Graduate Programme
"Molecular Biology & Biomedicine"

Master thesis

"*In silico* and *in vivo* characterization of the ecdysone importer Oatp74D in Lepidoptera."

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Heraklion
November 2021

Acknowledgments

First of all I would like to thank the committee, Professor John Vontas (Agricultural University of Athens, IMBB-FORTH), Res. Inga Siden-Kiamos (IMBB-FORTH, University of Crete) and Res. Pavlos Pavlidis (ICS-FORTH, University of Crete) for the supervision, the advice and the corrections of my thesis report. Moreover I would like to thank Dr. Shane Denecke and Phd candidate George Samantsidis for supervising and advising during the whole period I was doing my master in Molecular Entomology / Pesticide Science Lab (Vontas Group). Also, I would like to thank all the members of the lab and of the Evolab for the help and the wonderful collaboration and particularly Panos Ioannidis, Jason Charamis, Eva Skoufa and Rafaela Panteleri for providing me with useful tips and guidelins and making lab time amusing. Next, I owe my thanks to Yannis Livadaras (IMBB/FORTH) for performing all the injections to the *Drosophila* and *S. frugiperda* embryos. Finally, and most importantly, I am grateful to my friends, especially Maria, to Fotis and of course to my family for always supporting me. Without them this year would not be the same in many ways.

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Abstract

Insect pests damage agricultural production to a great extent, imposing significant costs to global economy. At the same time, the intensive use of insecticides often brings about populations resistant to the existing insecticides. At present, two major lepidopteran pests are *Helicoverpa armigera* and *Spodoptera frugiperda*. These species are considered to be so destructive, because they are polyphagous, invasive, widely distributed and have already developed resistance to existing insecticides via target-site mutations. Thus, the discovery of new targets for drug development, in order to control their populations, is of utmost importance.

Recent research in the model insect *Drosophila melanogaster* reports the role of a plasma membrane transporter referred as Ecdysone Importer (EcI), also known as organic anion transporting polypeptide 74D (Oatp74D), in the development of *D. melanogaster.* Ecdysone is a steroid hormone that regulates several physiological processes. When ecdysone enters the cell it binds and activates an intracellular nuclear receptor (EcR) which is then able to induce the transcription of several genes essential for the development of the organism. It was shown that *EcI* is necessary for ecdysone signaling. This transporter belongs to a specific subclade of the Solute Carrier O (SLCO) family of transporters, which is well represented in insects while being absent in mammals. These two characteristics of *EcI*, the essentiality for the development of the organism and the insect specificity, render it a potential target for the development of insecticides. However, further analysis of this transporter is needed in pest species to prove whether it is appropriate as a drug target.

In this study, we constructed a phylogenetic tree of the SLCO transporters of several ecdysozoa, organisms that utilize ecdysosteroids. The gene family tree provided information on which species have potential orthologs of *Oatp74D* and showed that this gene probably appeared before the evolution of arthropods, but after the evolution of ecdysozoa. After finding the potential orthologs of *Oatp74D* in the pests of our interest, we proceeded to functional characterization of the lepidopteran *Oatp74D*. By making use of CRISPR Cas9 system we managed to generate mosaic knockouts of *Oatp74D* in *S. frugiperda* embryos

and found that this gene is necessary for the development of the organism at embryonic and larval stages.

Περίληψη

Τα έντομα παράσιτα βλάπτουν την αγροτική παραγωγή σε μεγάλο βαθμό, επιβάλοντας σημαντικά κόστη στην παγκόσμια οικονομία. Την ίδια στιγμη, η εντατική χρηση εντομοκτόνων οδηγεί συχνά σε πληθυσμούς ανθεκτικούς στα υπάρχοντα εντομοκτόνα. Τη σημερινή εποχή, δύο σημαντικά παράσιτα-λεπιδόπτερα είναι η *Helicoverpa armigera* και η *Spodoptera frugiperda.* Αυτά τα είδη θεωρούνται τόσο καταστροφικά, επειδή είναι πολυφάγα, εισβάλλουν σε νέες καλλιέργειες, εντοπίζονται σε μεγάλο εύρος περιοχών και έχουν ήδη αναπτύξει ανθεκτικότητα σε υπάρχοντα εντομοκτόνα, μέσω σημειακών μεταλλαγών. Επομένως, η εύρεση νέων στόχων για ανάπτυξη εντομοκτόνων, για την αντιμετώπιση αυτών των πληθυσμών, είναι ύψιστης σημασίας.

Πρόσφατη έρευνα στο έντομο-μοντέλο *Drosophila melanogaster* αναφέρει το ρόλο ενός μεμβρανικού μεταφορέα γνωστό ως Ecdysone Importer (EcI) ή αλλιώς organic anion transporting polypeptide 74D (Oatp74D), στην ανάπτυξη της *D. melanogaster.* Η εκδυσόνη είναι στεροειδής ορμόνη που ρυθμίζει διάφορες φυσιολογικές διεργασίες του οργανισμού. Μόλις η εκδυσόνη εισέλθει στα κύτταρα, προσδένεται και ενεργοποιεί ενα ενδοκυττάριο πυρηνικό υποδοχέα (EcR), ο οποίος μετά είνια ικανός να επάγει την μεταγραφή διάφορων γονιδίων αναγκαίων για την ανάπτυξη του οργανισμού. Έχει δειχθεί οτι το *EcI* είναι απαραίτητο για τη σηματοδήτηση από εκδυσόνη. Ο μεταφορέας αυτός ανήκει σε ένα συγκεκριμένο υποκλάδο της Solute Carrier O (SLCO) οικογένειας μεταφορέων, που εμφανίζεται εκτενώς στα έντομα αλλά απουσιάζει από τα θυλαστικά. Αυτά τα δύο χαρακτηριστικά του *EcI,* η αναγκαιότητά του για τον οργανισμό και η ειδικότητα υπαρξής του στα έντομα, τον καθιστούν ένα πιθανό στόχο για την ανάπτυξη εντομοκτόνων. Ωστόσο, περαιτέρω ανάλυση αυτού του μεταφορέα χρειάζεται να γίνει στα έντομα-παράσιτα προκειμένου να δειχθεί αν είναι κατάλληλος ως στόχος.

Σε αυτή τη μελέτη, κατασκευάσαμε ένα φυλογενετικό δέντρο των SLCO μεταφορέων διάφορων εκδυσόζωων, οργανισμών που χρησιμοποιούν εκδυστεροειδή. Το δέντρο αυτής της οικογένειας γονιδίων παρείχε πληροφορίες σχετικά με το ποια είδη έχουν πιθανά

ορθόλογα του *Oatp74D* και έδειξε ότι αυτό το γονίδιο πιθανότατα εμφανίστηκε πριν την εξέλιξη των αρθρόποδων, αλλά μετά την εξέλιξη των εκδυσόζωων. Μετά τον εντοπισμό των πιθανών ορθόλογων του *Oatp74D* στα υπό μελέτη παράσιτα, προχωρήσαμε σε λειτουργικό χαρακτηρισμό του αντίστοιχου γονιδίου στα λεπιδόπτερα. Χρησιμοποιώντας το CRISPR Cas9 σύστημα καταφέρμε να δημιουργήσουμε μοσαϊκά knockouts του *Oatp74D* σε έμβρυα *S. frugiperda* και βρήκαμε ότι το συγκεκριμένο γονίδιο είναι αναγκαίο για την ανάπτυξη του οργανισμού σε εμβρυικό και λαρβικό στάδιο.

1. Introduction

1.1 Lepidoptera as agricultural pests

1.1.1 Agricultural Pests

Global population is continuously increasing and is estimated to reach 9.7bn in 2050 and 10.9bn in 2100 accodring to United Nations. This has created a concern about food production, which needs to grow proportionally in order for the population to sustain this increase (Stefanis, 2014). While demand on food production is growing, destructive animal species continue causing damage to agriculture. It is referred in the literature that crop pests are responsible for 30-40% of crop losses (Flood, 2010) and many of them are likely to become the more invasive in coming years (Bebber, Holmes and Gurr, 2014). For all these reasons, food security and particularly control of pest populations is a challenge that must be overcome.

1.1.2 *H. armigera* and *S. frugiperda*

Lepidoptera constitute a diverse order of crop pests. Even though adult moths and butterflies are usually beneficial insects that feed on nectar and act as pollinators, caterpillars (larval stages of Lepidoptera) are typically voracious feeders of plant material. The order of Lepidoptera includes two major global pests, *H. armigera* (Flood, 2010; Cunningham and Zalucki, 2014) and *S. frugiperda* (Luginbill, 1928). *H. armigera* is widespread (Fig.1A) throughout Europe, Africa, Asia, and Australia (Zalucki *et al.*, 1986) and has recently been detected in the Americas (Kriticos *et al.*, 2015). Some of its most significant hosts are cotton, oilseeds, corn and several vegetables (Luginbill, 1928). (Natália A Leite *et al.*, 2014) reports that *H. armigera* lead to a loss of more than US$2 billion to the Brazilian agriculture. Another pest of corresponding economic significance is fall armyworm (FAW), *S. frugiperda*. FAW is a cosmopolitan pest, present in 107 countries worldwide (*EPPO*, 2021a), (Fig.1B). Its high reproduction capacity, polyphagy and ability to migrate long-distance regions render it a very invasive pest (Barros *et al.*, 2010; Westbrook *et al.*, 2016). FAW feeds on 353 known plant species (*EPPO*, 2021b) including corn, rice, maize, wheat and cotton. According to (Hruska

and Gould, 1997) maize yield suffered a loss up to 73% when culture was infested by *S. frugiperda.* Taking all these into account, in the context of food security, the control of these two lepidopteran pests (Fig.2) is of utmost importance.

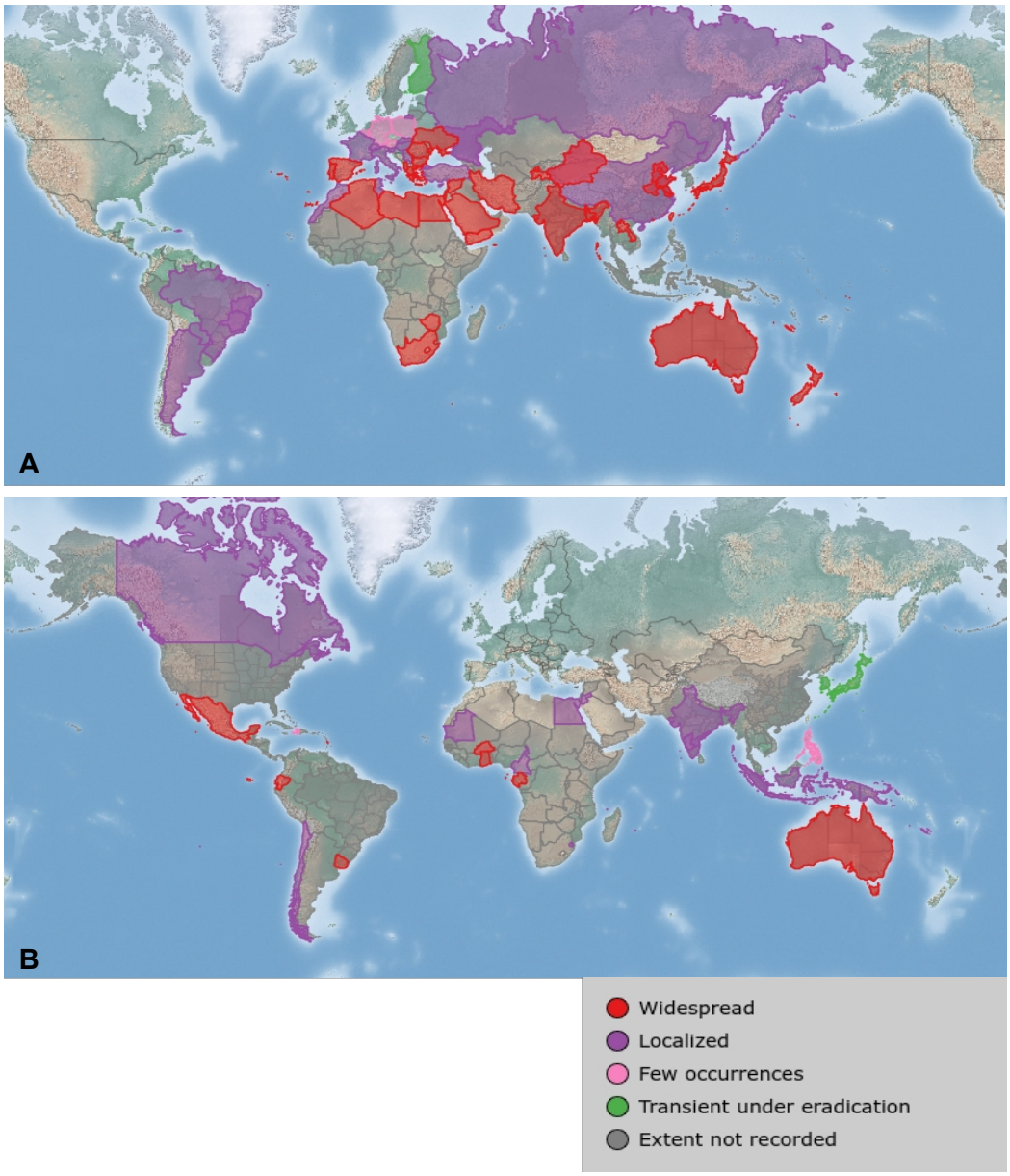


Figure 1: Global distribution of *H. armigera* and *S. frugiperda*

Colors represent the extent of distribution as shown on the table. (A) Distribution of *H. armigera.* (B) Distribution of *S. frugiperda.* Adapted from CABI (2021).

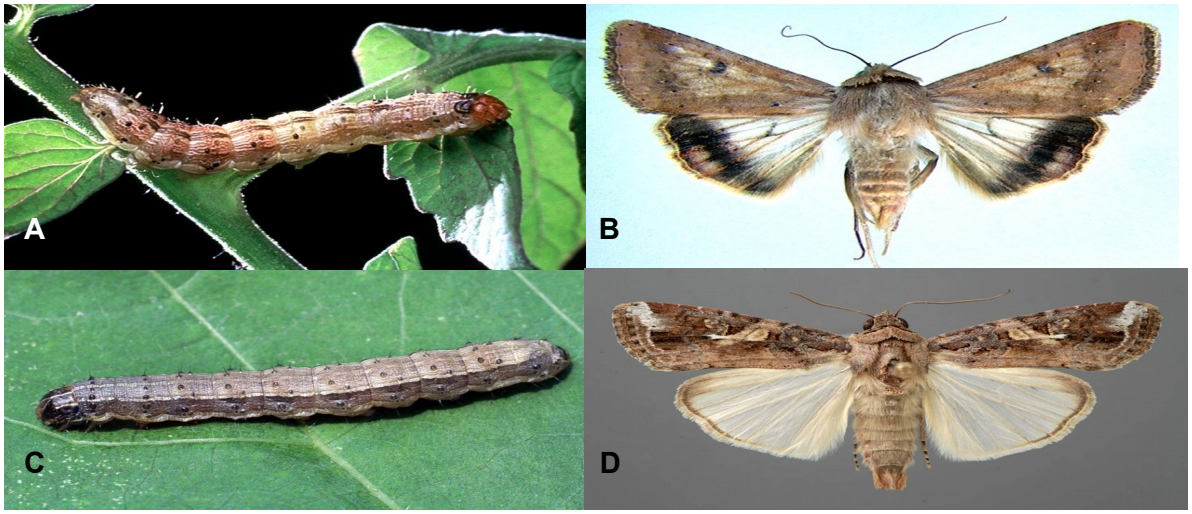


Figure 2: Lepidopteran pests: *H. armigera* and *S. frugiperda*

Larval (A, C) and adult (B, D) stages of *H. armigera* and *S. frugiperda* respectively. Adapted from CABI (2021).

1.1.3 Pest control: Use of insecticides

In order to efficiently control pest populations, which destabilize food production and thus global economy, significant research effort has been spent. Non-chemical methods like genetically-modified crops and Sterile Insects Techniques are applied, but on a relatively small scale due to regulation, scale-up, and efficacy issues. Chemical methods have been widely used for several decades, and have a strong track record of real world efficacy. They are intensively applied globally and new insecticides are constantly being developed.

A limitation inherent to chemical pesticides is the ability of insects to develop insecticide resistance. With the term “insecticide resistance” we refer to the heritable shift in the tolerance of the pest population expressed in natural or chemical compounds, leading to a repeated failure to achieve the desired level of control (Insecticide Resistance Action Committee, https://www.irac-online.org/). Historically, resistance has started developing very soon after the introduction of a pesticide; the two-spotted spider mite (Tetranychus urticae) was the first greenhouse pest to develop resistance back in 1949 (Chattopadhyay, Banerjee and Mukherjee, 2017). Since then, every new insecticide entry has been accompanied by resistance development because of the genetics of heritable resistance and the intensive

repeated application of pesticides. More precisely, in cases of pre-adaptation resistance alleles exist naturally in populations and can be part of their genetic variation. The use of insecticides exerts selection pressure to pest population, which enables some initially very rare, pre-adapted insects with resistance alleles, to survive and pass on their offspring the resistance trait. However, there are cases in which the resistant allele does not pre-exist, but emerges over time due to a novel mutation. While continuing to administer insecticides with the same mode of action, selection of resistant individuals continues to increase the proportion of resistant insects in the population, while the insecticide removes susceptible individuals (Liu, 2015). Thus, under this permanent selection pressure, resistant insects outnumber susceptible ones and the insecticide is no longer effective.

1.2 Insecticide targets

1.2.1 Need for new targets

The relatively limited number of available molecular targets for insecticides, along with the emergence of resistance and the regulatory limitations imposed by public health and environmental concerns, have generated an urging need to identify new potential insecticide targets.

H. armigera and *S. frugiperda* have already developed resistance against existing insecticides and target-site mutations are among the main

mechanisms of resistance that have been identified. In *H. armigera* the first cases of insecticide resistance were observed since 1980 (Ahmad and McCaffery, 1988; Armes *et al.*, 1992) and today there are still cases of resistance evolving (Nimbalkar *et al.*, 2009; Mironidis *et al.*, 2013). Concerning *S. frugiperda,* 144 cases of insecticide resistance have been identified globally, based on the Arthropod Pesticide Resistance Database (APRD) (*APRD*, 2021). Among the 41 different active substances affected, 45% of the cases belong to proteins produced by *Bacillus thuringiensis* (Bt), 26% and 19% to insecticides targeting the voltage-gated sodium channel (VGSC), and acetylcholinesterase (AChE), respectively (Boaventura *et al.*, 2020). The economic impact of *H. armigera* and *S. frugiperda* on

agriculture in combination with the continuous arise of insecticide resistance cases renders the development of new insecticides to control their populations necessary.

1.2.2 Insecticide target characteristics

The term insecticide target refers to a molecule, usually a protein, in the body of the pest that is intrinsically associated with a vital process. Insecticides, either as agonists or as inhibitors, disrupt the proper function of the target protein and thus the vital processes it mediates, thereby causing lethality to the pest. For this reason, the physiological role of a potential target needs to be known.

Insecticidal molecules usually reach their target via spray or plant mediated applications and can efficiently kill the pest. The efficacy of an insecticide strongly depends on its ability to reach the sufficient rate and amount in pest's body. In cases where the target is not directly accessible by the insecticide, limitations leading to low bioavailability may reduce the drug's efficacy (Bonning and Chougule, 2014). Thus, easily accessible targets, usually transmembrane proteins on epithilial tissues, are preferred.

In the process of drug discovery, druggability, which is the ability to design a drug for the potential target, needs to be considered. Many drugs act as inhibitors that imitate the structure of the natural substrate and bind to the target with higher affinity than this of the natural substrate. In terms of druggability, major efforts are being made to optimize drug development. Based on (Zheng *et al.*, 2006) there are specific structural and physicochemical properties of high-affinity binding sites that render a target protein druggable. In cases that the protein structure is unknown, analysis on hydrophobicity, protein length, mean pI, membrane location, specific post-translational modifications and protein motifs can be done in order to predict whether a potential target is likely to be druggable (Bakheet and Doig, 2009).

Apart from essential, accessible and druggable, an insecticide target needs to be species-specific to the desired extent. Insecticides that are carried by air or water may kill non-target species such as pollinators. This sequentially may lead to a gradual destabilization of the local ecosystem which could damage not only the environment but also the agricultural economy. A well-known example of such case is DDT. DDT was the first

synthetic insecticide that was intensively used, as it was believed to be of low toxicity towards humans (Jarman and Ballschmiter, 2012). It took only a few years until the chronic toxicity of DDT to non-target species, such as reptiles and mammals, was identified (Carson, 1962) Since then, potential off-target effects of insecticides are examined thoroughly. The specificity of a drug is determined based on its commercial use. Insecticides that kill more than one pest species, but not beneficial ones, provide a wider variety of applications and thus are preferred.

1.2.3 Oral insecticides and midgut targets

As mentioned above, insecticide targets need to be sufficiently accessible by the pesticide. Many insecticides are oral and are typically delivered via feeding. After they arrive at the alimentary canal (gut) and reach the midgut, they penetrate in order to enter the hemocoel (Fig.3). Then, they transfer through the hemocoel until they reach the tissues (nerve, muscle, etc.) which they specifically target. Different types of insecticides use a variety of mechanisms in order to enter or cross the midgut epithelium (Denecke *et al.*, 2018) (Fig.3).This step of gut penetration is the critical step which determines whether lethality will be achieved or not, because it affects bioavailability and thus, the final concentration of the insecticide in target tissues. The immediate contact of gut tissues with oral insecticides, in combination with the limitations of drugs' efficiency due to gut penetration render the proteins of midgut epithelium ideal as insecticide targets, in terms of accessibility.

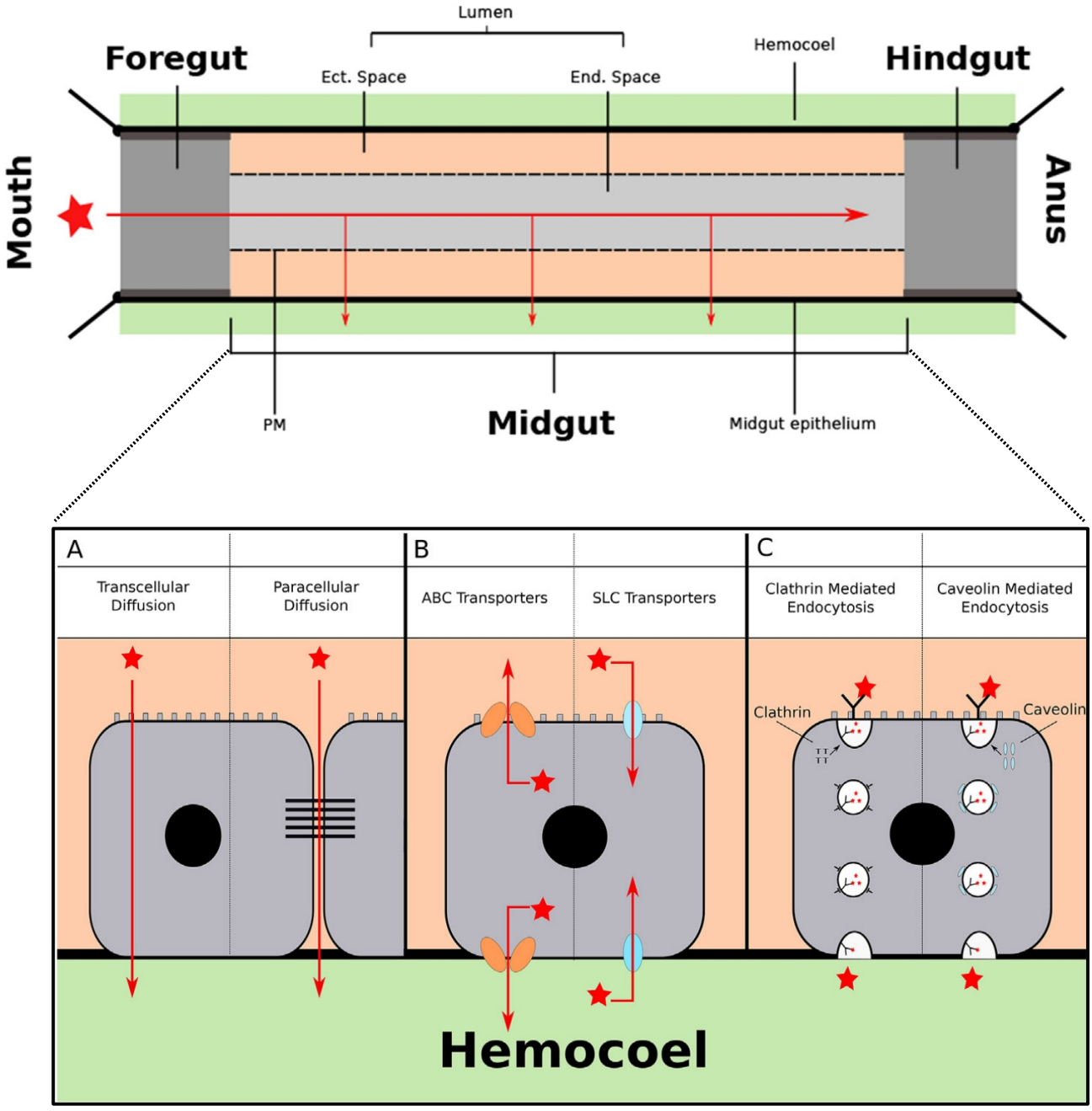


Figure 3: Rout of oral insecticides through the alimentary canal.

As an insecticide (red star) enters the gut, it arrives to the midgut and penetrates it via different pathways (red lines) to enter the hemocoel (green). The penetration pathway depends on the nature of the insecticide and can include transcellular or paracellular diffusion (A), active or passive protein transportation (B) or endocytosis. Adapted from (Denecke *et al.*, 2018)

1.3 Ecdysone: Endocrine control of development

1.3.1 Insect development

The key behind finding a new or innovative control strategy against insect pests is strongly connected with the available knowledge in organismal and physiological level. Understanding of fundamental processes like molting and metamorphosis that undergo development in insects is important in order to efficiently control pest populations. The life cycle of insects includes a number of transition stages characteristic for every species (Wolfgang and Riddiford, 1981; Esperk, Tammaru and Nylin, 2007). The hatching of the egg is followed by larval stages which are named instars. During transitions between larval stages, insects shed parts of their body and this periodic process is known as molting or ecdysis. The last molting in insects life leads to adult stage and is called metamorphosis. All these molting events are necessary for insects because they enable them to grow in size. More specifically, insects are covered by a rigid structure, exoskeleton, which protects and supports their body. However, this rigid structure constrains growth and changes in morphology. Thus, in order for the insect to grow in size and finally transform to adult, exoskeleton needs to be shed and replaced at different time points during development.

1.3.2 Hormonal regulation

Research on the mechanisms that regulate stage transitions in insects development began almost a century ago (Kopec, 1917). Major efforts have been made on this field and it is known today that insect developmental processes are under strict hormonal regulation. The key hormones and neuropeptides that act as regulators are prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone (Fig.4A). PTTH is a neurosecretory hormone. It is produced by the medial neurosecretory cells of the brain and released in the hemolymph. In most insects the neurosecretory cells of PTTH are located in corpora cardiaca. However, exceptions have been found in some Lepidoptera (Frederik Nijhout, 1975) and cyclorrhaphan Diptera (Roberts, Gilbert and Bollenbacher, 1984), in which secretion occurs in the corpora allata and the ring gland respectively. When PTTH is released in the hemolymph it reaches the prothoracic gland (PG), an endocrine tissue, and

stimulates it to express the biosynthetic enzymes that will finally produce ecdysone (Rewitz *et al.*, 2006; Huang, Warren and Gilbert, 2008). Ecdysone is considered to be the master regulator of development in insects. However, the key event that determines the timing of developmental transitions is secretion of PTTH.

Ecdysone, when converted to its active form, has double role in insects' development, depending on its concentration. The first form of ecdysone produced by PG is relatively inactive. Through circulation ecdysone reaches peripheral tissues and becomes activated by a P450 monooxygenase named Shade (Petryk *et al.*, 2003). This active form of ecdysone is called 20-hydroxyecdysone (20E) and can activate transcription factors that regulate gene expression in target cells. At low concentration, 20E stimulates several issues, mostly gut, muscles and fat body, and induces mitosis (Kato and Riddiford, 1987; Quinn *et al.*, 2012; Nijhout *et al.*, 2014). In this process, the role of insulin is major as it initiates the uptake of nutrients and protein synthesis (Britton *et al.*, 2002; Léopold and Layalle, 2006). It is the combinatorial act of insulin and low amounts of 20E promotes cell proliferation and insect growth. At high concentration, 20E acts directly on epidermal cells and induces differentiation (Champlin and Truman, 1998; Smagghe *et al.*, 2005; Nijhout *et al.*, 2007). An entire sequence of cellular events constitute the process of molting: detachment of the epidermis from the overlying cuticle (apolysis), secretion of molting fluid into the intervening space, cell division, and differentiation of new epidermal organelles such as a sensory hair and its socket (Nijhout, 1994). *In vitro* experiments have shown that ecdysone is both necessary and sufficient to stimulate molting (Marks, 1972; Noriaki Agui, 1973). Apart from molting, *in vivo* studies in *Drosophila melanogaster* have found that ecdysone regulates growth in insects (Mirth and Riddiford, 2007; Colombani, Andersen and Léopol, 2012).

As mentioned above, molting is a periodic phenomenon in the life of insects, that is regulated by oscillations in ecdysone production. Every molt corresponds to an ecdysone pulse (Fig.4B) and every pulse can be divided in three phases, a preparatory phase, a cuticle induction phase and an ecdysis phase (Nijhout, 1994). These phases are characterized by different levels of E or 20E which lead to differential gene expression (Clever and Karlson, 1960).

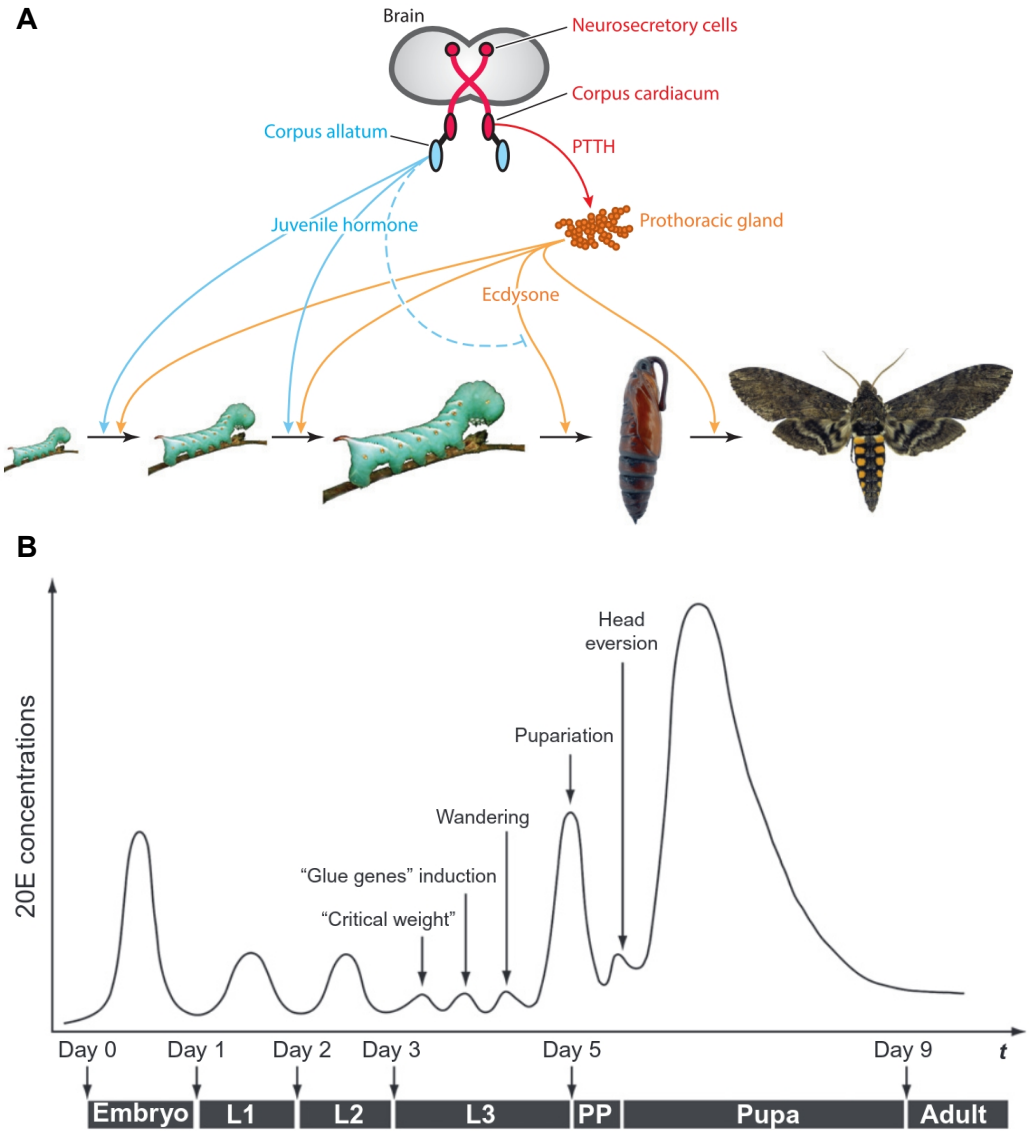


Figure 4: Endocrine regulation of insects development

(A) Prothoraciotropic hormone (PTTH) produced by the brain and released from the corpora cardiaca induces the production of ecdysone from the prothoracic gland. Oscillations in the concentration of ecdysone direct periodic molts in the life cycle of insects. The nature of molts, growing or metamorphic, is determined by juvenile hormone (JH). JH is released by corpora allata and during its presence metamorphosis is suppressed. In the last larval instar JH inhibits ecdysone and pupation can occur only when JH is cleared. Adapted from (Nijhout and Callier, 2015) (B) Ecdysone pulses direct developmental transitions from stage to stage. The highest peaks of ecdysone during insects life are at embryonic stage, before pupation and during the pupal stage. Adapted from (Ou and King-Jones, 2013)

1.3.3 Ecdysone signaling

Ecdysone is a steroid hormone fundamental for insects development. Steroid hormones constitute a group of lipophilic molecules that control various biological processes by regulating gene expression (Sapolsky, Romero and Munck, 2000; Rhen and Cidlowski, 2005; Oakley and Cidlowski, 2011). It is well known that, the endocrine system of organisms undergoes strict regulation. Destabilization of its proper function and specifically of steroid hormones homeostasis can lead to a variety of pathological processes, such as inflammatory disorders (Clemons and Goss, 2001; Rhen and Cidlowski, 2005; Attard, Cooper and de Bono, 2009). However, few studies on this field have been made on non-mammalian species. Steroid hormones are highly conserved among animals and plants. The general mechanism of their function includes entry to the cell, binding to a nuclear receptor that is located intracellularly and formation of an active complex, which can enter the nucleus and act as transcription factor (Mangelsdorf *et al.*, 1995; Nilsson *et al.*, 2001; McKenna and O'Malley, 2002; Kirst and Thummel, 2005; Evans and Mangelsdorf, 2014). Until recently it was believed that lipophilic steroid hormones can freely enter the cell by simple diffusion (Nussey and Whitehead, 2001; Alberts et al., 2015; Urry et al., 2017). Even though several studies have suggested protein mediated transportation of these hormones, no paradigm proving this mechanism existed (Milgrom et al., 1973; Rao et al., 1976; Pietras and Szego, 1977). It was in 2018 that (Okamoto *et al.*, 2018) proposed a model of facilitated diffusion of ecdysone in *D. melanogaster* by a membrane transporter, named Ecdysone Importer (EcI), also referred as organic anion transporting polypeptide 74D (Oatp74D). This non-conventional model (Fig.5) suggests that ecdysone is transported to the cytoplasm of target cells by EcI and there, its active form 20E binds ecdysone receptor (EcR), which is a nuclear receptor. This active complex enters the nucleus and forms an heterodimer with another nuclear receptor named Ultraspiracle (Usp) and regulates the transcription of genes necessary for developmental processes like molting and metamorphosis (Thummel, 1996; Riddiford, Cherbas and Truman, 2000; Yamanaka, Rewitz and O'connor, 2013). In this study it was shown that EcI is necessary for the development of *D. melanogaster.*

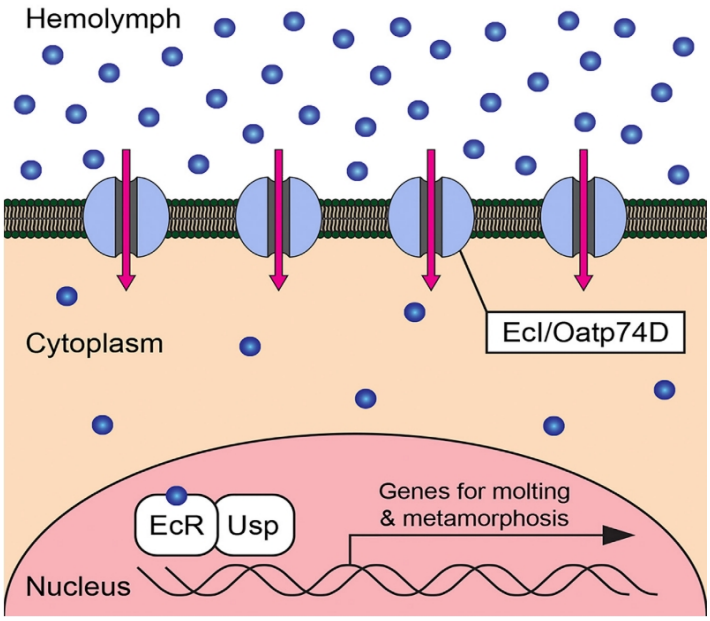


Figure 5: Facilitated Diffusion Model of Ecdysteroid Uptake by Target Cells

EcI/Oatp74D is required for facilitating cellular uptake of ecdysteroids (blue circles) from the hemolymph into cytoplasm down the concentration gradient. The nuclear receptor complex composed of EcR and Usp regulates the transcription of genes for molting and metamorphosis in the nucleus. Reprinted from (Okamoto *et al.*, 2018)

1.4 SLCO transporters and Oatp74D as a novel insecticide target

1.4.1 SLCO family

The ecdysone importer (EcI), also referred as Oatp74D, belongs to the SLCO (former SLC21) family which consists of organic anion transporting polypeptides (OATPs). SLCO transporters are only a few members of the solute carrier (SLC) superfamily which consists of hundreds of membrane transporters ubiquitously expressed across the tree of life. Classification of SLC members is done based on protein function and not on gene homology. SLCs transport a wide range of substrates that can be either hydrophobic or lipophilic to several degrees (Hediger *et al.*, 2004). Concerning the function of OATPs, it is known that they mediate the sodium-independent transportation of various substrates such as nutrients, ions and xenobiotics, via facilitated diffusion or via taking advantage of existing

electrochemical gradient, without hydrolyzing ATP (Hagenbuch and Stieger, 2013a). Until now, major efforts have been made for the functional characterization of mammalian SLCs as drug targets (Estudante *et al.*, 2016). Humman Oatps are also studied intensively, due to their pharmacological significance (Zhang *et al.*, 2006, 2008; Huang *et al.*, 2008; Giacomini *et al.*, 2010; Zhang, Huang and Lesko, 2011; Fenner *et al.*, 2012; Tweedie *et al.*, 2013). Analysis have shown that these transporters affect the uptake and deposition of a wide array of drug compounds (Schulte and Ho, 2019), while their role as drug targets in human diseases, such as cancer, is emerging (Schulte and Ho, 2019). Although these transporters have been extensively studied in mammals, little is known about their role in other species. SLCs have only been systematically identified in *Drosophila melanogaster*, *Aedes aegypti* and *Anopheles gambiae* (Höglund *et al.*, 2011; Elbourne *et al.*, 2017), while non-mammalian OATPs have been analyzed mostly in *D. melanogaster (Torrie et al., 2004)*. However, most recent studies focus on the identification of SLC transporters as a whole on arthropod species. Better knowledge on the SLC superfamily will enable further analysis of arthropods physiology.

1.4.2 Structure of the SLCO transporters

Concerning the general structure of SLCOs, it is known that they have 12 transmembrane domains which from an aquatic pore for substrate's transportation and both their N- and C-terminal ends located on the cytoplasmic side (Jacquemin *et al.*, 1994; Kullak-Ublick *et al.*, 1995). Experiments using side-directed mutagenesis have shown that these transporters often undergo post-translational modifications such as N-glycosylation (Wang *et al.*, 2008; Yao *et al.*, 2012) and that the formation of disulfide bonds may be crucial for their proper targeting to the plasma membrane (Hänggi *et al.*, 2006). However, the absence of crystallographic structures for these transporters still remains a problem. Furthermore, there still remains much to be done in the way of genetic characterization of these genes, especially in insects.

1.5 Genetic tools

1.5.1 Reverse genetics using CRISPR-Cas9 technique

Reverse genetic approaches are widely used to interrogate the functions of genes of interest. For this reason, a variety of methods have been developed that enable the insertion, deletion or substitution of DNA sequences at precise locations in the genome. The most common strategy in order to assess the essentiality of a gene is to knock it out either in specific tissues or in the whole organism that is being examined. Knocking out genes has been significantly helped by the increasing efficiency of CRISPR (Clustered, regularly interspaced, short palindromic repeats)-Cas9 technology. In this technology, Cas9 endonuclease is guided by a 20nt single-guide RNA to generate sequence specific double stranded breaks (DSBs) (Jinek *et al.*, 2012; Wiedenheft, Sternberg and Doudna, 2012). DSBs stimulate the cell to recruit enzymes that will repair these breaks. DNA repair is done either through the Non-Homologous End Joining (NHEJ) pathway or through the Homologous-Directed Repair (HDR) pathway (Bassett *et al.*, 2013). In the first case, NHEJ pathway generates small random insertions or deletions. These often alter the open reading frame of the gene and result in a protein product that is totally different form the initial, thus leading to a knockout (Bibikova *et al.*, 2002). Knockout animals can be generated by directly injecting Cas9 and sgRNAs into the embryo. This may lead to mosaic animals that do not have a uniform phenotype, if mitotic events have occurred before the introduction of the reagents. However, mosaic knockout animals can still be informative when assessing the essentiality of a gene. If the reduction of a protein, but not its elimination, is lethal for the organism, this is a strong evidence that the knockout gene is essential.

1.6 Aim of this study

The scope of this study is the *in silico* and *in vivo* characterization of the lepidopteran Oatp74D as a novel insecticide target. First, a phylogenetic tree was constructed using all Oatp transporters of species from all orders of ecdysozoa, in order to unravel the evolution of *EcI* and determine when an *Oatp74D*-like gene evolved. Then, via injections in *S. frugiperda* embryos, CRISPR-Cas9 technology was applied to knockout *EcI*. Mortality was measured for mosaic knockout animals and the essentiality of *EcI* for *S. frugiperda* was assessed.

2. Materials and methods

2.1 Phylogenetic analysis

For the phylogenetic analysis the ecdysozoan species listed on Table 1 were selected. The reference gene annotations and proteomes of those species were downloaded from NCBI and were filtered in order to contain only one amino acid sequence per gene, representing the longest isoform. Then, the SLC_id pipeline (Denecke *et al.*, 2020) was applied on the filtered proteomes to select the OATP transporters of the selected ecdysozoa. Multiple sequence alignment was performed for the amino acid sequences of the identified OATPs (Table S1) with Mafft v7.310 (Katoh and Standley, 2013) using the default parameters. The produced alignments were automatically trimmed using Trimal (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). Finally, the phylogenetic tree was built under the maximum likelihood optimality criterion by making use of RaxML 8.2.11, with the parameter "-N 500" for 500 bootstraps (Stamatakis, 2014). The ggtree was visualized with the ggtree package in R (Stamatakis, 2014).

2.2 CRISPR knock out in *S. frugiperda*

2.2.1 *S. frugiperda* rearing

S. frugiperda population was raised at 25 °C with 51 ± 1 % humidity with an artificial diet under 16 hours/8 hours light/dark cycle. Male and female pupae were kept in cages at which they mated as adults. Adults were fed on 10% sugar water. This insect culture was grown from *S. frugiperda* eggs obtained from Bayer Crop Sciences (Monheim, Germany).

2.2.2 DNA extraction from *S. frugiperda*, PCR amplification, purification and sequencing of *Oatp74D* target sequence

Genomic DNA was extracted from individual *S. frugiperda* larvae using the cetyl-trimethyl-ammonium bromide extraction protocol (Doyle, 1190). PCR amplification of CRISPR target sequence of *S. frugiperda* oatp74D was done with primers Sf_OatP74D_CDS_F, Sf_OatP74D_CR_R (Table S2) using Phusion High-Fidelity DNA Polymerase (M0530S,

NEB). The conditions used were 98 °C for 30 sec of initial denaturation, followed by 30 cycles of denaturation at 98 °C for 10 sec, annealing at 61 °C for 30 sec and extension at 72 °C for 45 sec, followed by a final extension for 5 min. The PCR product was purified using NucleoSpin Gel and PCR Clean-up Kit, (Macherey – Nagel (Düren, Germany), 740609) following the manufacturer's instructions. The purified target region of *Oatp74D* was sequenced (Genewiz S.A.) (Fig.S1)

2.2.3 sgRNA synthesis

Each sgRNA template was generated by PCR in a total volume of 150µl, using Phusion High-Fidelity DNA Polymerase (M0530S, NEB) with a target specific forward primer (Sf_OatP74D_long_1, Sf_OatP74D_long_2, Sf_OatP74D_long_3, Sf_OatP74D_long_4) (Table S2) and a common Uni_R reverse primer as described in (Bassett and Liu, 2014). PCR products were purified using NucleoSpin Gel and PCR Clean-up Kit, (Macherey – Nagel (Düren, Germany), 740609) and DNA concentration was measured on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), using 1 µl DNA. Then, in vitro transcription with 300ng of each PCR template was done for the production of sgRNAs using T7 MEGAscript kit (Ambion). DNA was degraded using TURBO™ DNase (Ambion) and RNA was isolated by sodium acetate precipitation.

2.2.4 *In vitro* digestion of *Oatp74D* by Cas9

100ng of purified DNA of the CRISPR target sequence were incubated at 37^{o}C for 15min with Cas9 nuclease (NEB, M0386T) (100 nM) and sgRNA (90 nM) in 1x NEB buffer 3.1 (NEB, B7203S). A total volume of 20µl was used per reaction and the same mixture without adding any sgRNA was used as negative control. After 15min incubation, 1µl of Proteinase K (NEB, P8107S) was added to each reaction and samples were incubated for 10min at RT. Samples were loaded on 1.5% agarose gel and the digested DNA fragments were visualized by ethidium bromide straining.

2.2.5 Microinjections in *S. frugiperda* to generate CRISPR knock outs

S. frugiperda eggs were injected 2hrs post-hatching with an injection mix including 310ng/µl Cas 9 nuclease (NEB, M0386T) and 100ng/µl of each sgRNA diluted in 1x injection buffer (0.1 mM sodium phosphate buffer pH 6.8, 5 mM KCl). Injected eggs were kept in a sterile petri dish at 25°C until hatching.

2.2.6 Mutation analysis of *Oatp74D* in G0 mosaic *S. frugiperda*

To confirm mutations in *Oatp74D,* DNA was isolated from injected G0 survivors using the DNA extraction protocol described above. Genomic DNA of individual insects was amplified using Phusion High-Fidelity DNA Polymerase (M0530S, NEB) and the primers Sf_oatp74D_amplicon.seq.F, Sf_oatp74D_amplicon.seq.R (Table S2) flanking the CRISPR target sites. The conditions used were 98 °C for 30 sec of initial denaturation, followed by 30 cycles of denaturation at 98 °C for 10 sec, annealing at 57 °C for 30 sec and extension at 72 °C for 15 sec, followed by a final extension for 5 min. The PCR product was purified using NucleoSpin Gel and PCR Clean-up Kit, (Macherey – Nagel (Düren, Germany), 740609) following the manufacturer's instructions. The purified target region of *Oatp74D* of each individual was sequenced (Genewiz S.A.) (Fig.S2, Fig.S3).

3. Results

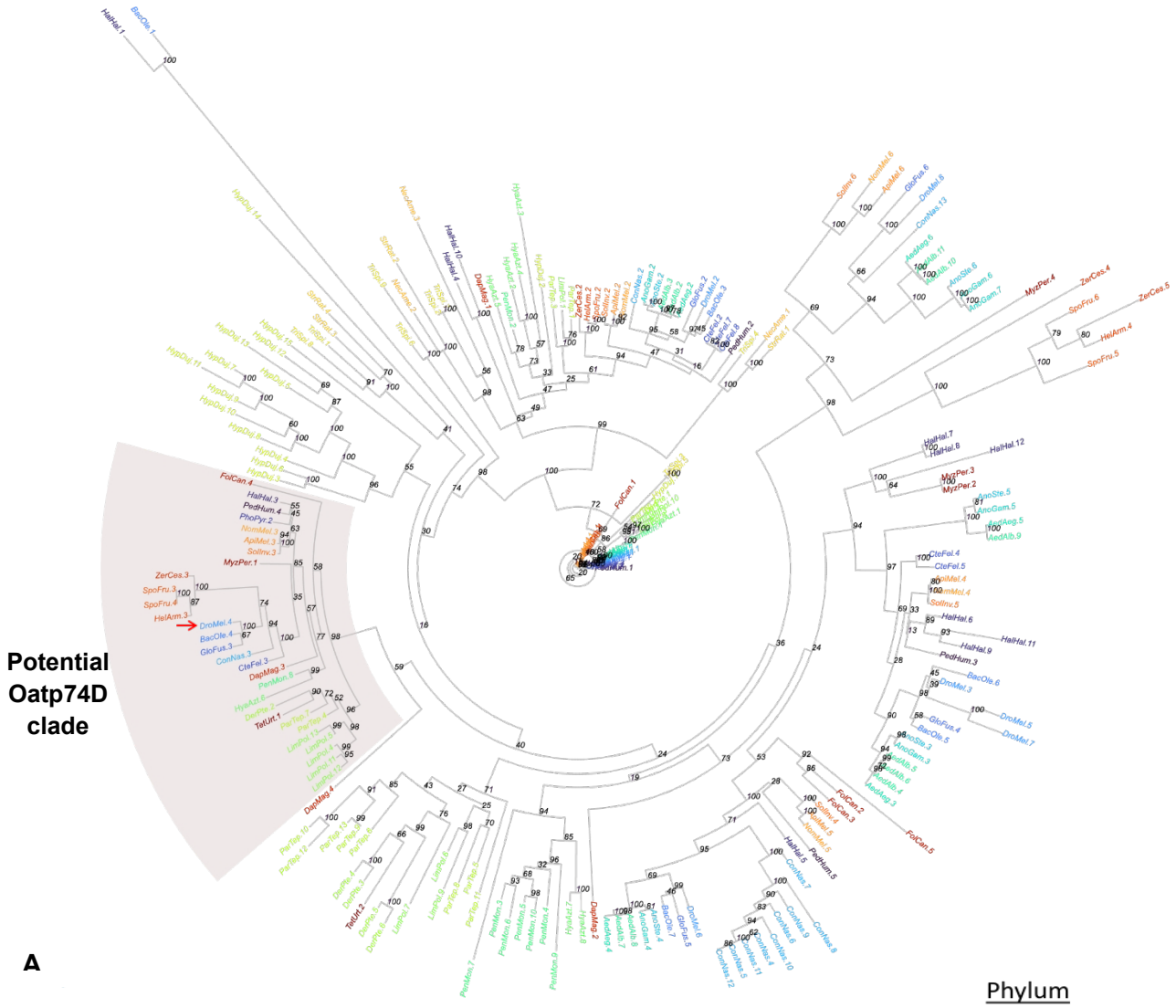
3.1. Evolution of *Oatp74D* among ecdysozoa

Organisms like arthropods and nemotodes that utilize ecdysosteroids taxonomically belong to ecdysozoa. Based on this knowledge and on the information that the arthropod *D.melanogaster* has an EcI, but the nematode *C. elegans* does not have any *Oatp74D* orthologs (Okamoto *et al.*, 2018), we aimed to identify the appearance of this gene during the evolution of ecdysozoan species. For this reason, a phylogenetic tree was constructed, using the SLCO transporters of species from all orders of ecdysozoa (Table 1, Table S1), (Fig. 7A). This enabled the identification of the potential Oatp74D clade (Fig. 7A), based on the functionally characterized OATPs of *D. melanogaster.* Moreover, a species tree was constructed using a wide range of 1:1 orthologous genes to show the evolutionary relationship of the selected taxa (Fig. 7B). Analysis of both trees revealed that all species represented on the potential Oatp74D clade of the phylogenetic tree, including *S. frugiperda* and *H. armigera,* are clustered on the species tree, on the clade of arthropods (Fig. 7B), which indicates that *Oatp74D* appeared at a common ancestor of Arthropoda.

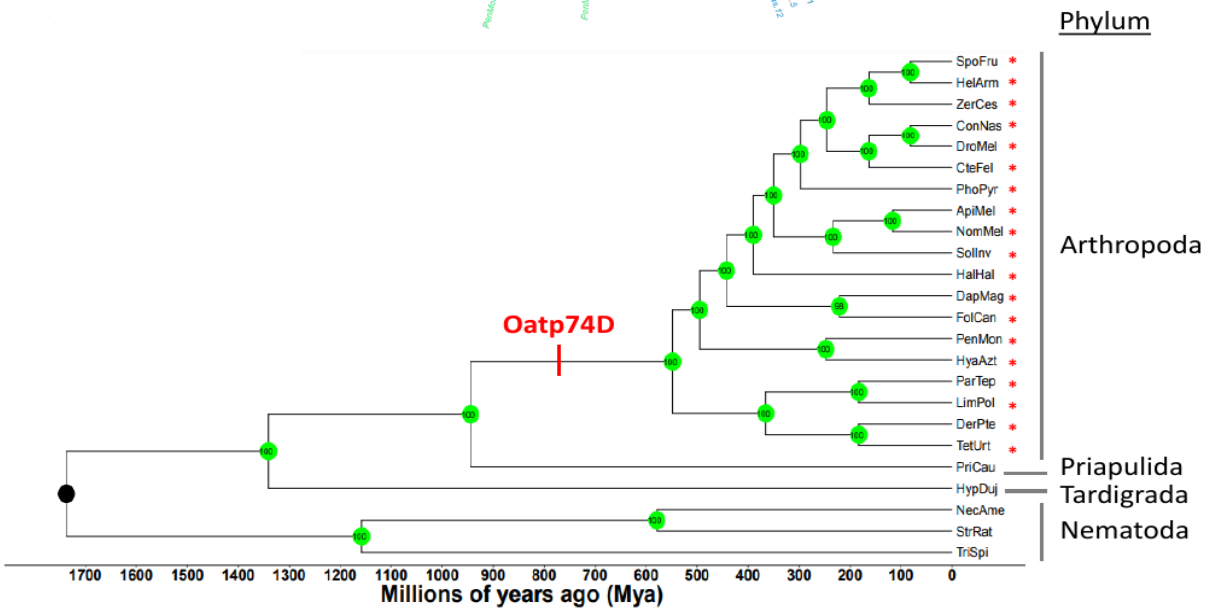
Table 1: Ecdysozoan species selected for the phylohenetic tree

Phylum	Coding letters	Species name (NCBI)
Arthropoda	AedAeg	Aedes aegypti (yellow fever mosquito)
	AedAlb	Aedes albopictus (Asian tiger mosquito)
	AnoGam	Anopheles gambiae (African malaria mosquito)
	AnoSte	Anopheles stephensi (Asian malaria mosquito)
	ApiMel *	Apis mellifera (honey bee)
	BacOle *	Bactrocera oleae (olive fruit fly)
	ConNas *	Contarinia nasturtii (swede midge)
	CteFel *	Ctenocephalides felis (cat flea)
	DapMag *	Daphnia magna
	DerPte *	Dermatophagoides pteronyssinus
	DroMel *	Drosophila melanogaster (fruit fly)
	FolCan *	Folsomia candida
	GloFus *	Glossina fuscipes
	HalHal *	Halyomorpha halys (brown marmorated stink bug)
	HelArm *	Helicoverpa armigera (cotton bollworm)
	HyaAzt *	Hyalella azteca
	LimPol *	Limulus polyphemus (Atlantic horseshoe crab)
	MyzPer *	Myzus persicae (green peach aphid)
	NomMel *	Nomia melanderi (Alkali bee)
	ParTep *	Parasteatoda tepidariorum (common house spider)
	PedHum *	Pediculus humanus (human louse)
	PenMon *	Penaeus monodon (black tiger shrimp)
	PhoPyr *	Photinus pyralis (common eastern firefly)
	SolInv *	Solenopsis invicta (red fire ant)
	SpoFru *	Spodoptera frugiperda (fall armyworm)
	TetUrt *	Tetranychus urticae (two-spotted spider mite)
	ZerCes *	Zerene cesonia (dogface butterfly)
Priapulida	PriCau	Priapulus Caudatus
Nematoda	NecAme	Necator americanus
	StrRat	Strongyloides ratti
	TriSpi	Trichinella spiralis
Tardigrada	HypDuj	Hypsibius dujardini

* (Species represented on the potential Oatp74D clade)


Potential
Oatp74D
clade

A


Phylum
SpoFru
HelArm
ZerCes
ConNas
DroMel
CteFel
PhoPyr
ApiMel
NomMel
SolInv
HalHal
DapMag
FolCan
PenMon
HyaAzt
ParTep
LimPol
DerPte
TetUrt
PriCau
HypDuj
NecAme
StrRat
TriSpi
Oatp74D
Arthropoda
Priapulida
Tardigrada
Nematoda
1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400 300 200 100 0
Millions of years ago (Mya)

B

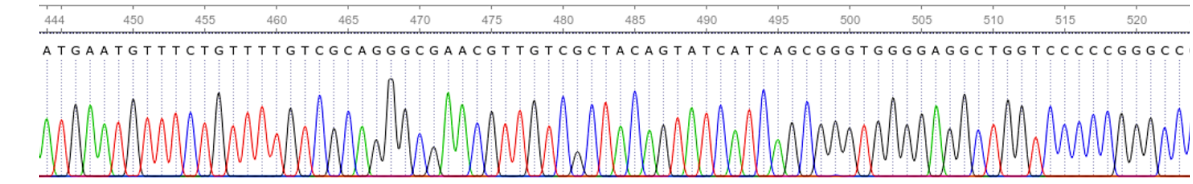
Figure 7: *Oatp74D* has potential orthologs among Arthropods

(A) Unrooted phylogenetic tree constructed using the amino acid sequences of the SLCO transporters from ecdysozoan species shown in Table 1. Gene IDs of the proteins used are listed on Table S1. Shaded area indicates the potential Oatp74D clade and red arrow indicates the EcI of *Drosophila*. (B) Unrooted species tree constructed using 1:1 orthologous proteins from the selected ecdysozoa. Red star marks species that are represented on the potential Oatp74D clade of the phylogenetic tree.

3.2. Oatp74D is necessary for the development of *S. frugiperda* at embryonic and larval stages

In order to assess the essentiality of the *Oatp74D* candidate genes in the two lepidopteran pests, we attempted to disrupt *Oatp74D* in *S. frugiperda.* After sequencing the first exon of *Oatp74D* of the *S. frugiperda* population rared in our lab (Fig. 8A, Fig. S1), CRISPR-Cas9 system was used combining 4 different sgRNAs (Table S2) that target the first exon of *Oatp74D* (Fig. 8B). Firstly, *in vitro* digestion of the DNA target sequence by Cas9 was done and proved that all sgRNAs and particularly the 1st, 2nd and 3rd can efficiently target and cleave the first exon of the gene (Fig. 8C). Then, 506 *S. frugiperda* embryos were injected with a mixture of Cas9 nuclease and sgRNAs, in order to generate mosaic knockouts of *Oatp74D.* As a negative control, mosaic knockouts of *scarlet* gene, which are known to normally develop to adults with mosaic patterns of yellow and wild type color ommatidia (Khan, Reichelt and Heckel, 2017), were generated in the same way.

Then, mortality and transition rates were measured for mosaic *oatp74D* and *scarlet* G0 animals (Fig.9). Measurements of transition rates showed that *oatp74D* knockouts appear to have significantly lower hatching rate than *scarlet*- knockouts (Fig.9A). Moreover, injected *oatp74D* hatched larvae seem to have higher mortality than *scarlet* hatched larvae during transition from L1 to L5 larval stage (Fig.9A). All injected animals of both genotypes that reached L5 larval stage managed to transform to pupae (Fig.9A) and then to adults. The fraction of dead *oatp74D* and *scarlet* animals that survived from injections was calculated end it was shown that *oatp74D* animals die significantly more compared to control animals (Fig.9B). To molecularly validate the lethal phenotype of *oatp74D* mosaic knockout animals, DNA from all dead *oatp74D* larvae and adult survivors was isolated and sequenced.



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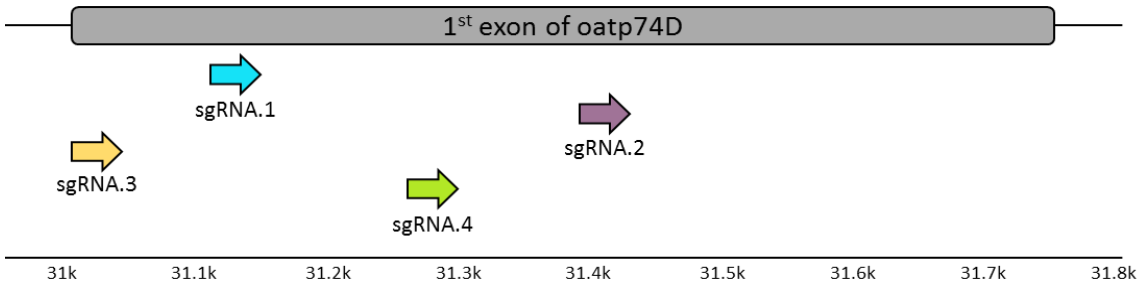

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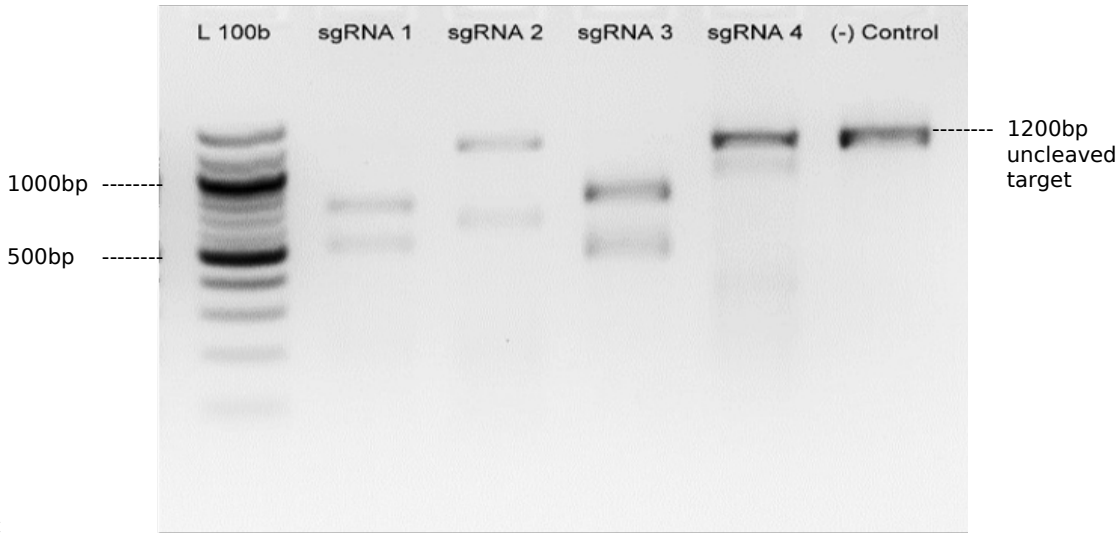
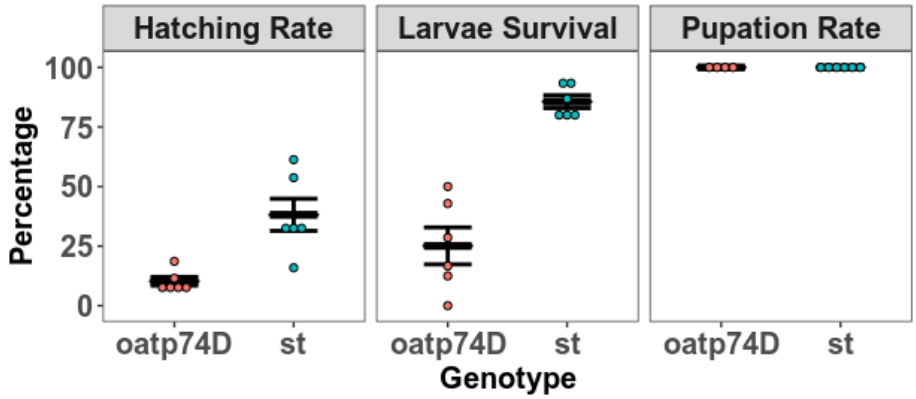


C

Figure 8: *In vitro* cleavage efficiency of Oatp74D by Cas9 using different sgRNAs

(A) Chromatogram of the sequence of the first exon of *Oatp74D* from *S. frugiperda.* (B) Schematic representation of the 4 sgRNAs used for CRISPR and the target region of *Oatp74D.* (C) *In vitro* digestion of the target sequence of *Oatp74D* by Cas9, using each sgRNA separately or no sgRNA as negative control. Multiple fragments of DNA below the uncleaved target proove that Cas9 can efficiently cleave *Oatp74D in vitro.*



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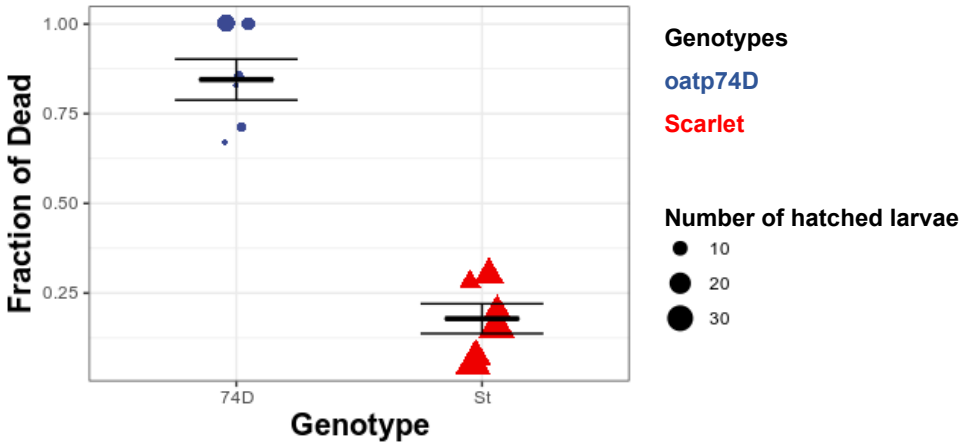

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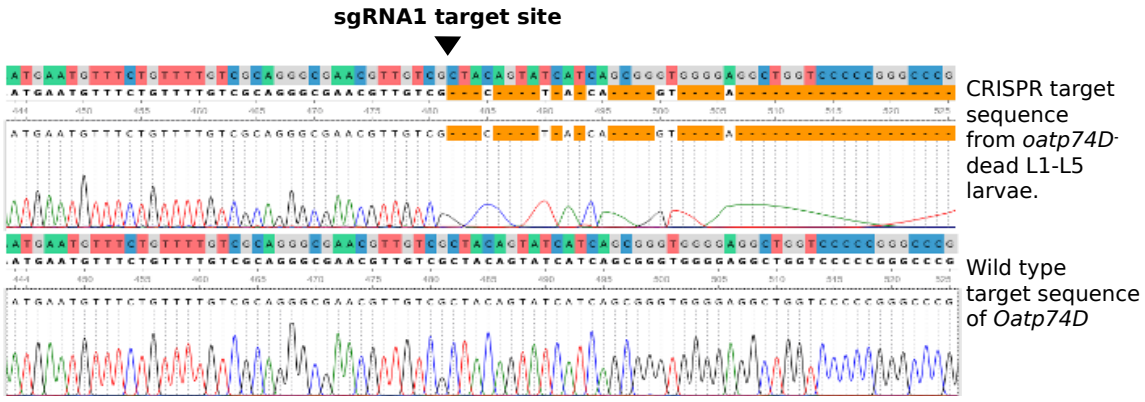

C

Figure 9: Oatp74D is necessary for the developemnt of *S. frugiperda* at embryonic and larval stages.

(A) Dot plot demonstrating transition rates of *oatp74D* and *scarlet* mosaic animals at different developmental stages. Every dot represents a plate of 80-100 injected embryos. Left, middle and right panel represent transtion rates from egg to L1 larva, L1 to L5 larvae and L5 larva to pupa respectively.

st, *scarlet* (B) Dot plot demonstrating overall mortality rates of *oatp74D* and *scarlet* mosaic animals. Every dot represents a plate of 80-100 injected embryos. The size of the dots is proportional to the number of hatched larvae from every plate. Bars represent 1.5 times the quartile range. ** p <0,01 from Weltch t-test compared to control (*scarlet*) (C) Chromatograms demonstarting aligned sequencing results (Fig.S2, Fig.S3) of animals bearing CRISPR-Cas9 generated knockout of *Oatp74D* (top) and wild type *Oatp74D (*bottom).

Sequencing results revealed that CRISPR events had occurred in all dead *oatp74D* larvae (Fig.S3), but not in injected animals that managed to reach adult stage (Fig.9C, Fig.S2). Taken toghether these results, *Oatp74D* appears to be necessary for the development of *S. frugiperda* at embryonic and larval stage. The fact that all survivors that reached L5 stage were found to be wild type indicates that animals lacking this gene cannot complete their life cycle and become adults. Also, pupation rate measured in this experiment is not indicative of the knockout phenotype as it was molecularly validated that animals used for this measurement were wild type.

4. Discussion

Insect pests damage agriculture imposing significant costs to global economy (Flood, 2010). *S. frugiperda* and *H. armigera* are two Lepidopteran pests that are considered to be of major economic importance (Flood, 2010; Cunningham and Zalucki, 2014; Luginbill, 1928) and there are several physiological factors that render them so destructive as well as the fact that they have already developed resistance to existing insecticides (Barros *et al.*, 2010; Westbrook *et al.*, 2016)(Boaventura *et al.*, 2020). Thus, it is of utmost importance to identify novel drug targets in order to control these populations. In this study, we focused on the characterization of the ecdysone importer Oatp74D in *S. frugiperda* as a putative target for the development of insecticides. The reasons we chose this protein were that it was recently identified to be necessary for the developement of *Drosophila* (Okamoto *et al.*, 2018), which is a model organism for studying insects, and also that the substrate of this transporter, ecdysone, is the key regulatory hormone in insects, but is not represented among mammals.

In order to characterize Oatp74D as a drug target in Lepidoptera we had to analyze whether homologous proteins exist in the pests of our interest and how species specific this transporter is. However, little is known about the evolution of this transporter. Although the function and phylogeny of OATPs have been extensively studied in mammals, due to their pharmacological significance (Zhang et al., 2006, 2008; Huang et al., 2008; Giacomini et al., 2010; Zhang, Huang and Lesko, 2011; Fenner et al., 2012; Tweedie et al., 2013; Hagenbuch and Stieger, 2013) concerning non-mammalian species there is limited information. Thus, taking into account that *Drosophila* has an *EcI* ortholog, but *C. elegans,* which is a nematode and taxonomically belongs to ecdysozoa, does not have one (Okamoto *et al.*, 2018), we aimed to identify the evolutionary boundaries of *EcI*. For this reason, we performed *in silico* analysis of this transporter (Fig.7), by selecting species that represent all phyla of ecdysozoa (Table 1) and found that Oatp74D-like orthologs (Fig.7B, Table S1) are arthoropod specific (Fig.7B). Our results agree with previous phylogenetic analysis of this transporter (Okamoto *et al.*, 2018) and give a more detailed image of the number of potential orthologs several significant species have, including disease vectors and agricultural pests. Interestingly, apart from identifying *Oatp74D* potential orthologs in *S. frugiperda* and *H. armigera*, we found that

mosquitoes do not have any *Oatp74D* orthologs, athough they are arthropods. In agreement with this result, a very recent study reports the existence of additional ecdysone importers EcI-2, EcI-3 and EcI-4 in *Aedes aegypti,* with EcI-2 being necessary for the development of the organism (Hun *et al.*, 2021). The fact that these transporters exist in *D. melanogaster,* but do not have dominant role in its development, exemplifies well why further functional analysis of Oatp74D is needed to be done in the two lepidopteran pests to assess its essentiality.

In order to identify whether Oatp74D is necessary for *S. frugiperda* we attempted to disrupt it by using CRISPR-Cas9 system. *In vivo* analysis, revealed the significance of this transporter in the development of *S. frugiperda* and showed that mosaic animals partially lacking this transporter die at embryonic and larval stages (Fig.9A). Increased embryonic lethality observed in *oatp74D* animals is consistent with previous studies which analyze the impact of inhibiting ecdysteroidogenesis in *Bombyx mori* and *D. melanogaster* during embryogenesis (Gilbert, 2004; Niwa *et al.*, 2004). In agreement to our results, embryonic lethality was also observed in *Aedes aegypti* when the dominant ecdysone receptor EcI-2 was knocked out. This observation is reasonable based on existing knowledge in insects hormonal regulation (Ou and King-Jones, 2013), since ecdysone levels increase periodically during insects development and in particularly high levels during embryogenesis. The overall increased mortality (Fig.9B) in *oatp74D* mosaic *S. frugiperda* indicates that Oatp74D has dominant role in the development of this insect. However, further *in vitro* and *in vivo* characterization of this transporter is needed to elucidate its exact role in the physiology of *S. frugiperda.* Genetic complementation in *Drosophila eci* background, which is known to be lethal (Okamoto et al., 2018), using the lepidopteran transporters, could provide information on whether these proteins share the same role with the ecdysone importer of *Drosophila.*

The essentiality of Oatp74D in the development of this pest demonstrated by our results renders it a potential drug target. However, in terms of insecticides development, species-specificity needs to be considered in order to avoid harming other species by the application of insecticides. The absence of Oatp74D from non-arthropod species significantly eliminates possible off-target species. Even though OATPs share a very conserved structure (Jacquemin *et al.*, 1994; Kullak-Ublick *et al.*, 1995), differences in critical regions of these proteins between species can be used in order to design drugs that specifically target and

inhibit Oatp74D transporters of *S. frugiperda* and *H. armigera.* The predicted structure of *Drosophila's* EcI (AlphaFold, 2021) in combination with computational tools that predict proteins druggability (Bakheet and Doig, 2009) can significantly facilitate drug development. Moreover, transcriptomic analysis in *H. armigera* and *D. melanogaster* has revealed high expression levels of Oatp74D in the gut (Ioannidis *et al*., 2021; Okamoto et al., 2018). This characteristic of Oatp74D in combination with its localization on cell membrane, renders it ideal target for the development of oral insecticides that will directly act on it. However, further analysis to identify the localization of this transporter on the apical and/or basal membrane of gut cells would be useful. The development of *in vitro* systems that will enable screening of drug compounds would also contribute in insecticide development. In conclusion, our results suggest that Oatp74D can be used as a putative drug target in Lepidoptera, while further analysis on this transporter will provide beneficial information about insect physiology and facilitate the development of novel drug compounds.

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Supplementary material

Table S1: Gene IDs of the proteins represented on the phylogenetic tree

NCBI Species Name	Phylogenetic Tree Ortholog	NCBI Gene ID
Aedes aegypti (yellow fever mosquito)	AedAeg.1	5572461
	AedAeg.2	5572460
	AedAeg.3	5571480
	AedAeg.4	5569501
	AedAeg.5	5574116
	AedAeg.6	5569498
Aedes albopictus (Asian tiger mosquito)	AedAlb.1	109407612
	AedAlb.2	109420505
	AedAlb.3	115255819
	AedAlb.4	109430555
	AedAlb.5	115260123
	AedAlb.6	115254608
	AedAlb.7	115258143
	AedAlb.8	109622884
	AedAlb.9	109430556
	AedAlb.10	115258139
	AedAlb.11	109622883
Anopheles gambiae (African malaria mosquito)	AnoGam.1	3291790
	AnoGam.2	1275562
	AnoGam.3	1277224
	AnoGam.4	1279467
	AnoGam.5	1277223
	AnoGam.6	1279468
	AnoGam.7	1268676
Anopheles stephensi (Asian malaria mosquito)	AnoSte.1	118511774
	AnoSte.2	118511767
	AnoSte.3	118514332
	AnoSte.4	118513303
	AnoSte.5	118514334
	AnoSte.6	118513299
Apis mellifera (honey bee)	ApiMel.1	409670
	ApiMel.2	107965041
	ApiMel.3 *	**100577409**
	ApiMel.4	409650

	ApiMel.5	409649
	ApiMel.6	726470
Bactrocera oleae (olive fruit fly)	BacOle.1	106616904
	BacOle.2	106617570
	BacOle.3	106616361
	BacOle.4 *	**106614116**
	BacOle.5	106627822
	BacOle.6	106627796
	BacOle.7	106615295
Contarinia nasturtii (swede midge)	ConNas.1	116337258
	ConNas.2	116336926
	ConNas.3 *	**116338554**
	ConNas.4	116348979
	ConNas.5	116348207
	ConNas.6	116338087
	ConNas.7	116338010
	ConNas.8	116344156
	ConNas.9	116340604
	ConNas.10	116338269
	ConNas.11	116348981
	ConNas.12	116348209
	ConNas.13	116338038
Ctenocephalides felis (cat flea)	CteFel.1	113377690
	CteFel.2	113374981
	CteFel.3 *	**113376710**
	CteFel.4	113368246
	CteFel.5	113368247
	CteFel.6	113374973
	CteFel.7	113374977
	CteFel.8	113374982
Daphnia magna	DapMag.1	116929909
	DapMag.2	116917617
	DapMag.3 *	**116925549**
	DapMag.4	116932711
Dermatophagoides pteronyssinus	DerPte.1	113795043
	DerPte.2 *	**113795044**
	DerPte.3	113798940
	DerPte.4	113798932
	DerPte.5	113789382
	DerPte.6	113789383

Drosophila melanogaster (fruit fly)	DroMel.1	34268
	DroMel.2	33927
	DroMel.3	37545
	DroMel.4 *	**39954**
	DroMel.5	37543
	DroMel.6	34660
	DroMel.7	37544
	DroMel.8	34662
Folsomia candida	FolCan.1	110854518
	FolCan.2	110851675
	FolCan.3	110858407
	FolCan.4	110846602
	FolCan.5	110851674
Glossina fuscipes	GloFus.1	119633159
	GloFus.2	119633088
	GloFus.3 *	**119632634**
	GloFus.4	119636045
	GloFus.5	119635482
	GloFus.6	106680829
Halyomorpha halys (brown marmorated stink bug)	HalHal.1	106688263
	HalHal.2	106687457
	HalHal.3 *	**106685704**
	HalHal.4	106688306
	HalHal.5	106687037
	HalHal.6	106680829
	HalHal.7	106688159
	HalHal.8	112211002
	HalHal.9	106680831
	HalHal.10	106688311
	HalHal.11	106689936
	HalHal.12	112210027
Helicoverpa armigera (cotton bollworm)	HelArm.1	110378947
	HelArm.2	110380717
	HelArm.3 *	**110377536**
	HelArm.4	110376784
Hyalella azteca	HyaAzt.1	108672586
	HyaAzt.2	108674364
	HyaAzt.3	108674376
	HyaAzt.4	108674365
	HyaAzt.5	108674366

	HyaAzt.6 *	**108670996**
	HyaAzt.7	108683227
	HyaAzt.8	108675222
Hypsibius dujardini	HypDuj.1	BV898_11360
	HypDuj.2	BV898_04971
	HypDuj.3	BV898_05593
	HypDuj.4	BV898_07405
	HypDuj.5	BV898_10426
	HypDuj.6	BV898_00949
	HypDuj.7	BV898_10403
	HypDuj.8	BV898_01504
	HypDuj.9	BV898_10404
	HypDuj.10	BV898_10405
	HypDuj.11	BV898_16684
	HypDuj.12	BV898_10407
	HypDuj.13	BV898_10410
	HypDuj.14	BV898_01677
	HypDuj.15	BV898_00899
Limulus polyphemus (Atlantic horseshoe crab)	LimPol.1	106460006
	LimPol.2	106470936
	LimPol.3	106469001
	LimPol.4 *	**106458779**
	LimPol.5 *	**106467456**
	LimPol.6	106462005
	LimPol.7	106465503
	LimPol.8	106473599
	LimPol.9	106478780
	LimPol.10	106475309
	LimPol.11 *	**106474691**
	LimPol.12 *	**106471432**
	LimPol.13 *	**106476799**
Myzus persicae (green peach aphid)	**MyzPer.1 ***	**111036156**
	MyzPer.2	111041601
	MyzPer.3	111027674
	MyzPer.4	111029574
Necator americanus	NecAme.1	25345720
	NecAme.2	25342589
	NecAme.3	25340106
Nomia melanderi (Alkali bee)	NomMel.1	116426241
	NomMel.2	116426190

	NomMel.3 *	**116431007**
	NomMel.4	116426239
	NomMel.5	116426118
	NomMel.6	116426168
Parasteatoda tepidariorum (common house spider)	ParTep.1	107445148
	ParTep.2	107437706
	ParTep.3	107437086
	ParTep.4 *	**107443800**
	ParTep.5	107442146
	ParTep.6	107442145
	ParTep.7 *	**107437715**
	ParTep.8	107456980
	ParTep.9	107441394
	ParTep.10	107450808
	ParTep.11	107442140
	ParTep.12	107442139
	ParTep.13	107441395
Pediculus humanus (human louse)	PedHum.1	8239843
	PedHum.2	8230524
	PedHum.3	8231563
	PedHum.4 *	**8239866**
	PedHum.5	8239301
Penaeus monodon (black tiger shrimp)	PenMon.1	119590666
	PenMon.2	119581163
	PenMon.3	119573412
	PenMon.4	119576950
	PenMon.5	119575357
	PenMon.6	119576958
	PenMon.7	119586931
	PenMon.8 *	**119572367**
	PenMon.9	119573367
	PenMon.10	119577366
Photinus pyralis (common eastern firefly)	PhoPyr.1	116165455
	PhoPyr.2 *	**116168080**
	PhoPyr.3	116165826
Solenopsis invicta (red fire ant)	SolInv.1	105203485
	SolInv.2	105202696
	SolInv.3 *	**105203812**
	SolInv.4	105196352
	SolInv.5	105196351

	SolInv.6	105196350
Spodoptera frugiperda (fall armyworm)	SpoFru.1	118278495
	SpoFru.2	118274558
	SpoFru.3 *	**118271297**
	SpoFru.4 *	**118278121**
	SpoFru.5	118278212
	SpoFru.6	118278156
Strongyloides ratti	StrRat.1	36375791
	StrRat.2	36380248
	StrRat.3	36373428
	StrRat.4	36375784
Tetranychus urticae (two-spotted spider mite)	TetUrt.1	107366484
	TetUrt.2	107360496
Trichinella spiralis	TriSpi.1	10910173
	TriSpi.2	10911142
	TriSpi.3	10912168
	TriSpi.4	10910086
	TriSpi.5	10912156
	TriSpi.6	10904092
	TriSpi.7	10911118
	TriSpi.8	10910174
	TriSpi.9	10904090
Zerene cesonia (dogface butterfly)	ZerCes.1	119829798
	ZerCes.2	119829434
	ZerCes.3 *	**119829759**
	ZerCes.4	119839114
	ZerCes.5	119839086

***** (Genes represented on the potential Oatp74D clade)

Table S2: Sequences and uses of oligonucleotides

Prmers renamed	Sequence	Use
Sf_OatP74D_CDS_F	ATGGATAGACGGCCAATAAAA	sequencing of CR. target
Sf_OatP74D_CR_R	CCATGTAAAGTGGTGACTGCC	sequencing of CR. target
Sf_oatp74D_amplicon.seq.F	CAGGTTTGTAAATACCTAGTG	amplicon sequencing of CR. target
Sf_oatp74D_amplicon.seq.R	GACCACACCCACCGCCAGCAC	amplicon sequencing of CR. target
CRISPR universal	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA synthesis
Sf_OatP74D_long_1	GAAATTAATACGACTCACTATAGGTCGCTACAGTATCATCAGCGTTTTAGAGCTAGAAATAGC	sgRNA synthesis
Sf_OatP74D_long_2	GAAATTAATACGACTCACTATAGGAGCAGGAACGGCTAGCAACGTTTTAGAGCTAGAAATAGC	sgRNA synthesis
Sf_OatP74D_long_3	GAAATTAATACGACTCACTATAGGTCGTTACACTGACGAAGTGTTTTAGAGCTAGAAATAGC	sgRNA synthesis
Sf_OatP74D_long_4	GAAATTAATACGACTCACTATAGGCGCGGGCACCGGCCGCGAGTTTTAGAGCTAGAAATAGC	sgRNA synthesis
Sf_OATP74D_CDS_Vd.NotI_F	GAATTGGGAATTCGTTAACAGATCTGCGCGGCCGCATGACGGCGAACGTTGTC	Cloning of Sf_oatp74D
Sf_OATP74D_CDS_Vd.XbaI_R	ATCCTCTAGAGGTACCCTCGAGCCGCTCTAGATCAGAGTTGTGTATCGGATGGGTTTG	Cloning of Sf_oatp74D
Sf_oatp74D_CDS_internal	CCT TGC TCC ACA CCA AAA TGT C	sequencing of Sf_oatp74D
Ha_oatp74D_CDS_internal	GTACGTCGACGATAATGTC	sequencing of Ha_oatp74D
Dm_oatp74D_CDS_internal	GACATCGTCCGAGATGGATTG	sequencing of Dm_oatp74D
Dm_oatp74D_NotI_F	GTACGCGGCCGCATGACGAAGAGCAATGGCGATG	Cloning of Dm_oatp74D
Dm_oatp74D_XbaI_R	GTACTCTAGACTAGACCGTCGTGTCCGGC	Cloning of Dm_oatp74D
pUAST_F	CAAGCGCAGCTGAACAAG	Diagnostics: Binds 176bp upstream of NotI in vector pUAST-attB
pUAST_R	GTCACACCACAGAAGTAAGGTTCC	Diagnostics: Binds 63bp downstream of NotI in vector pUAST-attB
pUAST_R_insert_seq2	CTCATCATCACTAGATGGC	Diagnostics: Binds ~220b downstream of XbaI in vector pUAST-attB
Dmel_OATP74D_XbaI_F	ATGACGAAGAGCAATGGCGATG	Cloning/Sequencing of Dm_oatp74D
Dmel_OATP74D_NotI_R	CTAGACCGTCGTGTCCGGC	Cloning/Sequencing of Dm_oatp74D

Figure S1: Sequencing results of the CRISPR target sequence of *Oatp74D* in *S. frugiperda*

```
NC_049711          CGGAATAACGTGTAATTGTTTGTCAGGTTTGTAAATACCTAGTGTTAAATGAATGTTTCTGTTTTGTCGC  31010
Sf_OatP74D_CDS_F   CGGAATAACGTGTAATTATTTGTCAGGTTTGTAAATACCTAGTGTTAAATGAATGTTTCTGTTTTGTCGC  31010
Sf_OatP74D_CR_R    CGGAATAACGTGTAATTATTTGTCAGGTTTGTAAATACCTAGTGTTAAATGAATGTTTCTGTTTTGTCGC  31010
                   ********************************************************************

NC_049711          AGGGCGAACGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCCGGGCCCGGTGACCGAGG  31080
Sf_OatP74D_CDS_F   AGGGCGAACGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCCGGGCCCGGTGACCGAGG  31080
Sf_OatP74D_CR_R    AGGGCGAACGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCCGGGCCCGGTGACCGAGG  31080
                   ********************************************************************

NC_049711          GTACCGACATGAAAGGTGTTGAGGCGACGCCAGAAGAGCAGGAACGGCTAGCAACCGGAAACAACAATGG  31150
Sf_OatP74D_CDS_F   GTACCGACATGAAAGGTGTTGAGGCTACGCCAGAAGAGCAGGAACGGCTAGCAACCGGAAACAACAATGG  31150
Sf_OatP74D_CR_R    GTACCGACATGAAAGGTGTTGAGGCTACGCCAGAAGAGCAGGAACGGCTAGCAACCGGAAACAACAATGG  31150
                   ************************ ****************************************

NC_049711          GTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCTCCGTGTTCCACTCGACG  31220
Sf_OatP74D_CDS_F   GTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCTCCGTGTTCCACTCGACG  31220
Sf_OatP74D_CR_R    GTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCTCCGTGTTCCACTCGACG  31220
                   ********************************************************************

NC_049711          CGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCTCACCTACTTCGTCAGTG  31290
Sf_OatP74D_CDS_F   CGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCTCACCTACTTCGTCAGTG  31290
Sf_OatP74D_CR_R    CGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCTCACCTACTTCGTCAGTG  31290
                   ********************************************************************

NC_049711          TGACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACGTTACTAGCGGCCACGGA  31360
Sf_OatP74D_CDS_F   TAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACGTTACTAGCGGCCACGGA  31360
Sf_OatP74D_CR_R    TAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACGTTACTAGCGGCCACGGA  31360
                   * ******************************************************************

NC_049711          AATAGGCCAGATATCTACAGCACTGATCCTGACGTACCTGGCGGGGCGCGGGGCACCGGCCGCGATGGATC  31430
Sf_OatP74D_CDS_F   AATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGGCACCGGCCGCGATGGATC  31430
Sf_OatP74D_CR_R    AATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGCACCGGCCGCGATGGATC  31430
                   ****************** ** **************************************************

NC_049711          GCCTGCATGATGATCGTGCTGGCGGTGGGAGTGGTGGGCTGCATCATGCCGCACCTTCTCTACGGCACTC  31500
Sf_OatP74D_CDS_F   GCCTGCATGATGATCGTGCTGGCGGTGGGTGTGGTCGGCTGCATCATGCCGCACCTCCTCTACGGCACTC  31500
Sf_OatP74D_CR_R    GCCTGCATGATGATCGTGCTGGCGGTGGGTGTGGTCGGCTGCATCATGCCGCACCTCCTCTACGGCACTC  31500
                   **************************** ***** ******************** *************

NC_049711          AGCTGCTCGAAGTGCACCAGGAAGCGCACCATGCCGGCGCCGGGCCTGTCTGCTACAGCTACCAGAACTC  31570
Sf_OatP74D_CDS_F   AGCTGCTCGAAGTGCACCAGGAAGCGCATCATGCCGGCGCCGGGCCTGTTTGCTACAGCTACCAGAACTC  31570
Sf_OatP74D_CR_R    AGCTGCTCGAAGTGCACCAGGAAGCGCATCATGCCGGCGCCGGGCCTGTTTGCTACAGCTACCAGAACTC  31570
                   **************************** ******************** ********************

NC_049711          CTCCGACTTGTGTGATGCCGCACACATCAAGAGCTCGACCACGCGGTCCTCCATCACCTCCGTTGTCATC  31640
Sf_OatP74D_CDS_F   TTCCGACTTGTGTGATGCCGCACACATCAAGAGCTCGACCACGCGGTCCTCCATCACCTCCGTCGTCATC  31640
Sf_OatP74D_CR_R    TTCCGACTTGTGTGATGCCGCACACATCAAGAGCTCGACCACGCGGTCCTCCATCACCTCCGTCGTCATC  31640
                   ********************************************************************** ******

NC_049711          CCATGGCTGTTTATTTGCCTGTTGATAGTGGGCGTGGGGCAGACTGGGATAGCCACGTTGGGCATTCCAT  31710
Sf_OatP74D_CDS_F   CCGTGGCTGTTTATTTGCCTGTTGATAGTGGGCGTGGGGCAGACTGGGATAGCCACGTTGGGC-------  31710
Sf_OatP74D_CR_R    CCGTGGCTGTTTATTTGCCTGTTGATAGTGGGCGTGGGGCAGACTGGGATAG-CACGTTGGGCATTCCGT  31710
                   ** ************************************************ **********

NC_049711          ACATAGACGACAACGTCGGCAGCAGGCAGTCACCACTTTACATGGG----------------------  31780
Sf_OatP74D_CDS_F   ------------------------------------------------------------------  31780
Sf_OatP74D_CR_R    ACATAGACGACAACGTCGG-NNCA-GCA-TCTCNTCTTTTNATAGGAAAAAANNGNGGCGGAATCTNNNN  31780
                   ** ************************************************ **********
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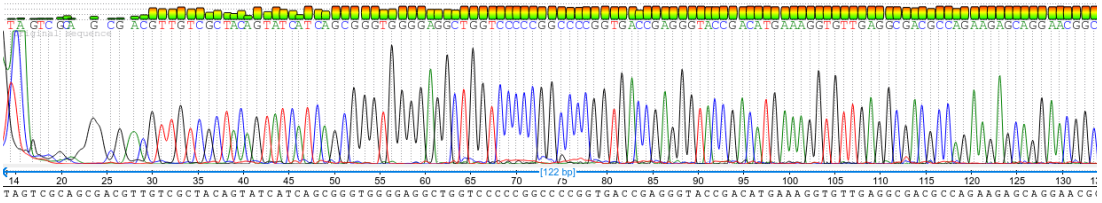
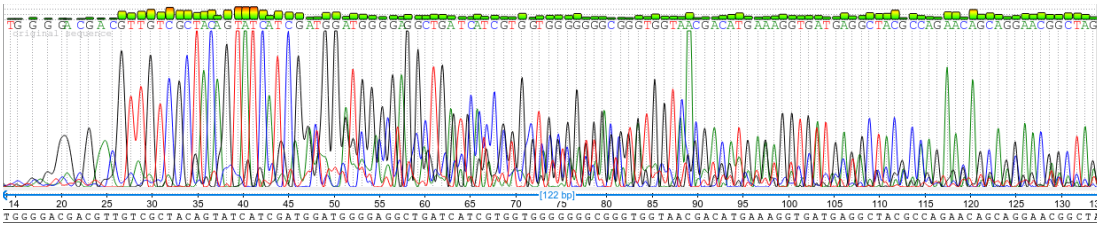
Figure S2: Sequencing results of the *Oatp74D* CRISPR target sequence from *S. frugiperda* injected dead larvae. Unclear chromatograms compared to the clear chromatograms of *wild type* organisms proove that CRISPR events have occurred in dead injected individuals.

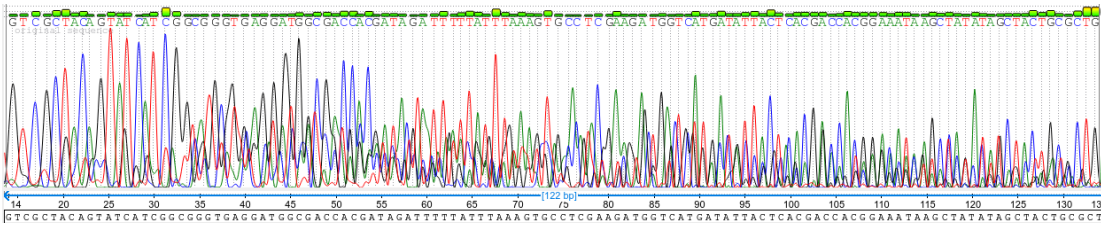
Wild type larva:



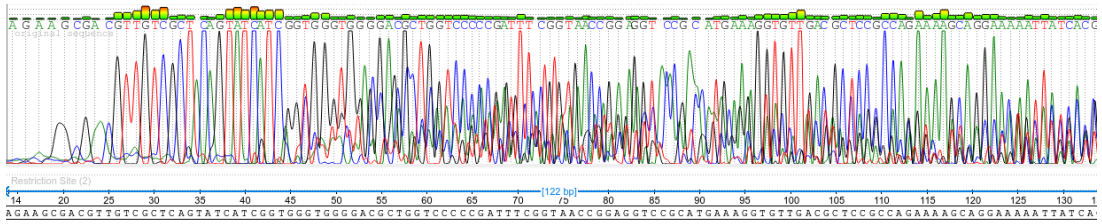
Injected oatp74D larva (1):



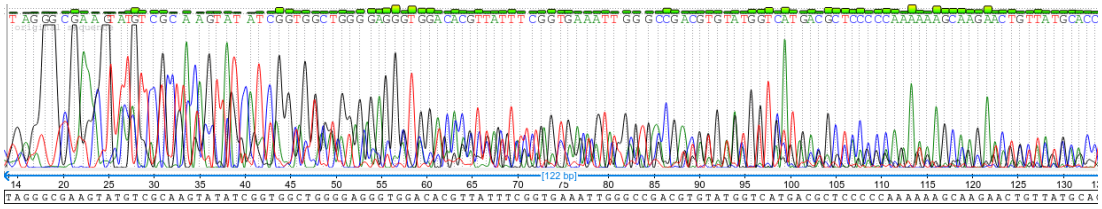
Injected oatp74D larva (2):



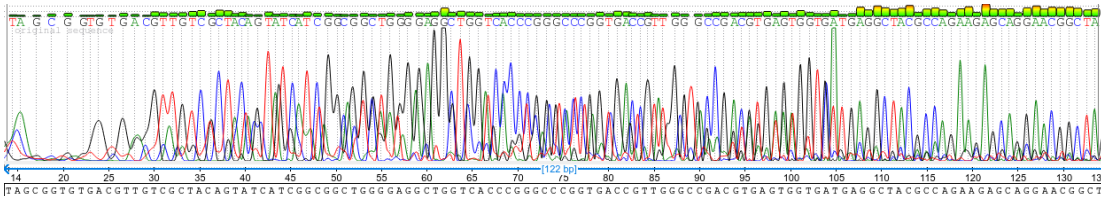
Injected larva (3):



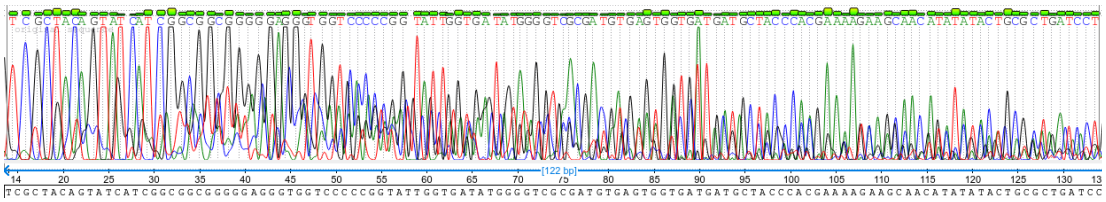
Injected larva (4):



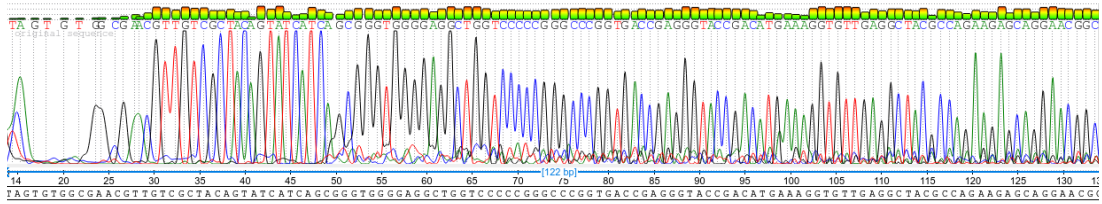
Injected larva (5):



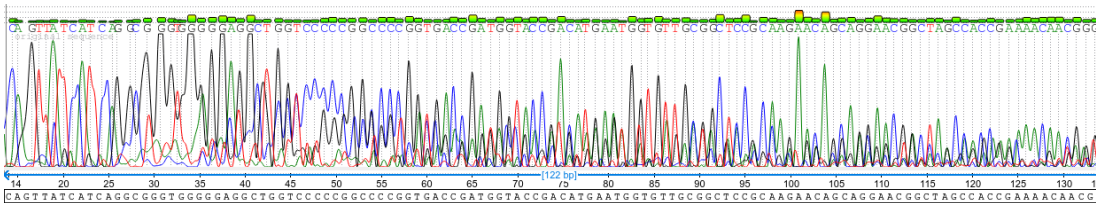
Injected larva (6):



Injected larva (7):



Injected larva (8):



Injected larva (9):

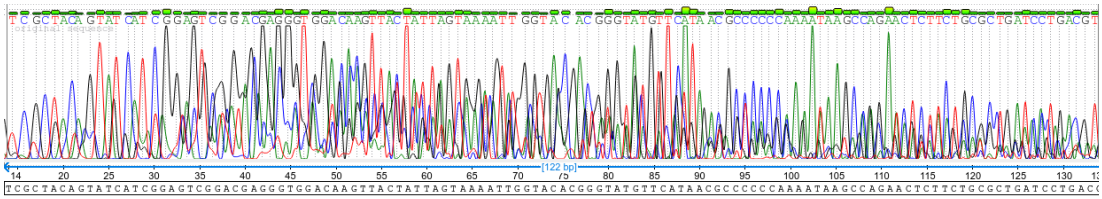


Figure S3: Sequencing results of the *Oatp74D* CRISPR target sequence from *S. frugiperda* injected adult survivors.

```
wt sequence     AGACTTCTGCTTATAGTCGCAGCGACGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCC  70
Adult1          ----------GAACGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCC              70
Adult2          ----------CGTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCC                 70
Adult3          ----------GTTGTCGCTACAGTATCATCAGCGGGTGGGGAGGCTGGTCCCCC                  70
                ******************************************

wt sequence     GGCCCCGGTGACCGAGGGTACCGACATGAAAGGTGTTGAGGCGACGCCAGAAGAGCAGGAACGGCTAGCA  140
Adult1          GGCCCCGGTGACCGAGGGTACCGACATGAAAGGTGTTGAGGCGACGCCAGAAGAGCAGGAACGGCTAGCA  140
Adult2          GGCCCCGGTGACCGAGGGTACCGACATGAAAGGTGTTGAGGCGACGCCAGAAGAGCAGGAACGGCTAGCA  140
Adult3          GGCCCCGGTGACCGAGGGTACCGACATGAAAGGTGTTGAGGCGACGCCAGAAGAGCAGGAACGGCTAGCA  140
                ************************************************************************

wt sequence     ACCGGAAACAACAATGGGTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCT  210
Adult1          ACCGGAAACAACAATGGGTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCT  210
Adult2          ACCGGAAACAACAATGGGTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCT  210
Adult3          ACCGGAAACAACAATGGGTCGCTGGACTGCAAGCACACGGCGGCCGACCCGCAGCCGCTGCAGGGCCGCT  210
                ************************************************************************

wt sequence     CCGTGTTCCACTCGACGCGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCT  280
Adult1          CCGTGTTCCACTCGACGCGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCT  280
Adult2          CCGTGTTCCACTCGACGCGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCT  280
Adult3          CCGTGTTCCACTCGACGCGGGTCTTCATGTTGGTGTTCCTGTCGGGCTGGATCCTGCAGGGCATGTTCCT  280
                ************************************************************************

wt sequence     CACCTACTTCGTCAGTGTAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACG  350
Adult1          CACCTACTTCGTCAGTGTAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACG  350
Adult2          CACCTACTTCGTCAGTGTAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACG  350
Adult3          CACCTACTTCGTCAGTGTAACGACCACAATAGAAAAATTATTTAAAGTAGAATCGAAGACGACGGGGACG  350
                ************************************************************************

wt sequence     TTACTAGCGGCCACGGAAATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGC  420
Adult1          TTACTAGCGGCCACGGAAATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGC  420
Adult2          TTACTAGCGGCCACGGAAATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGC  420
Adult3          TTACTAGCGGCCACGGAAATAGGCCAGATATCTACTGCGCTGATCCTGACGTACCTGGCGGGGCGCGGGC  420
                ************************************************************************

wt sequence     ACCGGCCGCGATGGATCGCCTGCATGATGATCGTGCTGGCGGTGGAATGTGGTCAA----------  490
Adult1          ACCGGCCGCGATGGATCGCCTGCATGATGATCGTGCTGGCGG-GGGGTGTGGTCCACGATCATGGCTGCG  490
Adult2          ACCGGCCGCGATGGATCGCCTGCATGATGATCGTGCTGGCGGGGGGGTTGTGGTCA----------  490
Adult3          ACCGGCCGCGATGGATCGCCTGCATGATGATCGTGCTGGCGGTGGGATGTGGTCA----------  490
                ******************************************* **  *******

wt sequence     ------------------------------------------------------------  560
Adult1          GCTGTTTTGGACTGCGAAAAGGTATTGAGGCCAGGTGCGGCGGGAGAATGAGGCAACGAGAAGATGAGCA  560
Adult2          ------------------------------------------------------------  560
Adult3          ------------------------------------------------------------  560

wt sequence     -  ----------  590
Adult1          CAAATAGCTGCGTAGGCAGGCGGGCATAAA  590
Adult2          ----------  590
Adult3          ----------  590
```