78	0.42	ab
NaCl	0	6.51	0.56	6.34	0.38	b
NaCl	10	6.67	0.66	6.38	0.38	b
NaCl	100	7.64	0.67	7.47	0.41	ab
NaCl	1000	8.29	0.78	8.14	0.43	a
Fructose	0	9.00	0.81	8.79	0.44	a
Fructose	10	8.65	0.68	8.43	0.43	a
Fructose	100	9.00	0.67	8.88	0.44	a
Fructose	1000	5.87	0.57	5.76	0.36	b
Sucrose	0	7.33	0.53	7.21	0.40	a
Sucrose	10	7.50	0.60	7.35	0.40	a
Sucrose	100	8.89	0.74	8.37	0.44	a
Sucrose	1000	5.89	0.59	5.55	0.36	b
Neem oil	0	13.70	1.12	13.18	0.55	a
Neem oil	0.1	9.91	0.81	9.64	0.46	b
Neem oil	1	5.89	0.54	5.62	0.36	c
Neem oil	10	3.50	0.28	3.34	0.28	d

Table S7. Pairwise comparison of the number of eggs laid by individual females in a oviposition substrate (filter paper). Observed data and estimated values from the GLM model are presented. Letters in the right column indicate significant differences among the species or stimuli (Tukey´s test, p < 0.05 after GLM). Corresponds with Figure 3 in the main text.

			mean SEM				
Stimulus	Species	Concentracion	Observed		Estimated		
KCl	*L. botrana*	0	0.29	0.02	0.29	0.02	a
KCl	*L. botrana*	10	0.19	0.02	0.19	0.02	b
KCl	*L. botrana*	100	0.26	0.02	0.26	0.02	ab
KCl	*L. botrana*	1000	0.27	0.02	0.27	0.02	a
KCl	*G. molesta*	0	0.24	0.02	0.24	0.02	a
KCl	*G. molesta*	10	0.25	0.02	0.25	0.02	a
KCl	*G. molesta*	100	0.28	0.03	0.28	0.02	a
KCl	*G. molesta*	1000	0.22	0.02	0.22	0.02	a
KCl	*C. pomonella*	0	0.24	0.02	0.24	0.02	b
KCl	*C. pomonella*	10	0.20	0.02	0.20	0.02	b
KCl	*C. pomonella*	100	0.33	0.02	0.33	0.02	a
KCl	*C. pomonella*	1000	0.23	0.02	0.23	0.02	b
NaCl	*L. botrana*	0	0.25	0.02	0.25	0.02	a
NaCl	*L. botrana*	10	0.23	0.02	0.23	0.02	a
NaCl	*L. botrana*	100	0.25	0.02	0.25	0.02	a
NaCl	*L. botrana*	1000	0.28	0.03	0.28	0.02	a
NaCl	*G. molesta*	0	0.25	0.02	0.25	0.02	a
NaCl	*G. molesta*	10	0.27	0.03	0.27	0.02	a
NaCl	*G. molesta*	100	0.24	0.03	0.24	0.02	a
NaCl	*G. molesta*	1000	0.24	0.02	0.24	0.02	a
NaCl	*C. pomonella*	0	0.20	0.02	0.20	0.02	b
NaCl	*C. pomonella*	10	0.19	0.02	0.19	0.02	b
NaCl	*C. pomonella*	100	0.28	0.02	0.28	0.02	a
NaCl	*C. pomonella*	1000	0.33	0.03	0.33	0.02	a
Fructose	*L. botrana*	0	0.25	0.02	0.25	0.02	ab
Fructose	*L. botrana*	10	0.25	0.02	0.25	0.02	ab
Fructose	*L. botrana*	100	0.31	0.02	0.31	0.02	a
Fructose	*L. botrana*	1000	0.19	0.03	0.19	0.02	b
Fructose	*G. molesta*	0	0.27	0.03	0.27	0.02	a
Fructose	*G. molesta*	10	0.28	0.03	0.28	0.02	a
Fructose	*G. molesta*	100	0.26	0.03	0.26	0.02	ab
Fructose	*G. molesta*	1000	0.19	0.03	0.19	0.02	b
Fructose	*C. pomonella*	0	0.28	0.02	0.28	0.02	a
Fructose	*C. pomonella*	10	0.28	0.03	0.28	0.02	a
Fructose	*C. pomonella*	100	0.27	0.03	0.27	0.02	a
Fructose	*C. pomonella*	1000	0.17	0.02	0.17	0.02	b
Sucrose	*L. botrana*	0	0.28	0.02	0.28	0.02	a
Sucrose	*L. botrana*	10	0.28	0.02	0.28	0.02	a
Sucrose	*L. botrana*	100	0.27	0.03	0.27	0.02	a
Sucrose	*L. botrana*	1000	0.17	0.02	0.17	0.02	b
Sucrose	*G. molesta*	0	0.25	0.03	0.25	0.02	ab
Sucrose	*G. molesta*	10	0.27	0.02	0.27	0.02	a
Sucrose	*G. molesta*	100	0.30	0.02	0.30	0.02	a
Sucrose	*G. molesta*	1000	0.18	0.02	0.18	0.02	b
Sucrose	*C. pomonella*	0	0.23	0.02	0.23	0.02	b
Sucrose	*C. pomonella*	10	0.22	0.01	0.22	0.02	b
Sucrose	*C. pomonella*	100	0.32	0.02	0.32	0.02	a
Sucrose	*C. pomonella*	1000	0.23	0.03	0.23	0.02	b
Neem oil	*L. botrana*	0	0.26	0.03	0.26	0.02	b
Neem oil	*L. botrana*	0.1	0.35	0.02	0.35	0.02	a
Neem oil	*L. botrana*	1	0.24	0.02	0.24	0.02	b
Neem oil	*L. botrana*	10	0.15	0.02	0.15	0.02	c
Neem oil	*G. molesta*	0	0.47	0.02	0.47	0.02	a
Neem oil	*G. molesta*	0.1	0.24	0.01	0.24	0.02	b
Neem oil	*G. molesta*	1	0.16	0.01	0.16	0.02	c
Neem oil	*G. molesta*	10	0.13	0.01	0.13	0.02	c
Neem oil	*C. pomonella*	0	0.50	0.02	0.50	0.02	a
Neem oil	*C. pomonella*	0.1	0.28	0.02	0.28	0.02	b
Neem oil	*C. pomonella*	1	0.16	0.02	0.16	0.02	c
Neem oil	*C. pomonella*	10	0.07	0.01	0.07	0.02	d

CHAPTER 4

Gustatory function of sensilla chaetica on the labial palps and antennae of three tortricid moths (Lepidoptera: Tortricidae)

Abstract

In adult Lepidoptera the labial palps are best known for their role in CO_2 detection, but they can also bear sensilla chaetica which function is unknown. The number and distribution of sensilla chaetica in labial palps was studied using bright field microscope. To determine if sensilla have a gustatory function, we performed single sensillum electrophysiology recordings from palp and antennal sensilla of adult moths of *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis and Shieffermüller). Each sensillum was stimulated with 3 doses of one of four test stimulus (sucrose, fructose, KCl and NaCl). Overall, responses (spikes/s^{-1}) increased with dose, and were higher in the palps than in the antennae, and higher to sugars than to salts. With sugars the response increased with concentration in the palp but not in the antenna. With salts there was a drop in response at the intermediate concentration. The number and position of sensilla chaetica on labial palps was variable among individuals. Sensilla were located in the most exposed areas of the palp. Differences in the distribution were detected between species. Such differences among species and between palps and antenna suggest that taste sensilla on the palps have an unforeseen role in adaptation.

Keywords: taste; electrophysiology; sucrose; fructose; NaCl; KCL; Tortricidae

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Introduction

Reception of gustatory stimuli (also referred to as gustation, taste or contact chemoreception) plays a vital role in many aspects of insect fitness, such as in food selection and oviposition choices (Cobb *et al.* 2009, Marion-Poll 2020). Tastants are detected by gustatory receptor neurons (GRNs), which are typically present in groups of 2 to 4 inside gustatory sensilla (Marion-Poll 2002). Gustatory sensilla have been found in almost any part of the adult body, especially those in direct contact with taste stimuli, such as the tarsi, mouthparts, food channel, ovipositor and antennae (Marion-Poll 2020). There are different morphological types of gustatory sensilla (Mitchell *et al.* 1999, Marion-Poll 2020). Sensilla chaetica are long, hair-like structures that can be differentiated from similar sensilla (e.g. sensilla trichoidea) by having thicker walls (Hallberg 1981, Mitchell *et al.* 1999) The present of a terminal pore in sensilla chaetica is usually associated with gustatory function and a flexible basal socked with tactile function(Mitchell *et al.* 1999). Sensilla chaetica typically have a contact chemoreception function, with two to four GRNs and one mechanosensory neuron which terminates in a tubular body at the base of the sensilla (Hallberg 1981, Koh *et al.* 1995).

Gustation in the mouthparts of adult insects is generally found in the galeae, in the maxillary palps of the maxilla, and in the labial palps of the labium. The labial palps of insects with standard chewing mouthparts (e.g., Coleoptera, Orthoptera) are usually small and they bear gustatory sensilla (Chapman & Ascoli-Christensen 1999, Ohmura *et al.* 2006, Seada & Hamza 2018). In insects with mouthparts modified for sucking and licking (e.g., Diptera, Hemiptera), the labium and associated labial palps retain their taste function (Powell *et al.* 2006, Montell 2009, Barrozo *et al.* 2017, Bestea *et al.* 2021). In Lepidoptera, the galeae have joined in a long and coiled proboscis, and in most lineages the rest of the mouthparts structures are largely reduced, except for the labial palps, which are relatively large and cover most of the front part of the insect head at each side of the proboscis (Krenn 2010). The labial palps of Lepidoptera present a cavity at the tip of the apical segment, the labial-palp pit organ (LPO), which contains sensilla that respond to changes in CO_2 concentration (Guerenstein & Hildebrand 2008). A ventral glomerulus in the antennal lobe receives the input of the CO_2 sensory cells. CO_2 detection may help Lepidoptera in host-plant selection (Guerenstein & Hildebrand 2008). In some moth species, the labial palps bear chaetica-like sensilla (Song *et al.* 2016, Hu *et al.* 2018), which often have a gustatory function in other insect appendages (Hallberg 1981, Koh *et al.* 1995, Mitchell *et al.* 1999). The presence of putative gustatory receptors of the sugar clade in the labial palps of *Helicoverpa armigera* (Hübner) (Guo *et al.* 2018) further indicates the presence of gustation in the labial palps, but as far as we know, there is no physiological evidence that the labial palps of Lepidoptera have a gustatory function. The antennae of Lepidoptera are well known their olfactory function performed by numerous sensilla trichoidea, but they also bear some gustatory sensilla chaetica in each flagellum (Marion-Poll 2020). In noctuid moths the gustatory function of antennal sensilla chaetica has been demonstrated (Calatayud *et al.* 2006, Jørgensen *et al.* 2007, Popescu *et al.* 2013). The gustatory function of sensilla chaetica on the antennae of tortricid moths (Roh *et al.* 2016, Ammagarahalli & Gemeno 2014, Godoy *et al.* 2019) remains to be shown.

The objective of this study is to determine if the sensilla present of the surface of the labial palp of moths respond to gustatory stimuli. To this end we performed electrophysiological recordings from adults of three tortricid moths, *Cydia pomonella* (L.)*, Lobesia botrana* (Denis and Shiffermüller) and *Grapholita molesta* (Busck), which are key pest species of fruit trees and vines worldwide (Ioriatti *et al.* 2011, Knight *et al.* 2019). Two sugars (fructose and sucrose) and two salts (KCl and NaCl) were tested at 3 concentrations. In addition, we tested the response of sensilla chaetica in the antennae. In order to determine the biological relevance of sugars in these species we also studied their effect on the adult longevity.

Material and methods

Insects

Larvae were reared on a semi-artificial diet modified from that of Ivaldi-Sender (1974) at 25°C under a 16:8 light:dark photoperiod. Pupae were sexed and kept in separate environmental chambers with unrestricted access to a 10% sucrose in water dilution. Before electrophysiological recordings, adults were anaesthetized with CO_2 to restrain them inside a modified pipette tip, with their head antennae and palps fixed with melted dental wax (ref: R3712-00, Leone s.p.a., Firenze, Italy). All insects tested were between 1 and 3 days old.

Labial palp sensilla

To investigate the location of sensilla chaetica in the labial palps, 10 palpi from different individuals were observed for each species and sex in a bright field microscope. We removed the scales, cleared the cuticle and stained the preparations according to George and Nagy (1984). Detached heads were boiled in 10% KOH until the scales fell off and then washed in distilled water and cleared in 2.5% bleach until they became transparent. After a second wash in distilled water, they were immersed in 0.5% crystal violet until the sensilla were clearly visible. Samples were placed on a drop of glycerol on a microscope slide under a cover slip.

The maximum length and width of the apical segment of the labial palp was measured with an eyepiece micrometer. To assess sensilla position, the apical segment was divided in 8 areas (Supplementary Fig. S1) resulting from the intersection of the 3 axes of symmetry (sagittal, transversal and frontal), and the number of sensilla in each area was recorded.

Electrophysiology

Gustatory stimuli [NaCl (CAS: 7647-14-5, ref: S7653-1KG), KCl (CAS:7647-14-5, ref: S7653-1KG), sucrose (CAS: 57-50-1, ref: S9378-1KG) and fructose (CAS: 50-48-7, ref: F0127-1KG), Sigma-Aldrich, Madrid, Spain] were diluted in deionized water. Sucrose and fructose dilutions contained 1 mM NaCl as electrolyte. Three concentrations of each stimulus (10, 100, 1000 mM) were prepared in 1 ml aliquots and kept at -20ºC. During the experiments, one aliquot of each concentration was defrosted and kept at 4ºC for up to one week.

For each individual, two to six sensilla chaetica from either the antenna or the apical segment of the labial palp were tested. The antenna was sampled up to the apical segment. On the antenna, sensilla chaetica were differentiated from sensilla trichoidea by their morphology (straighter and thicker), their orientation (perpendicular to the surface) and their optical characteristics (brighter under the stereo microscope). On the outside of the labial palp only sensilla chaetica were present. Only one stimulus was used for a given individual, starting from lowest to highest concentration. The contact with each sensillum was limited to 2 seconds, allowing 3 min between stimulations to avoid adaptation. Sugars stimuli were preceded by stimulation with the electrolyte (1 mM NaCl) as a control.

Electrophysiological recordings from sensilla chaetica were obtained using the tip recording technique (Hodgson *et al.* 1955) Glass capillary recording electrodes (Hirschmann Laborgeräte GmbH & Co, Germany) were pulled to obtain a 3.5 μm wide tip (PP-830, Narishige, Japan), which was filled with the test solution and connected to a 0.5 mm-wide platinum wire, fitted to a preamplifier probe (Taste Probe, Syntech, Germany). The recording electrode was placed over the sensillum tip using a micromanipulator (NMN-25, Narishige, Japan) under a stereomicroscope (Leica M125, objective 2x, oculars 25x, zoom range 0.8-10, Leica microsystems, Spain). The reference electrode was a sharpened tungsten wire (0.125-mm diameter, 99.98% purity, Advent Research Materials Ltd, UK), inserted into the eye with the help of a micromanipulator (UM-3C, Narishige, Japan). The recording electrode was filled with the stimulus solution just before stimulation. When the stimulation sequence from one concentration to the next took longer than two minutes, a new electrode was prepared.

The signal from the recording electrode was pre-amplified (10x) and filtered (10 Hz high-pass) using a Taste Probe amplifier (Marion-Poll & Pers 1996; Syntech, Germany) and further amplified (50x) and filtered (3000 Hz low-pass filter) (AC/DC differential amplifier, A-M systems Inc., WA, USA). The signal was digitalized and analyzed (Micro 1041-3 and Spike2, respectively, Cambridge Electronic Design Limited, UK), and spike detection was performed using dbWave software (Marion-Poll 1996).

Effect of water and sucrose on adult longevity

We determined the effect of water and sugar on the survival of 20-24 adults in order to assess the importance of these stimuli on adult fitness. To this mean, groups of 4 to 9 newly emerged adults of both sexes picked randomly from the colony, were placed in ventilated 100 mL plastic bottles after CO_2 anesthesia. Four bottles were prepared for each treatment: control (dry cotton swab), water: (a cotton swab soaked in distilled water) and sugar (cotton swab soaked in 10% domestic sucrose diluted in distilled water). Cotton was replaced regularly so that it was never dry. Bottles were inspected daily (with sporadic 2 to 3 day gaps for the longest-lived species, *C. pomonella*) until all individuals were dead, dead individuals were scored and removed.

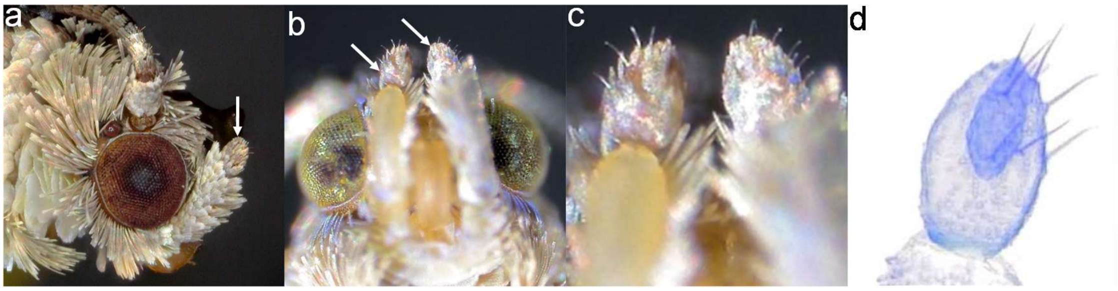

Figure 1. Labial palps of a female *G. molesta*. a) Lateral view, b) ventral view, scales have been partially removed from the right palp, c) close up of "b" showing sensilla chaetica clearly protruding among the scales from the third segment, d) third segment digested with KOH and stained with gencian violet to show the labial pit organ (elongated dark sac inside the palp) and the sensilla chaetica on the surface. White arrows in panels “a” and “b” indicate the apical segment of labial palp.

Data analysis

Statistical analyses were run in R 4.1.2 software (R Core Team, 2022). Generalized lineal models (GLM) were used for all the analyses with specific error families and link functions where needed. Pairwise comparisons used the Tukeys´s test of the package “emmeans”, unless specified otherwise. To analyse length and width of the apical segment of the labial palp a GLM with Gaussian error distribution was used. The total number of sensilla in the apical segment of the labial palps and the distribution of sensilla, within each species, among the delimited areas (Supplementary Fig. S1) was analysed with GLM with Poisson error distribution and a logit link. For spike counts analysis only the response to stimuli, and not to the 1mM NaCl electrolyte, was analysed. A *quasi*-Poisson error distribution was used because overdispersion was detected. A different model was run to compare the number of spikes between each sugar concentration and the electrolyte control using Dunnett test.

Model selection started from the simplest model containing no main effects, then main factors and interactions were added sequentially. For comparison between models, the likelihood ratio test (LRT) and the Akaike information criterion (AIC) were used, and models with lower AIC values and significantly different LRT were selected. The model with the best fit was used to conduct pairwise comparison between relevant groups of significant factors using estimated marginal means.

To check the relation between number of sensilla and the labial palp size, a Pearson correlation test was conducted between total number of sensilla and the surface of the labial palp. The area of the 3rd segment of the labial palp was estimated as the area of a cylinder with diameter equal to the maximum width of the segment and length equal to the length of the segment. The comparison of the sensilla distribution among the areas of a labial palp was conducted with a “vector GLM” using the package “VGAM”, an ANOVA was used to calculate the significance level of species and sexes.

The effect of diet on survival curves was calculated using the Kaplan-Meier method (Kaplan & Meier 1958), and a log-rank test was used to detect overall differences among curves. Log-rank test and Benjamini-Hochberg correction was used for multiple pairwise comparisons among curves.

Results

Labial palp sensilla

The labial palps were composed of three segments densely covered with scales, with sensilla protruding from the scales in the apical segment (Fig. 1). These sensilla have a typical chaetica morphology, they are hair-like structures relatively straighter and more rigid than sensilla trichoidea, and with a flexible socket at the base (Hallberg 1981, Koh *et al.* 1995, Mitchell *et al.* 1999). The second segment was the longest (*G. molesta* 356.5 µm, *L. botrana* 464 µm and *C. pomonella* 636 µm on average), it was curved upwards and projected forward on each side of the head (Fig. 1). The apical segment was oval, between 120 and 237 µm long and 60 to 143 µm wide depending on the species and sex (Table 1). The apical segment was longest in *C. pomonella*, followed by *L. botrana* and *G. molesta* and widest in *C. pomonella* than in the other two species. It was wider and longer in females than in males (Supplementary Table S1). The number of sensilla in the apical segment of the labial palp ranged between 5 and 15 (Table 1), and it was positively correlated with the area of the segment (Person correlation coefficient = 0.73, $t_{58} = 8.15$, $p < 0.001$). In general females had 1.3 more sensilla than males and the number of sensilla was 1.3 times higher in *C. pomonella* than in *G. molesta* (Supplementary Table S2).

Table 1. Dimensions and number of sensilla chaetica on the apical segment of the labial palp.

		Size (*µ*m, Mean ± SEM)		Number		
Species	Sex	Length	Width	Mean ± SEM	Min	Max
L. botrana	Female	237.00 ± 6.01	106.75 ± 1.79	10.00 ± 0.52	7	12
	Male	184.25 ± 2.56	65.00 ± 2.17	7.30 ± 0.37	5	9
G. molesta	Female	148.25 ± 4.79	94.00 ± 4.00	7.80 ± 0.61	5	10
	Male	120.50 ± 1.93	79.25 ± 1.35	7.40 ± 0.54	5	9
C. pomonella	Female	278.25 ± 7.59	142.25 ± 3.86	12.30 ± 0.54	9	15
	Male	202.00 ± 5.55	116.75 ± 2.14	8.70 ± 0.54	6	12

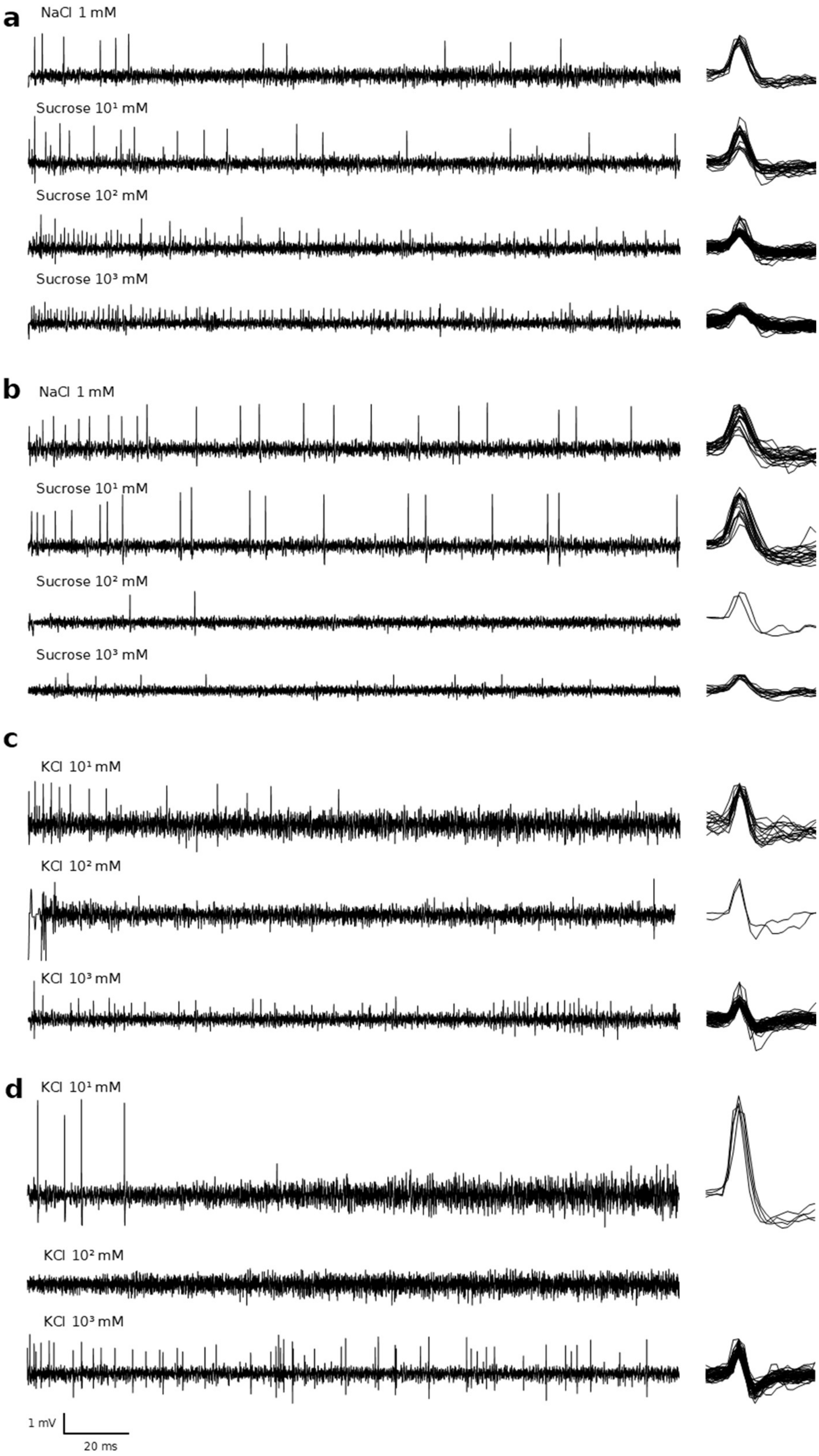

Figure 2. Representative single-sensillum recording traces of sensilla chaetica and on the right superimposed spikes detected in the recording (spike duration 6 ms). Each group (a-d) shows the response of the same sensillum to increasing concentrations of test stimulus. a) Labial palp of *C. pomonella* female, b) antenna of *C. pomonella* male, c) labial palp of *C. pomonella* female, and d) antenna of *L. botrana* male.

The position of the sensilla chaetica in the apical segment of the labial palp was highly variable between individuals and no consistent location could be determined for any sensillum. However, some general patterns could be observed. Sensilla tended to be located on the most exposed areas (of the resting palp). In addition, many sensilla occurred around the LPO opening (39% in *L. botrana,* 30% in *C. pomonella* and 23% in *G. molesta*), (Fig. 1, Supplementary Fig. S1). In all species, more than 80% of sensilla were located on the distal half of the segment and more than half of the sensilla (62% in *L. botrana*, 66% in *G. molesta* and 75% in *C. pomonella*) were lateral. The most substantial difference between species was found in the dorsal/ventral axis. In *G. molesta* 90% of the sensilla were ventral, while in the other two species the number of sensilla in the dorsal and ventral regions was similar (Supplementary Fig. S1, Supplementary Table S3). The results of the ANOVA of the distribution of the sensilla among the areas defined by the symmetry axes showed differences among species (Deviance = 106.28, df = 14, *p* [Chi] <0 .001), but not between sexes (Deviance = 11.53, df = 7, *p* [Chi] = 0.117) and no interaction between species and sexes was detected (Deviance = 10.23, df = 14, *p* [Chi] = 0.745). Within each species, the number of sensilla varied between areas. The ventro-lateral-distal area of the palp had the highest number of sensilla in all species, however substantial differences could be observed between species in the sensilla distribution among areas (Tukey: $p < 0.05$, Supplementary Fig. S1, Supplementary Table S3).

Effect of diet on survival

Diet had a significant impact on adult survival in all three species (Log-rank test: $p < 0.0001$), the longest survival time was detected in *C. pomonella* with access to sugared water (35 d) and the lowest in *G. molesta* without water (1 d). In the absence of water, median longevity decreased by 75% in *G. molesta,* 50% in *L. botrana* and 40% in *C. pomonella*. Addition of sugar to the diet increased median longevity in *G. molesta* and *C. pomonella* by 6 and 4.5 d respectively (Log-rank test: $p < 0.0001$, Supplementary Fig. S2).

Electrophysiology

For each combination of factors (stimulus, species, sex and appendage) between 16 and 34 sensilla from between 4 and 6 individuals were sampled, with a total of 3690 recordings from 1057 sensilla sampled from antenna and palp together (Supplementary Table S4). The highest number of spikes/s (68) was observed on the labial palp of a female *C. pomonella* in response to KCl*,* but in general the number of spikes was relatively low (2.84 on average) (Fig. 3). The average spike amplitude was relatively variable between recordings of the same sensillum and between sensilla (Fig. 2). Thus, although several spike amplitude classes could be detected in some recordings, we did not sort spikes. Instead, for the purpose of this study, we analysed the sum of all the spikes in a given recording, irrespective of whether they belong to one or more neuron types. Different number of spikes between electrolyte and sugars indicate that the electrolyte alone did not stimulate sensilla more than the test stimuli (Dunnett test: $p < 0.01$, Supplementary Table S5).

The model that best explained responses to stimuli contain all main factors with a 4th-order interaction (Likelihood-ratio test: $p < 0.01$, Supplementary Table S6). According to the ANOVA (Table 2), appendage (i.e., antenna or palp) explained 23.95% of the deviance, the interaction between stimulus and concentration explained 13.07% of the deviance, concentration 9.98%, stimulus 7.77%, and species 6.8%. The remaining 25 factors of the model together explained 33.56% of the deviance, each contributing 4.79% or less to the total deviance. On average, response was 2.76 times higher in palpi than in antennae, and the number of spikes increased with stimulus concentration (Tukey: $p < 0.05$, Table 3). There were 1.2 times more spikes/s to sugars (fructose and sucrose combined) than to salts (NaCl and KCl combined), and sucrose was the strongest of the four stimuli tested (4.30 ± 0.26 spikes/s) (Tukey: $p < 0.05$, Table 3, Fig. 3). *C. pomonella* produced 1.8 times more spikes/s than *G. molesta* (Tukey: $p < 0.05$, Table 3, Fig. 3). Sex had a significant, but very low, impact on the number of spikes (0.76 more in females than in males, Tukey: $p < 0.05$, Table 3), and so the two sexes were combined for graphic display (Fig. 3).

The effect of dose was different for salts and sugars. In salts the highest response occurred at 1M, then at 10 mM and the lowest at 100 mM (Tukey: $p < 0.05$, Supplementary Table S6, Fig. 3). With sugars, no differences were found among concentrations when pooling antennal and palp responses (Tukey: $p < 0.05$, supplementary Table S6). However, in antenna the response to sugars decreased as the dose increased, whereas in the palp responses increased with concentration (Tukey: $p < 0.05$, Supplementary Table S6, Figs. 2 and 3).

From highest to lowest, the mean responses to sugars and salts were as follows: sugars in palps (5.33 ± 0.27 spikes/s), salts in palps (3.60 ± 0.27 spikes/s), and sugars and salts in antennae (1.40 ± 0.11 and 1.81 ± 0.14 respectively) (Tukey: $p < 0.05$, Supplementary Table S6, Fig. 3). Responses to sugars in *C. pomonella* and *L. botrana* were in average 1.9 times higher than in *G. molesta,* but no differences were detected between species with salts. Females produced more spikes than males in *L. botrana* and *G. molesta* (Tukey: $p < 0.05$, Supplementary Table S6).

Table 2. Summary of the GLM model for the effect of species (*C. pomonella*, *G. molesta* and *L. botrana*), sex, stimulus (fructose, sucrose, NaCl and KCl), dose (10^1, 10^2, 10^3 mM), and appendage (antenna or labial palp) on the number of action potentials of sensilla chaetica. Terms have been ordered by their contribution to the model deviance (% Dev.).

Model term	Df	Deviance	Resid. Df	Resid. Dev	F	*p*(>F)	% Dev.	Cum. Dev.
NULL			3170	23956				
appendage	1	2125.63	3166	21073	334.77	<0.001	23.95	
stimulus*dose	6	1160.41	3130	16776	30.46	<0.001	13.07	37.02
dose	2	885.62	3161	19497	69.74	<0.001	9.98	47.00
stimulus	3	690.08	3163	20383	36.23	<0.001	7.77	54.77
species	2	612.75	3168	23343	48.25	<0.001	6.90	61.67
appendage*stimulus	3	422.64	3138	18122	22.19	<0.001	4.76	66.44
species*appendage*stimulus	6	378.64	3112	15984	9.94	<0.001	4.27	70.70
species*sex*stimulus	6	355.39	3122	16420	9.33	<0.001	4.00	74.71
species*stimulus	6	255.47	3151	18889	6.71	<0.001	2.88	77.58
species*appendage	2	240.03	3157	19145	18.90	<0.001	2.70	80.29
species*dose	4	192.58	3147	18697	7.58	<0.001	2.17	82.46
appendage*dose	2	185.58	3136	17936	14.61	<0.001	2.09	84.55
species*sex*appendage*stimulus	6	168.81	3073	15370	4.43	<0.001	1.90	86.45
sex	1	144.24	3167	23199	22.72	<0.001	1.62	88.07
species*stimulus*dose	12	140.23	3096	15769	1.84	0.037	1.58	89.65
species*sex	2	112.59	3159	19385	8.87	<0.001	1.27	90.92
species*sex*stimulus*dose	12	111.86	3057	15214	1.47	0.129	1.26	92.18
appendage*stimulus*dose	6	103.15	3079	15538	2.71	0.013	1.16	93.34
sex*stimulus*dose	6	90.26	3085	15642	2.37	0.028	1.02	94.36
sex*stimulus	3	89.22	3143	18544	4.68	0.003	1.01	95.37
species*appendage*stimulus*dose	12	87.17	3045	15127	1.14	0.319	0.98	96.35
species*appendage*dose	4	74.88	3108	15909	2.95	0.019	0.84	97.19
sex*appendage	1	63.07	3146	18634	9.93	0.002	0.71	97.90
species*sex*dose	4	56.60	3118	16363	2.23	0.064	0.64	98.54
sex*appendage*stimulus*dose	6	47.63	3039	15079	1.25	0.277	0.54	99.08
species*sex*appendage*dose	4	43.91	3069	15326	1.73	0.141	0.49	99.57
sex*appendage*stimulus	3	34.78	3093	15734	1.83	0.140	0.39	99.96
sex*appendage*dose	2	2.47	3091	15732	0.19	0.823	0.03	99.99
species*sex*appendage	2	0.72	3128	16775	0.06	0.945	0.01	100.00
sex*dose	2	0.05	3141	18544	0.00	0.996	0.00	100.00

Table 3. Pairwise comparison of the number of spikes produced by sensilla chaetica present on the antennae and labial palps of the three moth species. Observed data and estimated values from the GLM model are presented. Letters in the right column indicate significant differences among the levels of the second column for each treatment of the first column (Tukey´s test, $p < 0.05$ after GLM).

		Observed	Estimated	
		Mean ± SEM	Mean ± SEM	
Appendage	Palp	4.43 ± 0.19	2.93 ± 0.15	a
	Antenna	1.61 ± 0.09	0.80 ± 0.13	b
Concentration (mM)	10^3	4.33 ± 0.25	2.69 ± 0.18	a
	10^1	2.37 ± 0.12	1.69 ± 0.12	b
	10^2	2.30 ± 0.16	0.79 ± 0.18	c
Stimulus	Sucrose	4.30 ± 0.26	2.68 ± 0.21	a
	Fructose	2.33 ± 0.14	1.65 ± 0.14	b
	KCl	3.13 ± 0.24	1.24 ± 0.30	b
	NaCl	2.32 ± 0.20	1.00 ± 0.20	b
Stimulus group	Sugar	3.28 ± 0.15	2.10 ± 0.12	a
	Salt	2.72 ± 0.16	1.11 ± 0.18	b
Species	*C. pomonella*	4.09 ± 0.22	2.11 ± 0.25	a
	L. botrana	2.66 ± 0.16	1.51 ± 0.18	ab
	G. molesta	2.28 ± 0.17	1.12 ± 0.13	b
Sex	Female	3.38 ± 0.15	1.87 ± 0.16	a
	Male	2.63 ± 0.15	1.25 ± 0.17	b

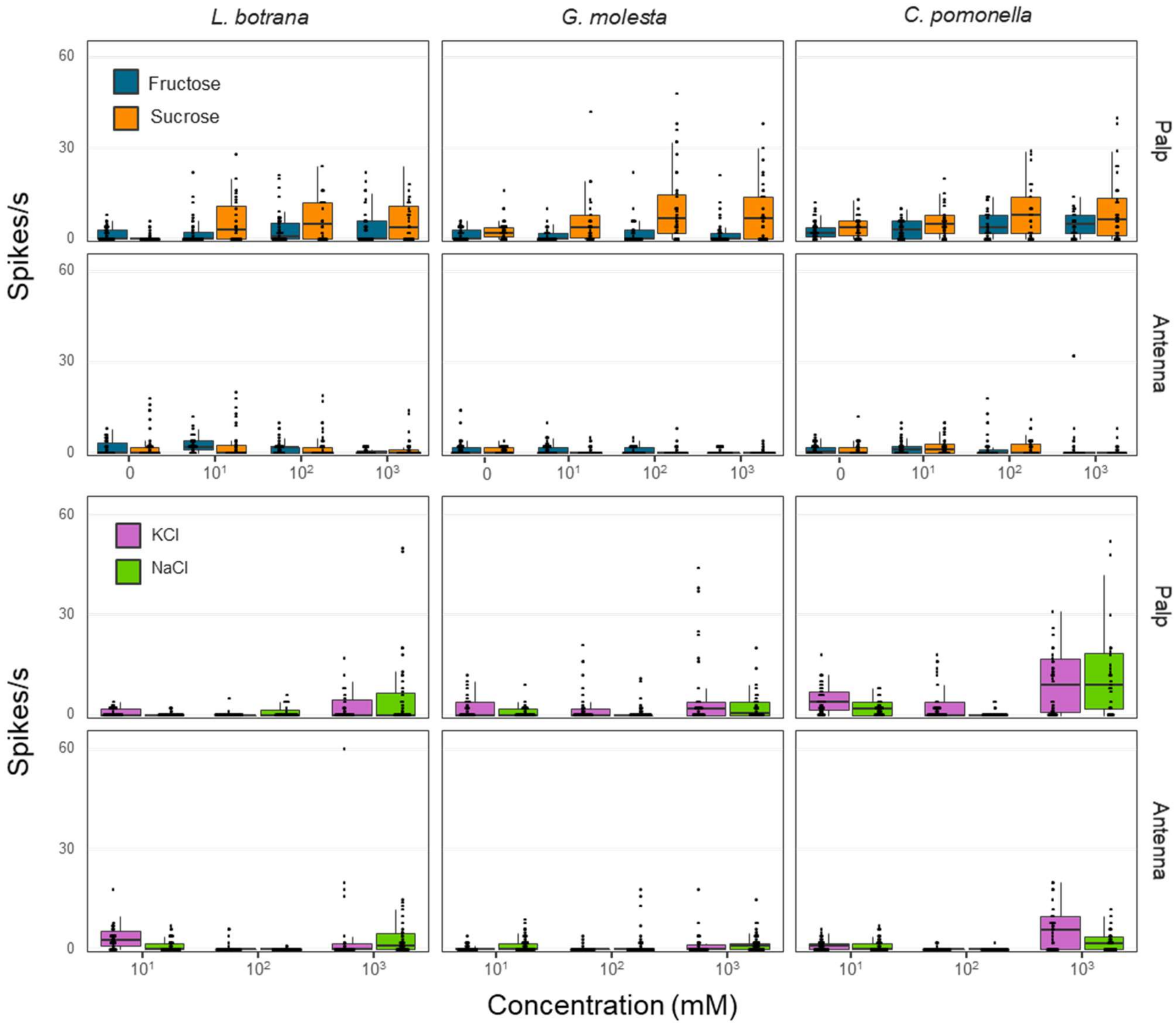

Figure 3. Response of gustatory receptor neurons of sensilla chaetica located on the apical segment of the labial palps (left) and on the antenna (right) of three tortricid moth species upon a 2 sec stimulation with three doses of NaCl, KCl, fructose or sucrose. N=16-34 sensilla of 4-6 individuals of each sex. Males and females have been combined in this plot. Box plots show median (horizontal line), first and third quartile (box), 1.5*inter-quartile range (error bars), and data points (•).

Discussion

We present evidence that the labial palps of moths have a gustatory function. Furthermore, we found significant differences between appendages, stimuli, species and sexes. As far as we know, this may be the first direct evidence that the labial palps of moths, or perhaps any Lepidoptera, have a gustatory function, while it is a common feature in other insect orders. The presence of a flexible socket at the base of the sensilla chaetica suggests mechanosensory function (Hallberg 1981, Mitchell *et al.* 1999), however observations should be conducted to confirm this role. We do not know the extent of gustation in the labial palps of Lepidoptera because reports of uniporous sensilla chaetica (i.e., with a putative gustatory function) on the external part of labial palps are scarce. Chaetica-like sensilla with a terminal pore have been reported in a species of the Cossidae family (Hu *et al.* 2018). Sensilla chaetica are described in other Cossidae and in members of the Tortricidae and Pyralidae families, but it is not indicated if they bear a pore at the tip, and thus they may not be gustative (Chen & Hua 2016, Song *et al.* 2016, Vargas-Fernández *et al.* 2018) In a Neopseustidae specie, aporous sensilla chaetica have been described (Faucheux 2008). Several studies on members of the Gelechiidae, Pyralidae, Sphingidae, Noctuidae, Plutellidae, Erebidae and Nymphalidae families fail to report sensilla chaetica or any other type of sensilla with a putative gustatory function in the labial palps (Bogner *et al.* 1986, Kent *et al.* 1986, Faucheux 1991, Krenn &Penz 1998, Zhao *et al.* 2013, Dong *et al.* 2014, Barcaba & Krenn 2015, Li *et al.* 2018, Chen *et al.* 2021, Abd El-Ghany & Faucheux 2022).

Obtaining electrophysiological recordings from sensilla chaetica of the labial palps and antenna was challenging because we experienced a persistent difficulty to make stable electric contacts with these sensilla, whereas it was relatively easy on sensilla styloconica of the proboscis (data not shown). Conditions improved when the humidity of the rearing chamber was increased, but consistent contacts were only obtained when the sensilla tip was wiped repeatedly with an empty glass capillary before making contact. Some authors have encountered similar problems in other insects and suggest that debris accumulates on the pore at the tip of the sensilla. Städler *et al.* (1987) report that low relative humidity reduced the quality of the recordings of *Rhagoletis cerasi* L. tarsi, and that flies that were allowed to walk on the plants were easier to record (implying that the sensilla were "unplugged" by the contact with the substrate). Canney and Gardner (1989) report that cleanness of the pore of sensilla chaetica improved the quality of electrophysiological recordings in *Ostrinia nubilalis* (Hübner).

Sugars and salts play a role on the fitness and behaviour of the three tortricid moths. Our longevity test confirms that adults of the three species do not live long without water and that sugars prolong longevity (Savopoulou-Soultani *et al.* 1998, Atanassov & Shearer 2005, Wenninger & Landolt 2011). When given access to flowers under laboratory conditions the longevity of *C. pomonella* increased with respect to a no-food control, but a dilution of sugar in water was even better than the flowers (Mátray & Herz 2021). Peach extrafloral nectar, a natural solution rich in sugars (Caldwell & Gerhardtt 1986), enhanced *G. molesta* adult longevity

(Atanassov & Shearer 2005). Despite the importance of sugar on adult fitness, only *L. botrana* has been observed feeding in the wild. It visits the flowers of tansy (*Tanacetum vulgare* L.) (a non-host plant located around the vineyards) at dusk to feed on the pollen and nectar (Gabel 1992). Sugars stimulate oviposition in *C. pomonella* and *L. botrana* (Lombarkia & Derridj 2002, Maher *et al.* 2006), and gustatory sensilla in the ovipositor of *L. botrana* detect fructose and sucrose (Maher *et al.* 2006). KCl is also perceived by sensilla in the ovipositor of *L. botrana,* and it affects oviposition (Maher *et al.* 2006). Sugars and salts could impact larval fitness too (Savopoulou-Soultani *et al.* 1994, Pszczolkowski & Brown 2003, Su *et al.* 2021). Therefore, the presence of taste receptors on antennae and palps could contribute to the detection of salts and sugars, but they may also be specialized on the detection of other stimuli not tested yet.

Gustatory sensilla of insects typically have 4 different GRNs, generally associated to different taste modalities (Mitchell *et al.* 1999, Marion-Poll 2020). GRNs in the antennae of adult Lepidoptera respond to sugars, water, salts, amino acids and bitter compounds (Calatayud *et al.* 2006, Jørgensen etl al. 2007, Popescu *et al.* 2013). The number of spikes that we observed in tortricids was relatively lower than what has been found in antennal sensilla of noctuid moths (Calatayud *et al.* 2006, Jørgensen etl al. 2007, Popescu *et al.* 2013). We also observed that the response to sugars in antennal sensilla decreased as concentration increased, contrary to what has been observed in *Chloridera* (formely *Heliothis*) *virescens* (Fabricus) and *Spodoptera littoralis* (Boisduval) (Jørgensen etl al. 2007, Popescu *et al.* 2013). Many noctuids are flower visitors, but this behaviour is rare in tortricids (Van Zandt *et al.* 2020), and so different sugar requirements between members of the two families may explain differences in sugar sensitivity. Water and salt intake regulation is crucial for animals to maintain osmotic homeostasis (Cobb *et al.* 2009, Kaushik *et al.* 2018), and in many insects some cells respond to low salt concentrations (water GRN) and some to high salt concentrations (salt GRN), which allows for fine detection of salts (Jørgensen etl al. 2007, Popescu *et al.* 2013, Kaushik *et al.* 2018). In our tests, the relatively high number sensilla responding to the electrolyte control, and the unusual shape of the dose-response curve to salts (with a drop at the intermediate concentration) suggests that palp and antennal sensilla chaetica of tortricids have a water and a salt GRN, which may help in salt detection.

There are few reports on the putative behaviours where Lepidoptera may use the gustatory sensilla detected on the labial palps. Grant (1987) observed the response of the tortricid moth *Choristoneura fumiferana* (Clemens) to a rubber septum loaded with female sex pheromone. The tips of the labial palps contacted with the septum ("the palps themselves pulled away from the head in lever-like fashion") right before attempting copulation (Figure 1 in Grant 1987). Also during courtship, *G. molesta* males and females perform a dance where there is mutual contact with the antennae and probably the palps too (Baker & Cardé 1979). Thus, gustatory receptors on palp and antennae may be used to detect conspecific signals, such as cuticular hydrocarbons, which often present sexual dimorphism in insects (Ferveur 2005, Gemeno *et al.* 2012). Gustatory function of the labial palps may also be related to oviposition choice behaviour because in the moth *Cactoblastis cactorum* (Berg) females actively touch the plant with the labial palps while searching for oviposition sites (Myers *et al.* 1981).

The discovery of gustation in the labial palps of moths may shed new light on the innervation of the labial palp nerves in the moth brain. Unilateral palp backfills typically reveal bilateral projections ascending to the antennal lobe where they stain a basal glomerulus, equivalent in both males and females, the "labial pit organ glomerulus" (LPOG) (Varela *et al.* 2009, Zhao *et al.* 2013, Pramod *et al.* 2020). This glomerulus receives the input from the CO_2 sensory neurons housed in the labial pit organ. However, palp backfills show additional arborizations in the suboesophageal ganglion (SOG) (Varela *et al.* 2009, Zhao *et al.* 2013). To clarify this point, Pramod *et al.* (2020) performed selective mass staining from both, the inside of the labial pit organ and the outer surface of the distal labial palp segment of *H. armigera* and compared their arborizations. LPO sensory neurons projected exclusively to the LPOG, whereas the non-LPO sensory neurons targeted the gnathal ganglion and the ventral nerve cord. It is very likely that the axons that do not innervate the LPOG correspond to mechano/gustatory neurons located on gustatory sensilla because the mechano/gustatory input from other cephalic appendages ends in the SOG (Mitchell *et al.* 1999, Marion-Poll 2020). Interestingly, an SEM picture of the labial palp of *H. armigera* does not show sensilla chaetica (Zhao *et al.* 2013). Sensilla chaetica are rather conspicuous and resilient (Ryan 2002) so it is unlikely that they are accidentally knocked off when scales were removed to observe the palps. If they did not fall during scale removal, then other type of sensilla may contain the palp neurons that innervate the SOG. Thus, further studies are required to determine which other Lepidoptera species have gustatory function in the labial palps.

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Supplementary material

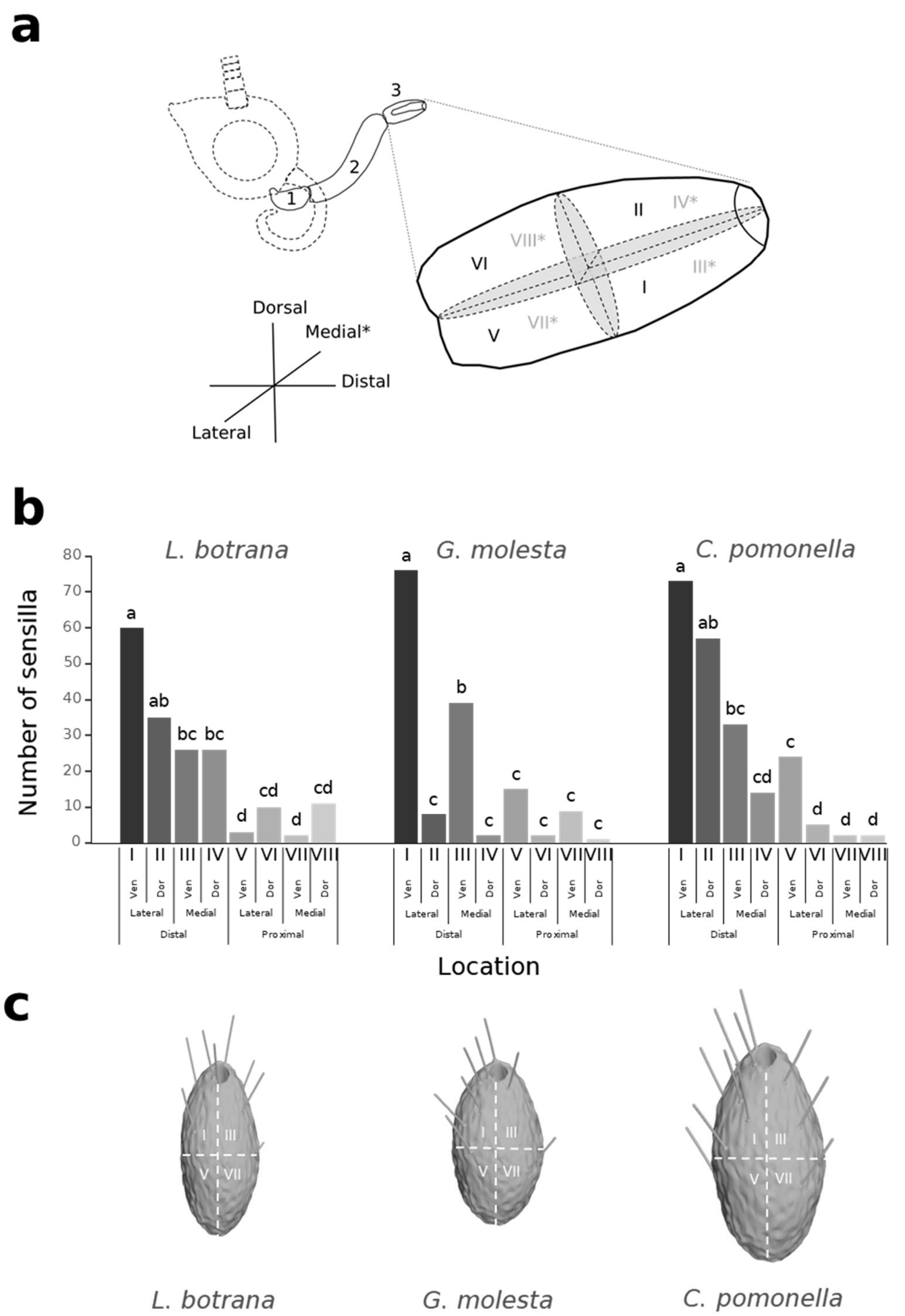

Figure S1. Number and location of sensilla chaetica in the apical segment of labial palps. A) Scheme showing the position of labial palp in the moth head and the areas used to count the sensilla based on the symmetry axes. B) Number of sensilla found in each of the areas. Different letters indicate significant differences among groups (Tukey´s test, P<0.05 after GLM). Males and females have been combined in this plot. C) Schematic diagram showing the sensilla distribution on a ventral view of the labial palps.

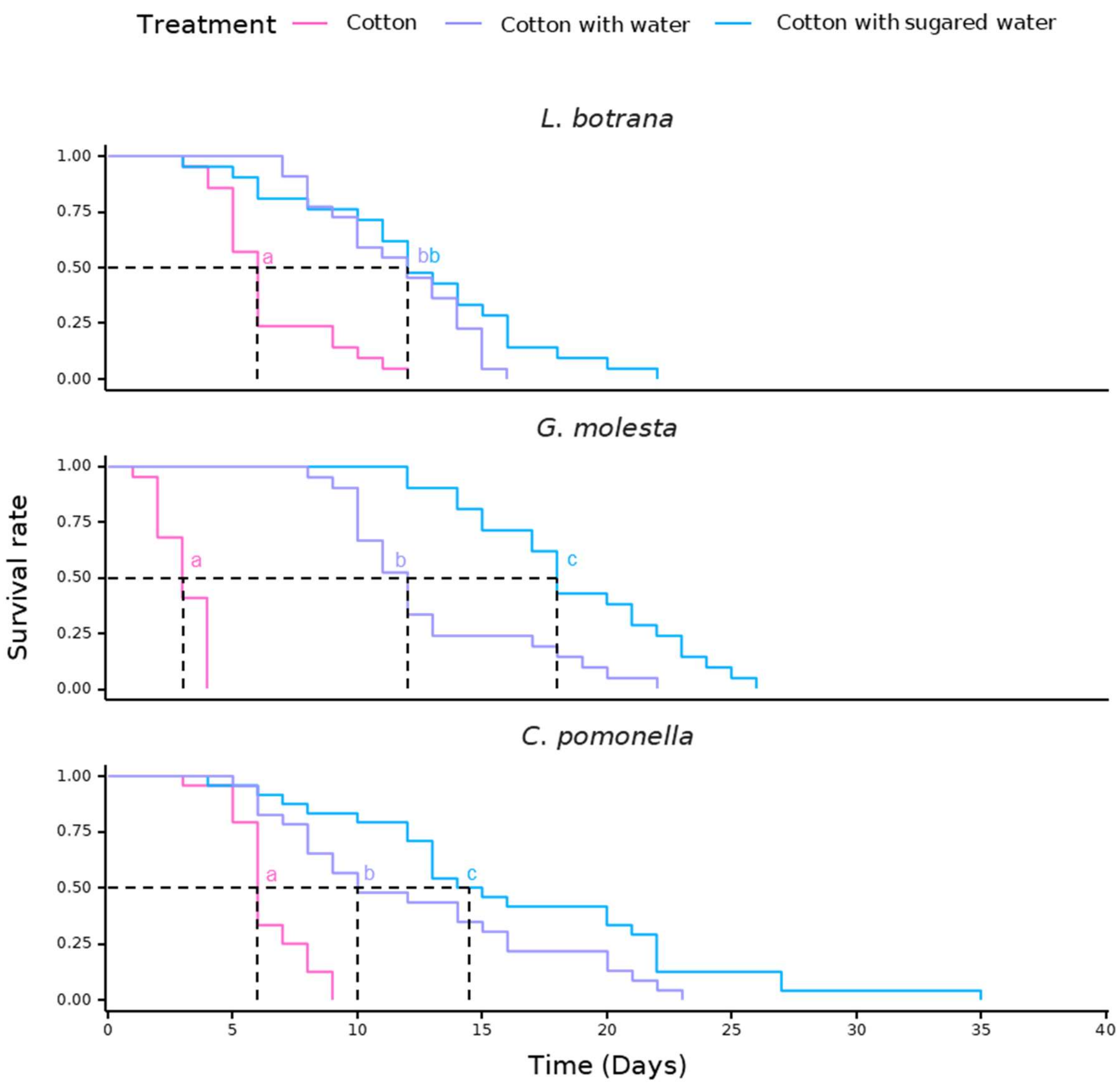

Figure S2. Survival curves of adults of *L. botrana, G. molesta* and *C. pomonella* subjected to different feeding regimes.

Table S1. Statistical analysis for the size (length and width) of the apical segment of the labial palp. a) Model selection showing the Akaike information criterion (AIC). b) Summary of the selected models. c) Pairwise comparison among species and between males and females showing observed values and those estimated by the models. Different letters in the column to the right indicate significant differences among groups (Tukey´s test, P<0.05 after GLM).

a

Model (Length)	AIC	DF model	DF residual deviance	Residual deviance	LRT P-value
Null	655	0	59	180144	-
Main effects	529	3	56	20077	<0.001
Main effects and 2nd-order interaction	512	5	54	14194	<0.001

Model (Width)	AIC	DF model	DF residual deviance	Residual deviance	LRT P-value
Null	567	0	59	42073	-
Main effects	456	3	56	5920	<0.001
Main effects and 2nd-order interaction	437	5	54	4073	<0.001

b

Length	Df	Deviance	Resid.Df	Resid.Dev	F	Pr(>F)
NULL			59	180144		
sp	2	119116.00	57	61028	226.58	<0.001
sex	1	40951.00	56	20077	155.79	<0.001
sp:sex	2	5883.00	54	14194	11.19	<0.001

Width	Df	Deviance	Resid.Df	Resid.Dev	F	Pr(>F)
NULL			59	42073		
Sp	2	24946.50	57	17127	165.39	<0.001
sex	1	11206.70	56	5920	148.60	<0.001
sp:sex	2	1847.70	54	4073	12.25	<0.001

c

Length	Observed			Estimated			
	Mean ± SEM			Mean ± SEM			
C. pomonella	240.13	±	9.87	240.13	±	3.63	a
L. botrana	210.63	±	6.83	210.63	±	3.63	b
G. molesta	134.38	±	4.06	134.38	±	3.63	c
Female	221.17	±	2.96	221.17	±	10.65	a
Male	168.92	±	2.96	168.92	±	6.82	b

Width	Observed			Estimated			
	Mean ± SEM			Mean ± SEM			
C. pomonella	129.50	±	3.63	129.50	±	1.94	a
G. molesta	86.63	±	2.66	86.63	±	1.94	b
L. botrana	85.88	±	4.98	85.88	±	1.94	b
Female	114.33	±	4.23	114.33	±	1.59	a
Male	87.00	±	4.19	87.00	±	1.59	b

Table S2. Statistical analysis for the number of sensilla in the apical segment of the labial palp. a) Model selection showing the Akaike information criterion (AIC). Main effects: species and sex. b) Summary of the selected model. c) Pairwise comparison among species and between males and females showing observed values and those estimated by the models. Different letters in the column to the right indicate significant differences among groups (Tukey´s test, P<0.05 after GLM).

a

Model	AIC	DF model	DF residual deviance	Residual deviance	LRT P-value
Null	280	0	59	37	-
Main effects	268	3	56	19	<0.001
Main effects and 2nd-order interaction	270	5	54	17	0.345

b

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL			59	37.09	
sp	2	9.57	57	27.52	0.008
sex	1	8.41	56	19.10	0.004

c

	Observed			Estimated			
	Mean ± SEM			Mean ± SEM			
Female	10.03	±	0.46	9.94	±	0.58	a
Male	7.80	±	0.30	7.73	±	0.51	b
C. pomonella	10.50	±	0.55	10.42	±	0.72	a
L. botrana	8.65	±	0.44	8.58	±	0.65	ab
G. molesta	7.60	±	0.40	7.54	±	0.61	b

Table S3. Statistical analysis for the effect of labial palp location on the number of sensilla chaetica in each moth species. a) Model selection showing the Akaike information criterion (AIC). Main effects: palp location. b) Summary of the selected models. c) Pairwise comparison among palp locations showing observed values and those estimated by the models. Different letters in the column to the right indicate significant differences among groups (Tukey´s test, P<0.05 after GLM). * The location code can be found in Supplementary Fig. S1.

a

Species	Model type	AIC	DF model	DF residual deviance	Residual deviance	LRT P-value*
L. botrana	Null	454	0	159	222	-
	Main effects	345	7	152	94	<0.001
G. molesta	Null	488	0	159	312	-
	Main effects	293	7	152	103	<0.001
C. pomonella	Null	542	0	159	301	-
	Main effects	362	7	152	107	<0.001

b

Species		Model Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
L. botrana	NULL			159	221.57	
	loc	7	123.66	152	97.91	<0.001
G. molesta	NULL			159	311.93	
	loc	7	208.53	152	103.40	<0.001
C. pomonella	NULL			159	301.09	
	loc	7	193.74	152	107.35	<0.001

c

		Observed			Estimated			
Species	Location*	Mean ± SEM			Mean ± SEM			
L. botrana	I	3.00	±	0.18	3.00	±	0.39	a
	II	1.75	±	0.20	1.75	±	0.30	ab
	IV	1.30	±	0.18	1.30	±	0.25	bc
	III	1.30	±	0.19	1.30	±	0.25	bc
	VIII	0.55	±	0.15	0.55	±	0.17	cd
	VI	0.50	±	0.14	0.50	±	0.16	cd
	V	0.15	±	0.08	0.15	±	0.09	d
	VII	0.10	±	0.07	0.10	±	0.07	d
G. molesta	I	3.80	±	0.24	3.80	±	0.44	a
	III	1.95	±	0.21	1.95	±	0.31	b
	V	0.75	±	0.14	0.75	±	0.19	c
	VII	0.45	±	0.20	0.45	±	0.15	c
	II	0.40	±	0.15	0.40	±	0.14	c
	VI	0.10	±	0.07	0.10	±	0.07	c
	IV	0.10	±	0.07	0.10	±	0.07	c
	VIII	0.05	±	0.05	0.05	±	0.05	c
C. pomonella	I	3.65	±	0.33	3.65	±	0.43	a
	II	2.85	±	0.26	2.85	±	0.38	ab
	III	1.65	±	0.22	1.65	±	0.29	bc
	V	1.20	±	0.21	1.20	±	0.24	c
	IV	0.70	±	0.18	0.70	±	0.19	cd
	VI	0.25	±	0.10	0.25	±	0.11	d
	VII	0.10	±	0.07	0.10	±	0.07	d
	VIII	0.10	±	0.07	0.10	±	0.07	d

Table S4. Number of moths and number of sensilla tested in the SSR experiment.

Species	sex	Stimulus	Appendage	N. moths	N. sensilla
L. botrana	Female	KCl	Palp	5	21
L. botrana	Female	KCl	Antenna	4	23
L. botrana	Female	NaCl	Palp	4	22
L. botrana	Female	NaCl	Antenna	4	24
L. botrana	Female	Fructose	Palp	4	22
L. botrana	Female	Fructose	Antenna	4	22
L. botrana	Female	Sucrose	Palp	4	20
L. botrana	Female	Sucrose	Antenna	4	22
L. botrana	Male	KCl	Palp	5	25
L. botrana	Male	KCl	Antenna	4	19
L. botrana	Male	NaCl	Palp	4	24
L. botrana	Male	NaCl	Antenna	4	21
L. botrana	Male	Fructose	Palp	5	25
L. botrana	Male	Fructose	Antenna	4	20
L. botrana	Male	Sucrose	Palp	4	19
L. botrana	Male	Sucrose	Antenna	5	28
G. molesta	Female	KCl	Palp	4	22
G. molesta	Female	KCl	Antenna	4	23
G. molesta	Female	NaCl	Palp	4	23
G. molesta	Female	NaCl	Antenna	4	19
G. molesta	Female	Fructose	Palp	4	21
G. molesta	Female	Fructose	Antenna	4	21
G. molesta	Female	Sucrose	Palp	4	20
G. molesta	Female	Sucrose	Antenna	4	22
G. molesta	Male	KCl	Palp	4	20
G. molesta	Male	KCl	Antenna	4	16
G. molesta	Male	NaCl	Palp	4	19
G. molesta	Male	NaCl	Antenna	6	34
G. molesta	Male	Fructose	Palp	4	23
G. molesta	Male	Fructose	Antenna	5	29
G. molesta	Male	Sucrose	Palp	4	19
G. molesta	Male	Sucrose	Antenna	5	24
C. pomonella	Female	KCl	Palp	5	27
C. pomonella	Female	KCl	Antenna	5	24
C. pomonella	Female	NaCl	Palp	5	18
C. pomonella	Female	NaCl	Antenna	4	22
C. pomonella	Female	Fructose	Palp	4	21
C. pomonella	Female	Fructose	Antenna	4	24
C. pomonella	Female	Sucrose	Palp	4	18
C. pomonella	Female	Sucrose	Antenna	4	18
C. pomonella	Male	KCl	Palp	5	28
C. pomonella	Male	KCl	Antenna	4	20
C. pomonella	Male	NaCl	Palp	5	25
C. pomonella	Male	NaCl	Antenna	4	19
C. pomonella	Male	Fructose	Palp	4	20
C. pomonella	Male	Fructose	Antenna	4	20
C. pomonella	Male	Sucrose	Palp	4	20
C. pomonella	Male	Sucrose	Antenna	4	21

Table S5. Comparison between each of the 3 sugars concentrations and the electrolyte control for the number of spikes of sensilla chaetica on the antenna and labial palps. a) Model selection. Main paramete are: species, sex and appendage. b) GLM model summary. c) P-values of the Dunnett´s test after GLM ar shown. Comparison between each test concentrations (10^1, 10^2 and 10^3 mM) of sugars and the solv control in each.

a

Model	DF model	DF Resid. Dev.	Resid. Dev.	P-Val
Null		2075.00	13801.15	
Main effects	8	2067.00	10847.75	<0
Main effects and 2nd-order interaction	32	2043.00	9663.03	<0
Main effects and 2nd- and 3rd-order interaction	66	2009.00	9144.20	<0
Main effects and 2nd-, 3rd- and 4rt-order interaction	89	1986.00	8921.24	0
Main effects and 2nd-, 3rd- , 4rt- and 5th order interaction	95	1980.00	8829.49	0

b

	DF	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			2075	13801.20		
sp	2	167.87	2073	13633.30	15.81	<0.01
sex	1	198.92	2072	13434.40	37.47	<0.01
app	1	1798.54	2071	11635.80	338.74	<0.01
stim	1	452.33	2070	11183.50	85.19	<0.01
conc	3	335.74	2067	10847.70	21.08	<0.01
sp:sex	2	237.07	2065	10610.70	22.32	<0.01
sp:app	2	186.93	2063	10423.80	17.60	<0.01
sex:app	1	61.48	2062	10362.30	11.58	<0.01
sp:stim	2	131.27	2060	10231.00	12.36	<0.01
sex:stim	1	0.13	2059	10230.90	0.02	0.87
app:stim	1	167.69	2058	10063.20	31.58	<0.01
sp:conc	6	50.73	2052	10012.50	1.59	0.15
sex:conc	3	1.36	2049	10011.10	0.09	0.97
app:conc	3	296.01	2046	9715.10	18.58	0.00
stim:conc	3	52.05	2043	9663.00	3.27	0.02
sp:sex:app	2	73.04	2041	9590.00	6.88	<0.01
sp:sex:stim	2	94.83	2039	9495.20	8.93	<0.01
sp:app:stim	2	108.52	2037	9386.60	10.22	<0.01
sex:app:stim	1	0.49	2036	9386.20	0.09	0.76
sp:sex:conc	6	53.81	2030	9332.30	1.69	0.12
sp:app:conc	6	84.80	2024	9247.50	2.66	0.01
sex:app:conc	3	16.88	2021	9230.70	1.06	0.36
sp:stim:conc	6	8.27	2015	9222.40	0.26	0.96
sex:stim:conc	3	37.21	2012	9185.20	2.34	0.07
app:stim:conc	3	40.97	2009	9144.20	2.57	0.05
sp:sex:app:stim	2	56.89	2007	9087.30	5.36	0.00
sp:sex:app:conc	6	17.52	2001	9069.80	0.55	0.77
sp:sex:stim:conc	6	53.07	1995	9016.70	1.67	0.13
sp:app:stim:conc	6	62.59	1989	8954.10	1.96	0.07
sex:app:stim:conc	3	32.90	1986	8921.20	2.07	0.10
sp:sex:app:stim:conc	6	91.74	1980	8829.50	2.88	0.01

c

Appendage	Stimulus	Species	Sex	10^1 mM	10^2 mM	10^3 mM
Antenna	Fructose	*L. botrana*	Female	0.552	0.980	0.088
			Male	0.474	0.567	0.889
		G. molesta	Female	0.998	0.865	0.337
			Male	0.421	0.113	0.118
		C. pomonella	Female	0.999	0.393	0.393
			Male	0.769	0.340	0.036
	Sucrose	*L. botrana*	Female	0.453	0.998	0.673
			Male	0.998	0.998	0.487
		G. molesta	Female	0.930	0.851	0.564
			Male	0.840	0.787	0.634
		C. pomonella	Female	0.995	0.144	0.925
			Male	0.421	0.474	0.559
Palp	Fructose	*L. botrana*	Female	0.245	0.028	0.026
			Male	0.342	0.845	0.096
		G. molesta	Female	0.990	0.554	0.737
			Male	0.805	0.991	0.999
		C. pomonella	Female	0.866	0.765	0.631
			Male	0.729	0.099	0.180
	Sucrose	*L. botrana*	Female	<0.001	<0.001	<0.001
			Male	0.222	0.134	0.203
		G. molesta	Female	0.474	0.016	0.003
			Male	0.013	<0.001	0.032
		C. pomonella	Female	0.328	0.003	0.002
			Male	0.660	0.197	0.086

Table S6. Model selection and pairwise comparisons for the number of spikes produced by sensilla chaetica on the labial palps and antennae. The main effects were sex, species (*C. pomonella*, *G. molesta* and *L. botrana*), appendage (antenna or palp), stimulus (fructose, sucrose, NaCl and KCl, or sugars and salts combined), and concentration (10^1, 10^2 and 10^3 mM). a). Models were compared with ANOVA (p-values of the likelihood ratio test, LRT). b) Pairwise comparison among concentrations for salts and sugars. c) Pairwise comparison among salts and sugars for palp and antenna. d) Pairwise comparison among salts and sugars for each species. e) Pairwise comparison between sexes for each species. f) Pairwise comparison between appendages for salts and sugars.

a

Model	DF model	DF Res. Dev.	Res. dev.	LRT P-value
Null	0	3170	23956	-
Main effects	9	3161	19497	<0.001
Main effects and 2nd-order interaction	40	3130	16776	<0.001
Main effects and 2nd- and 3rd-order interaction	91	3079	15539	<0.001
Main effects and 2nd-, 3rd- and 4rt-order interaction	131	3039	15079	0.001
Main effects and 2nd-, 3rd- , 4rt- and 5th order interaction	143	3027	14994	0.314

b

		Observed			Estimated			
mM	Stimulus	Mean ± SEM			Mean ± SEM			
Salts	10^1	1.86	±	0.12	1.34	±	0.15	b
	10^2	0.97	±	0.12	0.26	±	0.12	c
	10^3	5.34	±	0.41	3.96	±	0.26	a
Sugars	10^1	2.89	±	0.22	2.13	±	0.19	a
	10^2	3.67	±	0.28	2.40	±	0.22	a
	10^3	3.28	±	0.27	1.82	±	0.21	a

c

		Observed			Estimated			
Appendage	Stimulus	Mean ± SEM			Mean ± SEM			
Palp	Sugars	5.33	±	0.27	4.34	±	0.22	a
	Salts	3.60	±	0.27	1.97	±	0.17	b
Antenna	Sugars	1.40	±	0.11	1.02	±	0.10	c
	Salts	1.81	±	0.14	0.63	±	0.19	c

d

		Observed			Estimated			
Stimulus	Species	Mean ± SEM			Mean ± SEM			
Salts	*C. pomonella*	4.15	±	0.34	1.52	±	0.33	a
	L. botrana	2.09	±	0.23	0.99	±	0.22	a
	G. molesta	1.89	±	0.20	0.91	±	0.19	a
Sugars	*C. pomonella*	4.02	±	0.27	2.93	±	0.24	a
	L. botrana	3.23	±	0.23	2.30	±	0.21	a
	G. molesta	2.66	±	0.27	1.38	±	0.17	b

e

		Observed			Estimated			
Species	sex	Mean ± SEM			Mean ± SEM			
L. botrana	Female	3.39	±	0.22	1.98	±	0.28	a
	Male	1.95	±	0.24	1.15	±	0.19	b
G. molesta	Female	2.64	±	0.24	1.60	±	0.18	a
	Male	1.94	±	0.24	0.78	±	0.16	b
C. pomonella	Female	4.07	±	0.31	2.17	±	0.35	a
	Male	4.11	±	0.31	2.06	±	0.30	a

f

			Observed			Estimated			
Stimulus	Appendage	mM	Mean ± SEM			Mean ± SEM			
Salts	Antenna	10^1	1.69	±	0.16	1.27	±	0.21	b
		10^2	0.43	±	0.12	0.07	±	0.06	c
		10^3	3.33	±	0.37	2.76	±	0.30	a
	Palp	10^1	2.03	±	0.18	1.41	±	0.22	b
		10^2	1.50	±	0.21	0.95	±	0.18	b
		10^3	7.28	±	0.71	5.68	±	0.41	a
Sugars	Antenna	10^1	1.83	±	0.19	1.44	±	0.21	a
		10^2	1.40	±	0.18	1.12	±	0.18	ab
		10^3	0.97	±	0.21	0.66	±	0.14	b
	Palp	10^1	4.05	±	0.39	3.16	±	0.34	b
		10^2	6.15	±	0.51	5.12	±	0.41	a
		10^3	5.79	±	0.48	5.07	±	0.39	a

GENERAL DISCUSSION

Agriculture and pest management practices must to face the growing social concern about food safety and environmental preservation, clearly expressed in the SDGs (UN 2023). Much progress have been made in pest management, the introduction of IPM has helped to rationalize pest control, implement new control methods (e.g. mating disruption, mass trapping) and reduce counter-productive practices (e.g. over fertilization that increase aphid infestations). However, agriculture environments are constantly evolving, causing new challenges for pest management. New agriculture practices are introduced to increase production that can enhance the performance of secondary pest. For example, *G. molesta* was considered a secondary pest in apples in Girona province, but in recent years it has become an important problem (Escudero-Colomar *et al.* 2012, 2019). Probably due to the increased focus of farmers in the province on apple production, which has resulted in a great reduction of peach orchards, the main host of *G. molesta*. Global climate change is also modifying the behaviour and distribution of some pest (Skendžić *et al.* 2021), and facilitating the introduction of new invasive pest (Schneider et al 2022) together with international trade (Paini *et al.* 2016). This thesis sought to produce basic and applied knowledge to improve pest management and facilitate the development of novel control methods with the overall goal of reducing the negative effects of pest management while also limiting the losses produced by pests on agriculture production in order to address the new challenges that threaten the advancements made in IPM programs.

Two key aspect of IPM have been studied in this thesis, pest population dynamics and behaviour manipulation. Proper comprehension of pest population dynamics is especially important to schedule control methods for the time when they will have the maximum effect. Additionally, good monitoring of pest population levels helps reduce unnecessary treatments. A better understanding of the dynamics of pest populations can also aid in the implementation of novel control methods that need more accuracy to have the intended outcome.

Alternative control methods are needed to reduce the current amount of insecticide used in fruit crops and contribute to limit the negative impact of agricultural practices. Behavioural manipulation linked to olfaction perception is already exploited (e.g. mating disruption, attract and kill, push pull). But other types of stimuli, such as non-volatile chemicals, also have a great potential to be used in IPM (e.g. Arnault *et al.* 2016). Gustation in insects is not only limited to taste perception, it plays an important role in many behaviours (e.g. oviposition, food selection, inter-individual recognition). Further exploring the gustation system in insects may help introduce more gustation-based control methods into IPM. However, insect behaviour is very complex, so a thorough understanding of its functioning is needed before it can be implemented to avoid unexpected effects.

G. molesta population dynamics

Moth population dynamics is usually studied in a limited number of orchards (e.g. Kovanci & Walgenbach 2005, Damos & Savopoulou-Soultani 2010, Ahn *et al.* 2012, Damos *et al.* 2018), even with the handicap of insecticide sprays, which can produce a sudden drop in the population's levels and distort the observations. In fact, no important differences were detected in the phenology of *C. pomonella* between commercial and abandoned orchards (Joshi *et al.* 2016). This type of study relies on data from a small number of orchards deeply monitored to ensure its accuracy, and these data may not be fully representative of the whole region. In Catalonian orchards, *G. molesta* is routinely monitored. The approach used in Chapter 1 applies this data to the study of pest population dynamics. The data from standard commercial orchards needs to be processed carefully to avoid introducing neglectful data; nevertheless, it proved reliable as it detected the five generations that normally occur in the Lleida province. Later in the season, generations were more difficult to determine because adult flight periods are prone to overlap (Russell 1986, Damos & Savopoulou-Soultani 2010). The principal setback of using this type of data was that the start of the first generation was not always possible to identify. It is our responsibility to convince the technical personnel responsible for placing the monitoring traps to do it earlier so the first generation, which is also key for better monitoring (Rice *et al.* 1982).

The results showed a clear difference in population dynamics between the studied areas. In the Girona province, four generations were observed, while in the Lleida province, there were five, despite moths being captured for a similar time period in both provinces. The number of generations of *G. molesta* can vary between areas from 3 to 5 generations, from the coldest areas to the warmest areas (Croft *et al.* 1980, Rice *et al.* 1982, Kanga *et al.* 2003, Ahn *et al.* 2012). The meteorological conditions in the analysed provinces are marginally different (Table 1 in Chapter 1), however using a phenology model (Croft *et al.* 1980) to compare years and areas revealed that the Girona population had a longer generation time (~900DD). Moreover, the first moth's captures were recorded later (in DD) in the Girona province than in the Lleida province, suggesting a possible difference in response to cold temperatures between both populations. *G. molesta* shows genetic variability between close populations (Torriani *et al.* 2010, Damos *et al.* 2014), and adaptation to local temperature regimes has been reported (Ahn *et al.* 2012). However, the results of Chapter 2 did not show any difference between populations in their development time at any temperature regime tested (constant and field temperatures), rejecting this hypothesis in this case.

Host plant interaction

Another factor that could account for the difference found between population dynamics in the Girona and Lleida provinces in Chapter 1 could be the host species, which have a great impact on insect development (Schoonhoven *et al.* 2005). Myers *et al*. (2006) reported a lower larval survival in apples than in peaches. Similar, the population level detected was generally lower in the Girona province than in Lleida; being apples its main host in Girona, while peaches are its main host in Lleida. Although other factors, such as different mating disruption coverage between both provinces, could also influence the number of captures, the development time of *G. molesta* larvae is also affected by host species, being generally higher in apples than in peaches (Myers *et al.* 2007, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang et al, 2016, Sarker *et al.* 2021). The development time recorded in laboratory conditions in Chapter 2 confirms these observations. Also in Chapter 2, generation time was longer in the Girona province where *G. molesta* larvae feed upon apples, although the increment was 50 DD higher than in Chapter 1. Host species can also affect pupa and adult stages (Myers *et al.* 2006, Silva *et al.* 2010, Najar-Rodriguez *et al.* 2013, Yang et al, 2016, Sarker *et al.* 2021); therefore, part of the difference detected may be a consequence of the different stages measured. While in Chapter 1, the whole generation time was analysed, in Chapter 2, larval development time alone was recorded (at laboratory conditions).

Another factor that is not usually taken into account is the effect of excising the fruit from the tree. In Chapter 2, significant differences were detected between detached fruits at constant temperatures, detached fruits at field conditions, and attached fruits at field conditions. Such differences could be a consequence of the different maturation rates of attached vs. detached fruits (Lin & Walsh 2008; Paul *et al.* 2012, Fernández-Cancelo *et al.* 2022) and also of plant-induced defence (Mithöfer & Boland 2012, Mauch-Mani *et al.* 2017). Nevertheless, recording the air temperature in the field could have introduced some inaccuracy because it is not perfectly correlated with the actual temperature experienced by the larvae inside the fruits (Saudreau *et al.*, 2009). The actual factors that cause the increase in development time in attached fruits could not have been properly identified in this thesis; more studies should be conducted on this topic to identify them. However, if the effect of attached fruits is confirmed, it can have deep implications for development studies. Regardless, the results obtained are crucial for creating a new phenology model and help to partially explain why the population dynamics of *G. molesta*, which mainly develops on apples in the Girona province, were not predicted or explained by the model now in use in Catalonia, that of Croft, 1980, which was built on the basis of the development of *G. molesta* at constant temperatures and in a colder climate (Amat *et al.* 2021).

Oviposition behaviour

Manipulating oviposition behaviour has a great potential in the studied species as the placement of eggs by females is key to the future larval performance, and therefore they need to be properly positioned by females (Mayhew 2001, Benda *et al.* 2011). The improvement of the precision in monitoring pest populations, such as the intended in Chapters 1 and 2 can help to improve new control methods based on organic compounds that a low persistence in the field like neem oil (Campos *et al.* 2016, Kumar *et al.* 2022). Neem oil is extracted from neem tree

(*A indica*), it has been used in India for traditional plant protection and is currently receiving more attention (Kumar *et al.* 2022). Neem oil contains at least 100 biologically active compounds (Mordue & Blackwell 1993, Campos *et al.* 2016, Kumari & Kaushik 2016), which can produce insect toxicity (e.g. Pinheiro *et al.* 2009, Hasan & Ansari 2011), antifeedant response (e.g. Saxena *et al.* 1981, Prianto *et al.* 2019) and also oviposition deterrents (e.g. Naumann & Isman 1995, Erler 2004). Results from Chapter 3 showed that neem oil have a great potential to be used as oviposition deterrent of the studied species, especially for *G. molesta* and *C. pomonella*, which showed a clear deterrence. For *L. botrana* more experiments should be conducted to elucidate the factors driving the stimulation detected in the lowest concentration at the highest concentration.

Neem oil also has some volatile compounds (Swapna Sonala *et al.* 2018) that could give olfactory cues to the females. However the setup used to test the oviposition, which had 3 different sources in a confined space and with no constant airflow, should limit the possibility of females to follow olfactory cues. Unfortunately, the test designed to confirm that females were not following olfactory cues could not be done. Many oviposition arenas can be found in the literate to test non-volatiles compounds, but they are mainly designed for no-choice or dual-choice experiments (e.g. Lombarkia & Derridj 2002, 2008, Maher *et al.* 2006, Rid *et al.* 2018). Our setup allows for multiple comparisons at the same time. Even in its extreme simplification, we consider this setup can mimic more accurately the natural conditions where females encounter gradients of concentrations. Neem oil results confirms that the setup is useful to detect strong effects, however the differences in sugars and salts were very low and sometimes they contradict previous literature in the same species (Lombarkia & Derridj 2002, 2008, Maher *et al*. 2006). Differences in the oviposition setup (no-choice and dual-choice vs multiple-choice) and stimuli substrate (impermeable vs porous materials) could also have altered the results. Carrying out tests under more natural conditions is needed to demonstrate the potential of neem oil to manage *C. pomonella* and *G. molesta.*

Gustation perception

Chemical perception is the first step of the response to chemicals, therefore, electrophysiology recordings from sensilla chaetica can contribute to understand the behaviours associate to gustation, however the integration in the central nervous system also plays and important role in gustation behaviours (Scott 2018) and direct correlations between sensilla detection and behaviour may not be easily identified. It is the case between the behaviour results from Chapter 3 and electrophysiological responses from Chapter 4. Only *C. pomonella,* which showed the highest response to salt in labial palp sensilla, also had the biggest impact on the oviposition pattern in the present of salts. Unfortunately, no other correlation between spike frequency and behaviour could be detected. However, the lack of correlation may also be due to oviposition stimuli are detected elsewhere. In *L. botrana* sugar and salts are also perceived in ovipositor and tarsal sensilla (Calas *et al.* 2006, Maher *et al.* 2006). Moreover, proving direct relationship between sensilla stimulation and oviposition response is difficult to accomplish due to the need to block sensilla from other parts of the insect (e.g. Qiu *et al.* 1998).

Even though sensilla chaetica in labial palp and antennae may not be linked to oviposition behaviour they can mediate in other behaviours that can also be exploited in pest control. The tested species clearly benefit from ingesting salty water and sugar (Chapter 4, Savopoulou-Soultani *et al.* 1998, Atanassov & Shearer 2008, Wenninger & Landolt 2011, Mátray, S. & Herz 2021). Many moth species use nectar, which is rich in sugars, as an adult food source, however only one of the three test species (*L. botrana*) visits flowers (Gabel 1992), possibly to feed on nectar. Other moth species exhibit direct feeding behaviours upon antennal stimulation with sugars, this response was linked to response in the antennal sensilla (Ramaswamy 1988, Jørgensen *et al.* 2006, 2007, Jiang *et al.* 2015). On the contrary, all tested species show a low response to sugars in the antenna suggesting that they use the antenna for food searching. Tortricids rarely visit flowers for feeding (Van Zandt *et al.* 2020), of the tested species, only *L. botrana* has been observer near flowers of *Tanacetum vulgare* (L.) (Gabel 1992). Nonetheless it is a specie of the Asteracae family which have very small flowers and moths don't require extended reach to detected stimuli associated with nectar. The most noteworthy finding of Chapter 4 is the confirmation of the gustatory function of the sensilla chaetica present in the labial palps. Reports of putative gustatory sensillas (i.e. chaetica-like sensilla) in labial palps of Lepidoptera are scares (e.g. Chen & Hua 2016, Song *et al.* 2016, Hu *et al.* 2018, Vargas-Fernández *et al.* 2018), compared to the cases where studies of labial palps fail to report any putative gustatory sensilla chaetica (e.g. Bogner *et al.* 1986, Kent *et al.* 1986, Faucheux 1991, Krenn &Penz 1998, Zhao *et al.* 2013, Dong *et al.* 2014, Barcaba & Krenn 2015, Li *et al.* 2018, Chen *et al.* 2021, Abd El-Ghany & Faucheux 2022), nevertheless, no thorough search have been conducted to identify gustatory sensilla on labial palps. On the contrary, gustatory sensilla on labial palp is usual in most other insect orders (e.g., Coleoptera, Orthoptera, Diptera, Hemiptera; Chapman & Ascoli-Christensen 1999, Powell *et al.* 2006, Ohmura *et al.* 2006, Montell 2009, Barrozo *et al.* 2017, Seada & Hamza 2018, Bestea *et al.* 2021). Remarkably, the only two Lepidoptera families (Tortricidae and Cossidae) that have been probed gustatory function (Amat *et al.* 2022) or the presence putative gustatory sensilla (i.e. sensilla chaetica with a terminal pore; Hu *et al.* 2018) in the labial palps are members of ancient lineages (Kawahara & Breinholt 2014). Its seems feasible that the newest Lepidoptera lineages (which contain more nectivorous species; Van Zandt *et al.* 2020) have favoured the gustatory perception on the antennae, a more optimal place to sense flower nectar (Jørgensen *et al.* 2007), lessening the need for gustatory sensilla in labial palps.

The higher response found in Chapter 4, to both sugar and salts, in labial palps sensilla is a clear indicative that they perform a role in the studied species. However, the implications and actual function of the labial palp sensilla chaetica remains unknown. Not much is known about the use of labial palps in moths, beside their implication in CO_2 detection of the Labial Pit Organ (Guerenstein & Hildebrand 2008). Two trotricid species have been observed to make contact with labial palps during sexual behaviour (Baker & Cardé 1979, Grant 1987). Thus, sensilla chaetica on palp and antennae may be used to detect conspecific signals, such as cuticular hydrocarbons, which often present sexual dimorphism in insects (Ferveur 2005, Gemeno *et al.* 2012). Also, *Cactoblastis cactorum* (Berg) females actively touch the plant with the labial palps while searching for oviposition sites (Myers *et al.* 1981) and may use the gustatory sensilla to identify the substrate, if they bear any.

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CONCLUSIONS

The general principles of IPM described in Annex III of the EU Directive on the Sustainable Use of Pesticides (2009/128/EC) establish in their second point: "Harmful organisms must be monitored by appropriate methods and tools, when they are available", which has been the main objective of Chapters 1 and 2. The fifth point of the same directive establishes: "The pesticides applied... will have the least secondary effects on human health, non-target organisms, and the environment". In Chapters 3 and 4, the taste systems of three important pests have been explored to help develop new control methods based on specific behavioural manipulation, which will help achieve the fifth point above mentioned.

Chapter 1: Different population phenologies of *Grapholita molesta* (Busck) in two hosts and two nearby regions in the NE of Spain

1) The population dynamics of *G. molesta* have been assessed in the Girona and Lleida provinces:
   - Both provinces have a similar flight period from March until October, with a slight difference in the start, which happens 1-2 weeks later in Girona.
   - Generation time is ~90DD longer in Girona than in Lleida
   - Four flights are shown by *G. molesta* in the Girona province and five in Lleida.
   - Population levels were usually higher in Lleida than in Girona province.
2) The phenology model that is normally used in the whole of Catalonia fails to properly predict the phenology of *G. molesta* in Girona, even when considering the prolonged development time reported when feeding on apples.
3) The identification of four generations of *G. molesta* in the Girona province will help to improve the control methods and the IPM program.
4) The system used for analyzing the population dynamics (data from traps in commercial orchards and a phenology model to compare between years and regions) proved reliable and can facilitate the study of pest population dynamics.

Chapter 2: Host-Pest Interactions: Investigating *Grapholita molesta* (Busck) Larval Development and Survival in Apple Cultivars.

5) Individuals from Girona and Lleida populations showed a similar development time and survival in apples, suggesting that: (i) no significant adaptation of the Girona population to apples has occurred; (ii) *G. molesta* can easily adapt to secondary hosts.
6) The development time of larvae in detached apple fruits from local cultivars at constant temperatures was consistent with previous reports; however, it was significantly longer in

field detached fruits and especially in field attached fruits. Attached fruits also significantly reduced the survival of *G. molesta* larvae.

7) The different responses of *G. molesta* to the studied cultivars, both in terms of development time and survival, observed in field studies with fruits attached to the tree and detached from it, as well as in laboratory studies with fruits detached from the tree, point to a complex host-pest interaction. Further analysis is required of the effects of using studies on detached fruits to adjust phenology models.
8) The longer larval development time reported at field conditions further confirms the lack of fit of the phenology model to the specific conditions experienced by *G. molesta* in the Girona province. The data obtained during this thesis will help create a new phenology model.

<u>Chapter 3</u>: Oviposition by three tortricid moths on filter papers treated with different concentrations of sugars, salts or neem oil.

9) A quick and simple method has been developed and tested to study oviposition behaviour for different concentrations of non-volatile compounds.
10) Differential oviposition behaviour to different concentrations of the same stimuli should be considered in the development of oviposition based control strategies.
11) Neem oil seems a good candidate to further study its potential in pest management as an oviposition deterrent for *C. pomonella* and *G. molesta*.
12) Sugars and salts had a low impact on oviposition behaviour of all tested species in the conditions tested.

<u>Chapter 4</u>: Gustatory function of sensilla chaetica on the labial palps and antennae of three tortricid moths (Lepidoptera: Tortricidae).

13) The presence of sensilla chaetica with gustatory function in the labial palps has been confirmed for the first time in Lepidoptera.
14) The higher response of sensilla on labial palp than on antennae to the tested stimuli suggest that they perform a role in the perception system of the studied species. Further studies should confirm their role in insect behaviour.
15) Sucrose elicited the highest GRN response, suggesting that it may play a more important role than fructose for the studied species.
16) There does not seem to be a direct correlation between GRNs response to sugar and salt stimulation and oviposition behaviour to the same compounds.
17) The accumulated knowledge on the gustatory system of the studied species will facilitate the study of other compounds (e.g. secondary plant metabolites) that can be applied in pest control techniques.