University of Crete
Department of Biology

PhD Thesis

Characterization of insecticide resistance mechanisms
of the olive fly (*Bactrocera oleae*)
and its interactions with symbiotic bacteria of the species *Candidatus* Erwinia dacicola

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Πανεπιστήμιο Κρήτης
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Διδακτορική Διατριβή

Χαρακτηρισμός μηχανισμών ανθεκτικότητας
του δάκου της ελιάς (*Bactrocera oleae*) στα εντομοκτόνα
και των αλληλεπιδράσεών του με συμβιωτικά βακτήρια, του είδους *Ca*. Erwinia dacicola

Βενετία Κοΐδου

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Abstract

The olive fruit fly *Bactrocera oleae* (*B. oleae*) is considered the major insect pest in olive orchards, causing great damage in the quality and the quantity of the olive production worldwide. However, its pest management approaches have proven difficult and inefficient, for a variety of reasons, a fact that has brought about a need for alternative tools and approaches. The objective of this thesis is to provide new insights and characterize mechanisms, potentially implicated in insecticide resistance, one of the limiting factors for an efficient pest management, as well as in the interactions between the olive fly and its symbiotic bacteria, especially of the species *Candidatus* Erwinia dacicola (*Ca*. E. dacicola), towards the establishment of innovative olive fly control strategies, which will target the symbiotic relationship with the bacterial partners (dysbiosis).

The present study is divided in 3 sections. In the first, the aim was to develop and apply a highly precise genome editing tool for *Bactrocera oleae*, and particularly, a CRISPR/Cas9 technology-based approach. We chose to target the *scarlet* gene in *B. oleae,* which provides an easy to screen eye color phenotype, in order to demonstrate that this technology is applicable to this non-model organism. We report the development of a CRISPR/Cas9 gene editing tool, using the well-known eye color marker gene *scarlet*. Two synthetic guide RNAs targeting the coding region of the *scarlet* gene were synthesized and shown to work efficiently *in vitro.* These sgRNAs along with purified Cas9 protein were then micro-injected into early-stage embryos. Successful CRISPR- induced mutations of both copies of the *scarlet* gene showed a striking yellow eye phenotype, indicative of gene disruption. Multiple successful CRISPR events were confirmed by PCR and sequencing. The establishment of an efficient CRISPR/Cas9-based gene editing tool in *B. oleae* will enable the study of critical molecular pathways in olive fly biology and physiology. The availability of such a genetic tool will enable a better understanding concerning the potent roles of various genes and mechanisms (i.e. critical symbiosis-based interactions with bacterial partners, insecticide resistance), towards the future development and application of novel pest control strategies.

In the second section of this thesis, we investigated potential mechanisms which are implicated in insecticide resistance of *B. oleae.* Olive fly pest management in Greece has relied mostly on the use of chemical (small-molecule) insecticides. The long overuse of organophosphorus-based (OP) insecticides has resulted in the development of resistance to this compound. OPs target the acetylcholinesterase enzyme (AChE) and suppress its function, which is the hydrolysis of the neurotransmitter acetylcholine (ACh), in order to prevent neuro-toxicity and subsequent death of the insect. In the first part of this second section, we attempted the functional validation of a target site mutation in the AChE of *B. oleae,* namely Δ3Q, which has been associated with OP resistance in *B. oleae,* after field screens and *in vitro* experiments, since 2008. The deletion of these three amino acids in its last exon, has been proposed to provide a better anchoring of the enzyme on the cell membrane, and as a result, the available molecules that hydrolyze ACh and interact with the insecticide are increased, resulting in survival of the olive fly at higher doses of insecticide, conferring resistance to OP compounds. However, this hypothesis has not been supported with

in vivo evidence yet. The aim of this study was to investigate this hypothesis *in vivo*, and more specifically by introducing the Δ3Q mutation, with the CRISPR/Cas9 gene editing tool developed in the previous section, in a susceptible genetic background of a laboratory-reared *B. oleae* strain and study the mutant phenotype upon insecticide application.

A strategy was implemented in which purified and commercially available Cas9 protein along with multiple sgRNAs and a synthetic donor ssODN DNA template (IDT) were introduced into early embryos with microinjections, following the protocol that was established in the previous section, in order to knock-in the Δ3Q modification, by homologous recombination (HDR mechanism). The results showed that although *in vitro,* five out of the total seven sgRNAs direct the Cas9 to the desired sites, in order to cleave the DNA and potently integrate the donor template (including the Δ3Q instead of the WT 5Q in the end of AChE), the corresponding result was not accomplished *in vivo*; approximately 2,174 olive fly embryos were injected and sequenced in groups. Sequencing results did not show any DNA cleavage, suggesting an insufficient Cas9 RNP uptake by the oocytes, resulting in a micro-injection protocol with very low efficiency. We conclude that the knock-in method requires further improvements, in order to successfully introduce mutations and study them functionally with the CRISPR/Cas9 genome modification tool.

In the second part of the second section of this thesis, we searched for potent gene candidates, implicated in pyrethroid resistance of olive flies, using transcriptome sequencing on olive fly malpighian tubules (MTs). The aim of this study was to identify genes not immediately apparent in the already existing whole organism RNA sequencing data, through a gene expression comparison in MTs, one of the proposed detoxification tissues in insects, dissected out of pyrethroid resistant and susceptible olive flies. Sequencing of the extracted RNA was performed using the Illumina platform, in three biological replicates for each one of the two populations. Sequencing reads were then aligned back to the olive fly genome reference sequence, gene expression levels were calculated and the up- and down-regulated genes, both in resistant and susceptible samples were identified. As expected, many genes that are well known to be implicated in insecticide resistance were identified, such as cytochrome P450s (CYPs), glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs). Moreover, several other genes were also identified, but their role in insecticide resistance remains to be elucidated via further investigation.

The over-expression of two P450 genes (CYP4P6 and CYP6G28) that were highlighted in the RNAseq data, was quantitatively validated with qPCR analysis and the functional validation of one of them, through gene silencing upon dsRNA injections (using the RNA interference technology, RNAi), revealed a promising phenotype, upon insecticide treatment; specifically, a 30% down-regulation of the CYP4P6 gene (compared to the control levels of the gene) conferred a 21% mortality to the injected olive flies, upon α-cypermethrin application, compared to the control group (4%). However, despite the promising phenotype conferred upon silencing of the CYP4P6 gene, foreshadowing a possible implication of this gene in pyrethroid resistance, the season-dependent limitation factor of this pest species did not allow the export of a complete and statistically significant conclusion, within the framework of this PhD thesis.

A general enrichment in the transcription of several genes from the five major detoxification gene families was observed in the MT-specific RNA dataset of resistant olive flies, providing new insights for the detoxification of insecticides in this species. However, further functional characterization studies are required. Following the encouraging preliminary results reported in the current study (concerning the role of the CYP4P6 gene), which require further validation steps, the interesting gene cases can be further investigated with *in vitro* insecticide metabolism assays, in order to confirm a potent implication in the detoxification procedures of insecticides. Therefore, such knowledge may contribute in the development of effective strategies for controlling this destructive pest and protecting the olive trees, the cultivation of which is of great regional importance, based on improved molecular diagnostics tools.

Moreover, in the third section of this thesis, we discuss the unique ability of olive flies to utilize unripe olives for their development, which has been associated with interactions with symbiotic bacteria. In this chapter, the aim was to investigate and define critical aspects of the unique symbiotic relationship, between the olive fly host and the *Ca*. E. dacicola bacterial symbiont. The objective of the current approach was to acquire further information for this unique symbiosis, through microscopy and transcriptomics analysis. At first, the determination of the relative abundance of *Ca*. E. dacicola during the life cycle of the olive fly, from the larval to the adult stage, comparing flies developing in unripe and ripe olives, was performed using real time quantitative PCR (RT-qPCR) and the data revealed that the bacterial titer is fluctuating between different developmental stages. Furthermore, we report, through confocal scanning imaging techniques, the localization of the bacterial symbionts in a specific part of the olive fly midgut, namely the gastric caeca, during the larval stages of the host. Noticeably, gastric caeca transform morphologically, depending on the developmental stage of the larva. Afterwards, a pairwise comparison was set, in order to define critical aspects, concerning the transformation of the gastric caeca during development, at a gene level. Gastric caeca which were dissected out of second and third instar larvae, revealed many genes potentially involved in the olive fly development. Moreover, comparative analysis between gastric caeca from second instar larvae developing in olives as well as in artificial diet, identified genes of the host, which are potentially involved in the establishment and the regulation of this symbiosis, since wild-type animals contain huge numbers of the symbiont partner, while the laboratory-reared do not. Subsequently, significant changes in transcript expression levels were reported and a detailed analysis of the data was undertaken, focusing on certain groups of genes that potentially participate in the symbiosis, as well as in the developmental transformation of the gastric caeca.

The new insights that are reported in this study, concerning the olive fly development and its interaction with this vertically transmitted and obligate symbiont partner, *Ca*. E. dacicola, can be exploited for the future development of symbiosis-based pest management strategies, concerning the olive fly control. Particularly, a better understanding of this symbiotic relationship will serve as the basis for the future development of novel olive fly control approaches, targeting this or other bacterial partners, by using molecular and classical tools in smart applications. Taken together, the data that are provided through this work, namely the establishment of a precise genome editing tool for *B. oleae* (CRISPR/Cas9) and the resistance-specific and symbiosis-specific released gene datasets, generate the opportunity to address the molecular basis of insecticide

resistance and symbiosis with bacterial partners mechanisms of the olive flies, in a systemic manner and, moreover, utilize the acquired knowledge towards the development of innovative pest control strategies, which will go beyond the traditional approaches and that will efficiently control this destructive pest species.

Scientific fields: Insecticide resistance, symbiosis with bacterial partners
Keywords: *Bactrocera oleae*, olive fly, insecticide resistance, CRISPR/Cas9, xenobiotic detoxification, malpighian tubules, *Candidatus* Erwinia dacicola, symbiont interactions, gastric caeca, midgut, symbiocides

Περίληψη

Ο δάκος της ελιάς, *Bactrocera oleae* (*B. oleae*) αποτελεί τον πιο σημαντικό εχθρό της ελαιοκαλλιέργειας, προκαλώντας έντονη υποβάθμιση τόσο στην ποιότητα όσο και στην ποσότητα της ελαιοπαραγωγής παγκοσμίως. Ωστόσο, οι διαφορετικές στρατηγικές διαχείρισης του εντόμου έχουν αποδειχθεί δύσκολες και αναποτελεσματικές για διάφορους λόγους, γεγονός το οποίο έχει δημιουργήσει την ανάγκη για ανάπτυξη εναλλακτικών εργαλείων και προσεγγίσεων. Στόχος αυτής της διατριβής είναι να παράσχει νέες γνώσεις και να χαρακτηρίσει μηχανισμούς οι οποίοι πιθανώς να εμπλέκονται στην ανθεκτικότητα του δάκου στα εντομοκτόνα, έναν από τους βασικότερους περιοριστικούς παράγοντες όσον αφορά την αποτελεσματική διαχείριση των εντόμων, καθώς και στις αλληλεπιδράσεις μεταξύ του δάκου και των συμβιωτικών βακτηρίων του είδους *Candidatus* Erwinia dacicola (*Ca*. E. dacicola), αποβλέποντας στην ανάπτυξη μιας νέας γενιά καινοτόμων στρατηγικών ελέγχου του εντόμου, οι οποίες θα στοχεύουν στη διατάραξη της συμβιωτικής του σχέσης με τα βακτήρια (dysbiosis).

Η παρούσα μελέτη χωρίζεται σε 3 ενότητες. Στην πρώτη ενότητα, ο στόχος ήταν να αναπτυχθεί και να εφαρμοστεί ένα ακριβές εργαλείο τροποποίησης του γονιδιώματος στο δάκο, και συγκεκριμένα μια προσέγγιση βασισμένη στην τεχνολογία CRISPR/Cas9. Επιλέξαμε να στοχεύσουμε το γονίδιο *scarlet*, το οποίο προσφέρει έναν εύκολα ανιχνεύσιμο φαινότυπο στο χρώμα των ματιών του εντόμου, προκειμένου να αποδείξουμε ότι αυτή η τεχνολογία είναι εφαρμόσιμη σε αυτόν τον οργανισμό. Δύο sgRNA μόρια-οδηγοί μαζί με την ενδονουκλεάση Cas9 ενέθηκαν σε έμβρυα δάκου αρχικού σταδίου. Οι γονιδιακές τροποποιήσεις στο γονίδιο *scarlet*, οι οποίες δημιούργησαν έναν εντυπωσιακό φαινότυπο κίτρινων ματιών, επιβεβαιώθηκαν με PCR και αλληλούχηση. Η δημιουργία ενός αποτελεσματικού εργαλείου τροποποίησης γονιδίων, με χρήση της τεχνολογίας CRISPR/Cas9 για το δάκο της ελιάς, θα διευκολύνει τη μελέτη κρίσιμων μοριακών μονοπατιών στη βιολογία και τη φυσιολογία του εντόμου. Η διαθεσιμότητα ενός τέτοιου μοριακού εργαλείου θα επιτρέψει την καλύτερη κατανόηση των ρόλων διαφόρων γονιδίων και μηχανισμών (ανθεκτικότητα σε εντομοκτόνα, αλληλεπιδράσεις με συμβιωτικά βακτήρια κ.α.), με σκοπό τη μελλοντική ανάπτυξη και εφαρμογή νέων στρατηγικών διαχείρισης του δάκου.

Στη δεύτερη ενότητα αυτής της μελέτης, διερευνήσαμε μηχανισμούς που πιθανώς εμπλέκονται στην ανάπτυξη ανθεκτικότητας του δάκου σε διαφορετικά εντομοκτόνα. Η διαχείριση του δάκου της ελιάς στην Ελλάδα έχει βασιστεί κυρίως στη χρήση χημικών (μικρομοριακών) εντομοκτόνων. Η μακροχρόνια υπερβολική χρήση οργανοφωσφορικών εντομοκτόνων (OP) έχει δημιουργήσει σοβαρά προβλήματα ανάπτυξης ανθεκτικότητας σε αυτές τις χημικές ενώσεις. Τα OP στοχεύουν το ένζυμο ακετυλοχολινεστεράση (AChE) του εντόμου και καταστέλλουν τη λειτουργία του, η οποία είναι η υδρόλυση του νευροδιαβιβαστή ακετυλοχολίνη (ACh), με αποτέλεσμα να προκαλείται νευροτοξικότητα και επακόλουθος θάνατος του εντόμου. Στο πρώτο μέρος της δεύτερης ενότητας, επιχειρήσαμε το λειτουργικό χαρακτηρισμό μιας μεταλλαγής στόχου στην AChE του δάκου (απαλοιφή τριών γλουταμινών, Δ3Q), η οποία έχει συσχετιστεί με την ανθεκτικότητα του δάκου στα OP από το 2008, μέσα από πειράματα πεδίου και *in vitro* δοκιμές. Έχει προταθεί ότι η απαλοιφή αυτών των τριών αμινοξέων στο τελευταίο εξόνιο του γονιδίου, παρέχει καλύτερη αγκυροβόληση της πρόδρομης μορφής του ενζύμου στην κυτταρική μεμβράνη.

Το αποτέλεσμα είναι να αυξάνονται τα διαθέσιμα μόρια που υδρολύουν την ACh και αλληλεπιδρούν με το εντομοκτόνο. Με αυτόν τον τρόπο, ο δάκος επιβιώνει σε υψηλότερες δόσεις του εντομοκτόνου, αναπτύσσοντας ανθεκτικότητα σε αυτές τις ενώσεις. Ωστόσο, αυτή η υπόθεση δεν έχει υποστηριχθεί ακόμη με *in vivo* δεδομένα. Ο σκοπός αυτής της μελέτης ήταν να διερευνηθεί αυτή η υπόθεση *in vivo*, και πιο συγκεκριμένα με την εισαγωγή της μετάλλαξης Δ3Q, με το εργαλείο γονιδιακής τροποποίησης CRISPR/Cas9 το οποίο αναπτύχθηκε στην προηγούμενη ενότητα, σε ένα ευαίσθητο γενετικό υπόβαθρο ενός εργαστηριακού στελέχους δάκου, με στόχο την μελέτη του φαινοτύπου κατά την εφαρμογή OP εντομοκτόνου.

Εφαρμόστηκε λοιπόν μια στρατηγική κατά την οποία η καθαρισμένη και εμπορικά διαθέσιμη πρωτεΐνη Cas9 μαζί με sgRNA μόρια οδηγούς και ένα συνθετικό DNA δότη ssODN ενέθηκαν σε έμβρυα δάκου, σύμφωνα με τη μεθοδολογία η οποία αναπτύχθηκε στην προηγούμενη ενότητα, με σκοπό την ενσωμάτωση της μεταλλαγής Δ3Q, με ομόλογο ανασυνδυασμό (μηχανισμός HDR). Τα αποτελέσματα έδειξαν ότι αν και *in vitro*, πέντε από τα συνολικά επτά sgRNAs κατευθύνουν την Cas9 στις επιθυμητές θέσεις κοπής, προκειμένου να κατατμηθεί το DNA και δυνητικά να ενσωματωθεί το πρότυπο κομμάτι DNA (το οποίο περιλαμβάνει τη μεταλλαγή Δ3Q στο τέλος της AChE), το αντίστοιχο αποτέλεσμα δεν επιτεύχθηκε *in vivo*. 2,174 έμβρυα δάκου ενέθηκαν και αλληλουχήθηκαν σε ομάδες. Τα αποτελέσματα δεν έδειξαν κάποια κατάτμηση του DNA, υποδηλώνοντας την πιθανώς ανεπαρκή πρόσληψη του συμπλόκου Cas9/RNP από τα πολικά ή τα ωοθυλακικά κύτταρα του εμβρύου, γεγονός που υποδεικνύει τη χαμηλή απόδοση αυτής της μεθοδολογίας μικρο-ενέσεων σε αυτό το έντομο. Συμπερασματικά, η knock-in μέθοδος με χρήση του εργαλείου γονιδιωματικής τροποποίησης CRISPR/Cas9 χρήζει περαιτέρω βελτίωσης, προκειμένου να εισάγονται επιτυχώς μεταλλάξεις για το λειτουργικό χαρακτηρισμό γονιδίων και μηχανισμών.

Στο δεύτερο μέρος της δεύτερης ενότητας της διατριβής, ψάξαμε για υποψήφια γονίδια στο δάκο, τα οποία συμμετέχουν σε μηχανισμούς ανθεκτικότητας σε πυρεθροειδή εντομοκτόνα, μέσω RNA αλληλούχησης, ειδικά στα μαλπιγγιανα σωληνάρια (malpighian tubules, MTs) του εντόμου. Ο στόχος αυτής της μελέτης ήταν να ταυτοποιηθούν γονίδια τα οποία έχουν ιστο-ειδική έκφραση και ως εκ τούτου δεν θα ήταν εύκολο να ανιχνευτούν σε δεδομένα RNA αλληλούχησης ολόκληρου του οργανισμού. Η μελέτη αυτή επιτεύχθηκε μέσω σύγκρισης της γονιδιακής έκφρασης στα MTs, έναν από τους προτεινόμενους ιστούς αποτοξικοποίησης στα έντομα, σε έναν ανθεκτικό και έναν ευαίσθητο σε πυρεθροειδή πληθυσμό. Η αλληλούχηση του RNA πραγματοποιήθηκε χρησιμοποιώντας την πλατφόρμα Illumina, σε τρεις βιολογικές επαναλήψεις για κάθε έναν από τους δύο πληθυσμούς. Στη συνέχεια, οι αλληλουχίες RNA στοιχήθηκαν με τη διαθέσιμη αλληλουχία αναφοράς του γονιδιώματος του δάκου της ελιάς, υπολογίστηκαν τα επίπεδα γονιδιακής έκφρασης και ταυτοποιήθηκαν γονίδια τα οποία σημείωσαν υπερ-έκφραση ή υπο-έκφραση, τόσο στον ανθεκτικό όσο και στον ευαίσθητο πληθυσμό. Όπως ήταν αναμενόμενο, εντοπίστηκαν πολλά γονίδια τα οποία είναι ευρέως γνωστό ότι εμπλέκονται σε μηχανισμούς ανθεκτικότητας σε εντομοκτόνα, όπως οι P450 μονοοξυγενάσες (cytochrome P450s), οι S-τρανσφεράσες της γλουταθειόνης (GSTs) και οι UDP-γλυκουρονοσυλο-τρανσφεράσες (UGTs). Επιπλέον, εντοπίστηκαν πολλά άλλα γονίδια, των οποίων ο πιθανός ρόλος τους σε μηχανισμούς ανθεκτικότητας σε εντομοκτόνα μένει να αποσαφηνιστεί μέσω περαιτέρω έρευνας.

Η υπερ-έκφραση δύο γονιδίων μονοοξυγενασών P450 (CYP4P6 και CYP6G28) η οποία επισημάνθηκε στα δεδομένα RNA, επικυρώθηκε ποσοτικά με ανάλυση RT-qPCR και ο λειτουργικός χαρακτηρισμός ενός από αυτά, μέσω γονιδιακής σίγασης (με τη χρήση της τεχνολογίας παρεμβολής RNA, RNAi), απέδωσε έναν πολλά υποσχόμενο φαινότυπο, μετά από εφαρμογή βιοδοκιμής τοξικότητας με alpha-cypermethrin (πυρεθροειδές εντομοκτόνο). Συγκεκριμένα, η μειωμένη κατά 30% έκφραση του γονιδίου CYP4P6 (σε σύγκριση με τα επίπεδα έκφρασης του γονιδίου στα δείγματα μάρτυρες) επέφερε αύξηση στα επίπεδα θνησιμότητας (21%) στα ενεμένα άτομα, κατά την εφαρμογή του εντομοκτόνου, σε σύγκριση με τους μάρτυρες (4% θνησιμότητα). Ο συγκεκριμένος φαινότυπος συνηγορεί υπέρ μιας πιθανής συμμετοχής της CYP4P6 στο μηχανισμό ανθεκτικότητας του δάκου στα πυρεθροειδή. Ωστόσο, η περιορισμένη διαθεσιμότητα δάκων δεν επέτρεψε την εξαγωγή ενός στατιστικά σημαντικού συμπεράσματος, στα πλαίσια της παρούσας διδακτορικής διατριβής.

Συμπερασματικά, αρκετά γονίδια αποτοξικοποίησης καταγράφηκαν ως υπερ-εκφραζόμενα βάσει των δεδομένων RNAseq, μετά την αλληλούχηση των MTs από ανθεκτικά και ευαίσθητα σε alpha-cypermethrin άτομα δάκου, παρέχοντας νέες πληροφορίες σχετικά με την αποτοξικοποίση των εντομοκτόνων σε αυτό το είδος. Ωστόσο, περαιτέρω μελέτες λειτουργικού χαρακτηρισμού των γονιδίων κρίνονται απαραίτητες. Μετά τα ενθαρρυντικά πρώτα αποτελέσματα που αναφέρονται στην παρούσα διερεύνηση (σχετικά με τον πιθανό ρόλο του γονιδίου CYP4P6), τα οποία ωστόσο χρήζουν περαιτέρω επιβεβαίωσης, οι ενδιαφέρουσες περιπτώσεις γονιδίων μπορούν να διερευνηθούν με *in vitro* πειράματα μεταβολισμού εντομοκτόνων, προκειμένου να διαλευκανθεί η πιθανή συμμετοχή τους στους μηχανισμούς αποτοξικοποίσης των εντομοκτόνων στο δάκο. Η απόκτηση αυτής της γνώσης μπορεί να συμβάλει στην ανάπτυξη αποτελεσματικών στρατηγικών για την καταπολέμηση αυτού του επιβλαβούς εντόμου και την προστασία των ελαιόδεντρων, η καλλιέργεια των οποίων έχει μεγάλη οικονομική σημασία.

Στην τρίτη ενότητα αυτής της διατριβής, προσεγγίστηκε η μοναδική ικανότητα του δάκου της ελιάς να χρησιμοποιεί άγουρες ελιές για την ανάπτυξή του, η οποία έχει συσχετιστεί με αλληλεπιδράσεις με συμβιωτικά βακτήρια. Στόχος αποτέλεσε να αναδειχθούν και να διερευνηθούν κρίσιμες πτυχές της μοναδικής αυτής συμβιωτικής σχέσης, μεταξύ του ξενιστή και του κυρίαρχου συμβιωτικού βακτηρίου *Ca*. E. dacicola, μέσω πειραμάτων μικροσκοπίας και ιστοειδικής μεταγραφικής ανάλυσης. Αρχικά, επιχειρήθηκε ο ποσοτικός προσδιορισμός του *Ca*. E. dacicola, κατά τη διάρκεια του κύκλου ζωής του δάκου, από το στάδιο της προνύμφης μέχρι το ενήλικο στάδιο, συγκρίνοντας μύγες που αναπτύσσονται σε άγουρες αλλά και σε ώριμες ελιές. Η μελέτη αυτή έγινε με χρήση ποσοτικής PCR σε πραγματικό χρόνο (RT-qPCR) και έδειξε έντονη διακύμανση μεταξύ των διαφορετικών αναπτυξιακών σταδίων. Επιπλέον, μέσω τεχνικών συνεστιακής μικροσκοπίας σάρωσης (confocal scanning imaging analysis), απεικονίστηκε το τμήμα του μεσεντέρου του δάκου, οι επονομαζόμενες τυφλές γαστρικές απολήξεις (gastric caeca), το οποίο φιλοξενεί τα συμβιωτικά βακτήρια κατά τα προνυμφικά στάδια του εντόμου και το οποίο διαπιστώσαμε ότι υπόκειται σε μορφολογικό μετασχηματισμό, ανάλογα με το αναπτυξιακό στάδιο της προνύμφης. Στη συνέχεια, αναλύθηκαν τα αποτελέσματα δύο συγκρίσεων, προκειμένου να μελετηθούν γονίδια τα οποία σχετίζονται αφενός με τον μετασχηματισμό και την ανάπτυξη του γαστρικού τυφλού και αφετέρου με τη ρύθμιση της συμβιωτικής σχέσης του δάκου με το *Ca*. E. dacicola. Πραγματοποιήθηκε λεπτομερής ανάλυση των δεδομένων, εστιάζοντας σε ορισμένες

ομάδες γονιδίων που δυνητικά συμμετέχουν σε μηχανισμούς αυτής της συμβίωσης, καθώς και στον αναπτυξιακά ρυθμιζόμενο μετασχηματισμό του γαστρικού τυφλού.

Οι νέες πτυχές οι οποίες παρουσιάζονται στην παρούσα μελέτη, σχετικά με τις αναπτυξιακές μεταβολές του δάκου της ελιάς και την αλληλεπίδρασή του με τον μητρικά κληρονομούμενο και απαραίτητο συμβιώτη, *Ca*. E. dacicola, μπορούν να αξιοποιηθούν στη μελλοντική ανάπτυξη καινοτόμων στρατηγικών διαχείρισης του εντόμου, οι οποίες θα στοχεύουν αυτήν την ιδιόμορφη συμβιωτική σχέση. Πιο ειδικά, η κατανόηση αυτής της συμβίωσης θα αποτελέσει τη βάση για την εγκαθίδρυση νέων προσεγγίσεων διαχείρισης του δάκου, μέσω του *Ca*. E. dacicola ή άλλων συμβιωτικών βακτηρίων, με χρήση μοριακών και κλασικών εργαλείων σε έξυπνες εφαρμογές.

Συνολικά, τα δεδομένα που παρέχονται στα πλαίσια της παρούσας διδακτορικής διατριβής, δηλαδή η ανάπτυξη ενός ακριβούς εργαλείου γονιδιωματικής τροποποίησης για εφαρμογή στο δάκο (βασιζόμενο στο σύστημα CRISPR/Cas9) καθώς και τα δεδομένα αλληλούχησης RNA, τα οποία αναφέρονται στην ανάπτυξη ανθεκτικότητάς του σε εντομοκτόνα αλλά και στη συμβιωτικές του αλληλεπιδράσεις, παρέχουν πλέον τη δυνατότητα να μελετηθεί η μοριακή βάση διαφόρων μηχανισμών (ανθεκτικότητα σε εντομοκτόνα, συμβιωτικές σχέσεις με μικροοργανισμούς) με συστηματικό τρόπο. Η γνώση αυτή θα συμβάλλει στην ανάπτυξη καινοτόμων στρατηγικών καταπολέμησης του δάκου, οι οποίες θα διαφοροποιούνται από τις παραδοσιακές προσεγγίσεις και θα παρέχουν μια αποτελεσματική διαχείριση αυτού του καταστροφικού για την ελαιοπαραγωγή εντόμου.

Επιστημονικό πεδίο: ανθεκτικότητα σε εντομοκτόνα, συμβιωτικές αλληλεπίδραση με βακτήρια
Λέξεις κλειδιά: *Bactrocera oleae*, δάκος της ελιάς, ανθεκτικότητα σε εντομοκτόνα, CRISPR/Cas9, εργαλείο γονιδιωματικής τροποποίησης, αποτοξικοποίηση, μαλπιγγιανά σωληνάρια, *Candidatus* Erwinia dacicola, τυφλές γαστρικές απολήξεις, gastric caeca, μεσέντερο

PhD Title |
"Characterization of insecticide resistance mechanisms of the olive fly (*Bactrocera oleae*) and its interactions with symbiotic bacteria of the species *Ca*ndidatus Erwinia dacicola*".*

L i s t o f C o n t e n t s

List of figures

List of supplementary material

Chapter 1

General Introduction

1.1. *Bactrocera oleae;* the morphology and life cycle of the pest

The olive fly, *Bactrocera oleae (B. oleae),* is the most significant pest of olives. This insect species can be found in the geographical areas where olive trees (*Olea europaea L.*) are both indigenous and cultivated, including the Mediterranean basin, western Asia, Canary Islands, south and east Africa (**Figure 1.1**) and other areas like central U.S. (California) and Mexico, where it is considered an invasive species (Rice et al. 2003; F Nardi et al. 2005; Ashraf, Chaudhry, and Peterson 2021).

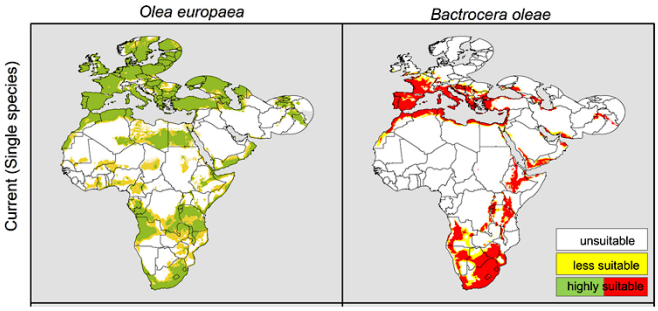

Figure 1.1. Current distributions of *O. europaea* and *B. oleae* in Africa, southern Europe and Asia. Map from Ashraf et al. 2021

Olive fly larvae are strictly monophagous, feeding exclusively on olive flesh, whereas adults are polyphagous, feeding on a variety of substrates including nectar, honeydew, fruit and plant exudates, bacteria, and bird feces (Tsiropoulos 1977; Sacchetti et al. 2014). The life cycle of the species coincides with the beginning of spring (for the northern hemisphere). Usually, in April, after mating, a large number of males appear, while females start laying during May. Adult females oviposit their eggs inside the olive fruit and the larvae develop by feeding on the fleshy mesocarp, causing great damage to the fruit (Fletcher 1987). Each female lays approximately 200 eggs. The duration of the olive fly life cycle varies from 22-26 days (summer) to several months (autumn-winter), depending on the season of the year (Daane and Johnson 2010).

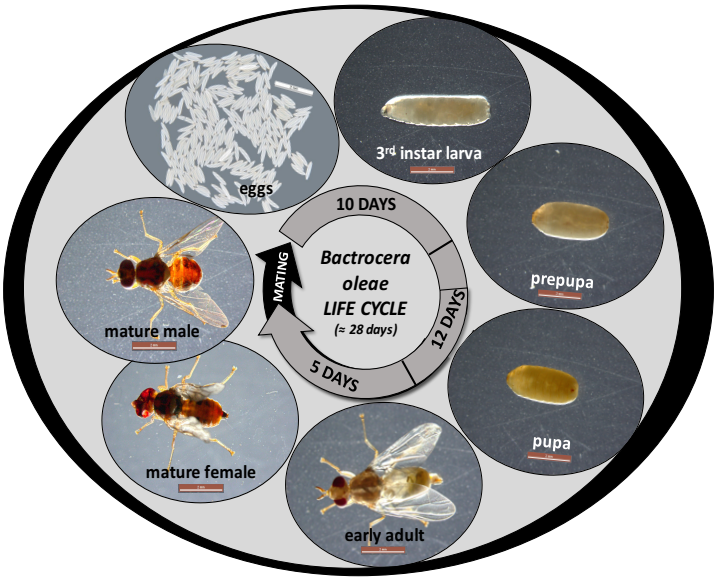

Figure 1.2. The life cycle of the olive fly, *Bactrocera oleae.*

Adult flies are 4-5 mm long and their characteristic small black spot located at the end of each wing, distinguishes them from other Tephritid species. Female adults carry an ovipositor for the oviposition of the eggs in the olives. This is accomplished, by making a puncture with the ovipositor into the skin of the olive, leaving one egg under the thin surface layer of the fruit. The egg is whitish and elongated, around 0.7 to 1.2 mm long and of 0.2 mm diameter. After a few days (2-3 in summer and around 10 during winter time) the hatched larva begins digging a

tunnel on the surface and moves to the depths of the fruit. During the three larval stages the color of the larva turns from white to white-yellowish and reaches the 7 mm length at late third instar stage. When in the last stage of the larval development, the larva moves to the surface of the fruit in order to prepare the hole for the adult exit. The duration of the larval stages is around 10-13 days in summer and around 20 days during winter (Fletcher 1987; Tzanakakis 2003).

The pupal stage takes place inside the puparium, an elliptical shell formed by the last exuvial transformation of the larvae, which is approximately 4 mm long and its color varies from creamy-white to yellow-brown according to the stage of maturation. The pupa remains dormant until the adult fly breaks the exuvia and emerges from the pupa, breaks the olive skin surface and the adult leaves the exit hole. The duration of the pupal stage in the summer is approximately 10 days. In late autumn and winter, the late third instar mature larva emerges from the olive and drops on the ground where pupation takes place and may last several months (**Figure 1.2**).

1.2. Damage caused by the olive fly

Heavy olive fly infestation affects both the quantity and the quality of olives and olive oil and reducing their value by up to 80% (Nobre 2019). In the Mediterranean countries, economic losses caused by *B. oleae* are annually estimated at 30% of total olive production; the consequent damage equating to economic losses has been estimated at more than 20 million euros just on the island of Crete, where olive trees represent the 65% of the agricultural land (Kampouraki et al. 2018b). At a world-wide level, the resulting damage caused by this pest has been estimated to be 5% of total olive production, corresponding to economic losses of approximately $ 800 millions/year and 100 million euros in 2019 within Greece (Konstantopoulos 2019).

As it concerns the effect of the olive fly infestation in the olive quantity, the detrimental effects caused by the olive fly include the premature fall of the olive fruits and the reduction of the olive weight, due to the feeding needs of the second and third instar larvae, inside the olive mesocarp (each larva consumes approximately 1/5 to 1/4 of the olive to develop (Fletcher 1987). The quality of the olive oil is also affected due to the negative alterations in the organoleptic properties of the virgin olive oil (voo). Recently it was shown that although a drastic decrease of the phenolic contents in olive oil obtained from infested olives is observed, compared to the control ones, a significant increase in the content of certain volatile compounds (such as (*E)*-hex-2-enal, ethanol, ethyl acetate, and β-ocimene) affects the exceptional voo aroma drastically (Notario et al. 2022).

1.3. Pest management approaches for olive fly control

The control of the olive fly has been based on several approaches, often applied under Integrated pest management (IPM) ("a careful consideration of all available plant protection methods and subsequent integration of appropriate measures, that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment", European Union Framework Directive on the Sustainable Use of Pesticides (Directive 2009/128/EC)) schemes (Barzman et al. 2015). The dynamic (in terms of design, implementation, and evaluation) IPM science-based decision-making process combines tools and strategies, including chemical insecticides and non-insecticidal applications, which are briefly described below.

1.3.1. Biotechnical pest management

This quite conventional control strategy includes a variety of attractive means that have been applied over the years, in order to lure olive flies and lead them to death. Since olive flies are being attracted by various compounds, the approaches that have been attempted mostly rely on trapping methods using baits, color traps and pheromone attractants (Daane and Johnson 2010).

Mass trapping techniques have been given special consideration since the early 1980s, as alternative control strategies, against the developing resistance to small-molecule insecticides (GE Haniotakis, Mazomenos, and Tumlinson 1977). Early studies revealed that sex pheromone produced by both female and male olive flies, can serve as an effective attractant substance to be employed in the traps (G. Haniotakis et al. 1986). Furthermore, comparative testing on the chemical attractants indicated that combining food and sex attractants in the same trap, higher effectiveness of the method is provided, compared to using either type individually (G. E. Haniotakis and Vassiliou-Waite 1987).

One of the first experimental trials in Greece, compared the most effective trap type in the most effective mass-trapping scheme, available until then and tested the efficacy in a pilot-scale study (Broumas et al. 2002). Since then, McPhail, Ecotrap, and yellow sticky traps have been used for monitoring fly activities and population levels. Plastic McPhail traps have been shown to be more efficient than the sticky ones, although when their color is yellow, high levels of beneficial insects have been reported to be caught (Neuenschwander 1982). However, yellow sticky traps, baited with a pheromone lure or ammonium bicarbonate, may be easier to use. Studies have shown that the "lure and kill" MT techniques that combine insecticide-baited traps, ammonia-releasing salts as a food attractant and a sex pheromone, result in a promising reduction in the active olive fly infestation (Petacchi, Rizzi, and Guidotti 2003; Speranza, Bellocchi, and Pucci 2004).

The fundamental concept behind these mass trapping strategies is that by removing a significant proportion of the olive fly population from the agroecosystem, the crop damage will be reduced. In many cases, the efficacy of such systems has not been fully established, and it has been difficult to reproduce results, due to the wide variation of the orchards, where these traps have

been located (Yasin et al. 2014). Although an effective “lure and kill” technique is expensive and unaffordable for producers yet, this field has continued developing and new approaches are being continually tested, aiming at reducing the massive insecticide usage (Daane and Johnson 2010).

1.3.2. Chemical control

In the early 1950s, organochlorines (OCLs) (dichlorodipehnyltrichlroethane, DDT), organophosphorus-based (OPs) (parathion, malathion), and carbamate-based (CARBs) insecticides were firstly applied for the chemical control of olive flies. The introduction of other OPs (dimethoate, fenthion), pyrethroids (α-cypermethrin, deltamethrin, β-cyfluthrin, λ-cyhalothrin), spinosyns (spinosad), and neonicotinoids (thiacloprid, acetamiprid) followed the next years (**Table 1.1**) (Vontas et al. 2011). Nowadays, the chemical management of this pest relies mainly on pyrethroids (lambda-cyhalothrin, deltamethrin, beta-cyfluthrin), spinosyns (spinosad), and rarely organophosphorus-based insecticides (phosmet), while the methods of application consist of cover and bait (insecticides mixed with an attractant) sprayings (Kampouraki et al. 2018a).

Chemical group	Active compound	Mode of action
organophosphates	Dimethoate	AChE inhibitor
	Phosmet	
	Fenthion	
	Malathion	
pyrethroids	α-cypermethrin	axonic excitotoxin targeting VGSC
	Deltamethrin	
	β-cyfluthrin	
	λ-cyhalothrin	
spinosyns	Spinosad	nACh & GABA receptor agonist
neonicotinoids	Thiacloprid	ACh receptor agonist
	Acetamiprid	

Table 1.1. The chemical compounds that have been used for the control of *B. oleae* in Greece, since 1950. Abbreviations**;** AChE: acetylcholinesterase, VGSC: voltage gated sodium channel, nACh: nicotinic acetylcholine, GABA: gamma - aminobutyric acid, ACh: acetylcholine.

Other studies have investigated the deterring action of kaolin-based particle film formulations, which are shown to significantly reduce the infestation levels on treated-trees, due to the residing clay or the lack of bacterial compounds on the olive surface. Although such traits of the olive fruits make the oviposition sites less attractive to the olive flies, suggesting a kind of behavioral resistance evolvement, the already reported results exhibit low levels of success in big areas of application (Saour and Makee 2004; Caleca et al. 2010; Pascual et al. 2010). Furthermore, the effect of Bt toxins derived from the bacterial species *Bacillus thurigiensis* on olive fly populations has been investigated, with not so encouraging results yet (Navrozidis et al. 2000).

1.3.3. The sterile insect technique (SIT)

SIT has been suggested as an alternative, species-specific and more environmentally friendly method for olive fly control since the early 1970s and is dependent upon the release of large numbers of sterilized insects in the field; when these insects mate with wild ones, the progeny are reproductively sterile, due to cytoplasmic incompatibility (CI), and as a consequence, the population is crashed down (Zervas and Economopoulos 1982). Although SIT has proven to be effective against other insect pests (Bourtzis and Vreysen 2021), the olive fly trials have yielded to poor results, due to several reasons, including the low quality of the mass-reared olive flies, the low competitiveness of the sterilized males when they were released in the field and the widespread concern about the impact of gamma-irradiation, used to induce the sterility (A. M. Estes et al. 2012). The promising SIT technique which has a few drawbacks, was followed by the recent advancements of RIDL (Release of Insects carrying a Dominant Lethal) technology (Ant et al. 2012a). The latter addresses some of these issues, by genetically manipulating the insect, but the efficacy and the efficiency of this method (especially the necessity for repeated insect releases) has to be validated in the field. However, the latest advances in the experimental methodologies that are being applid, offer significant opportunities for the development of effective SIT-based control approaches of the olive fly nowadays (Ant et al. 2012b; Genç et al. 2016; Ahmad et al. 2018; Deutscher et al. 2019; Choo et al. 2020).

1.3.4. Biological control

Classical biological control methods that use natural enemies of the olive flies (predators, parasitoids mostly of the species *Psyttalia lounsburyi* and *Psyttalia concolor,* as well as micro-organisms like *Bacillus thurigiensis* and *Beauveria bassiana*), were attempted throughout the twentieth century, but with low levels of success and high economical costs (Hepdurgun, Turanli, and Zümreoğlu 2009; Daane and Johnson 2010). Among the limitation factors of this approach are the mass rearing procedures of the parasitoid species (due to the difficult artificial rearing of the *B. oleae* host) and the lack of information on appropriate indigenous parasitoids, depending on the region (Daane and Johnson 2010). However, successful management of the olive fly populations with the use of endoparasitoids (Psyttalia humilis Silvestri, *P.* lounsburyi (Silvestri)) is being applied in California, since 2003 (Daane et al. 2015). The main reason about the lack of such effective approaches in the Mediterranean basin until now, is the absence of specialized indigenous parasitoids and the limitation of the imported from sub-Saharan Africa parasitoid species in adapting under the adverse heat conditions during summer (Hoelmer et al. 2011). The aforementioned approach consist of a huge field of research, which is still under exploration, and has the potential to provide new insights for a functional bio-control (Wang et al. 2021).

1.3.5. Pest control by targeting the bacterial symbionts (Dysbiosis)

As described in the previous sections, mass trapping, mating disruption technologies, Sterile Insect Technique (SIT-gamma irradiation-based), and chemical insecticides are the main means that have been applied in order to control *B. oleae* populations until now. On the other side, the

heavy use of insecticides in recent years has resulted in the development of insecticide resistance, which has negatively affected our ability to manage olive flies in this manner (Vontas et al. 2011; Pavlidi et al. 2018a; Kampouraki et al. 2018b). The until now inefficient management of olive fly populations in combination with the mandatory in the European Union (EU) guideline for a sustainable use of insecticides, under the directive 2009/128/EC, necessitates a clear requirement for alternative tools and strategies to control this pest species (Nobre 2019). The development of novel tools for understanding the physiology and the molecular biology of targeted processes and mechanisms is an important pre-requirement for such advances.

Due to the limited efficiency of the existing conventional olive fly management approaches, new generation techniques which will target the bacterial partners are now up for discussion and development (Nobre 2019). Targeting the endosymbiotic and vertically transmitted bacterial partner *Ca.* Erwinia dacicola appears to be a promising method for pest control and an ideally-suited and well-targeted decision, since this bacterial species represents the major obligate symbiont in olive fly (Kounatidis et al. 2009; Capuzzo et al. 2005; A. Estes et al. 2018), and it is localized in specialized insect tissues (A. M. Estes et al. 2009; Capuzzo et al. 2005). Furthermore, the way that it is exclusively maternally transmitted to the next generation is a critical point, which may provide several targets for the disruption of this association (A. M. Estes et al. 2009; Capuzzo et al. 2005). In addition, the recent advances of the genome and the RNA sequencing technologies have revealed many candidate gene targets, with a potential critical role in this symbiotic relationship, providing new perspectives in this field of science (A. Estes et al. 2018; Pavlidi et al. 2013; 2018b).

Symbiosis-based strategies are still underdeveloped; control methods that follow this strategy until now include approaches which aim to disrupt the *B. oleae* - *Ca.* Erwinia dacicola symbiotic relationship using copper or propolis (Bigiotti et al. 2019). Copper (Cu) fungicides are being extensively used since the early 1900s, to control fungal diseases affecting olive trees (Vitanovic 2012). Cu compounds tested in field trials, have suggested not only a direct action against the olive fly, but also an antibacterial activity against its associated bacterial partners (Belcari, Sacchetti, and Rosi 2005; Caleca et al. 2010). More recent studies have reported a reduction specifically in *Ca*. E. dacicola content, the major endosymbiont of the wild-type olive flies, and reduced egg production of the adult flies treated with Cu products (Bigiotti et al. 2019). However, the lack of specificity of these techniques, as well as the contaminating effects of copper due to the high Cu accumulation, which was observed in soils of treated olive tree crops, indicates an urgent requirement for reduction of the contamination risk, which affects initially but not only the soil microenvironment and necessitates the research of more targeted methods (Ballabio et al. 2018).

From a different point of view, a 3-D pharmacophore was developed recently, in a way which identifies the putative common binding interactions, in a series of *Ca.* Erwinia dacicola potential targets, aiming to reduce the concentration of the symbiont on adult flies (Konstantopoulos 2019); this model has integrated the molecular recognition patterns required for a low molecular weight inhibition drug, so that it will optimally interact with the olive fly's endosymbiotic bacteria, and is pending to be implemented in new control approaches.

1.4. Insecticide resistance

As previously analyzed, considering all the pest management approaches that have been developed and applied in olive fly control programs worldwide for many years, due to several reasons, the application of chemical (small molecule) insecticides, serves as the core and most robust means for the control of this destructive agricultural pest. Nowadays, basically pyrethroids (α-cypermethrin), spinosyns (macrocyclic lactone spinosad) and rarely OP-based compounds (phosmet) are being applied.

Generally, the application of an insecticide has several technical advantages, including efficacy and low management costs, as well as low labor and fuel costs (Cooper and Dobson 2007). However, the heavy use of them carries-out a number of well-known risks (Fletcher and Kapatos 1981). Among the negative consequences of the intense use of insecticides are the ecological disturbance of the predator-prey relationship in the system, which sometimes benefits the insect pests and causes an outburst of minor enemy populations. Furthermore, the harmful toxicological effects of these compounds on soil, water, and air pollution, as well as their entrance into the food chain as toxic residues, the suppression of the beneficial insects and the harmful impact on human health are critical drawbacks that should be taken under consideration (Cloyd 2012; Desneux, Decourtye, and Delpuech 2007; Varikou et al. 2020).

1.4.1. The development of insecticide resistance

Among the above drawbacks of the chemical insecticide (small-molecule) application, and one of the major issues to asses, which negatively impacts on the ability to efficiently control pest species, is the development of insecticide resistance in the populations. The term *resistance* describes “a state (genetically-based decrease in susceptibility) in which the insect population survives under doses of insecticide that would normally have killed it, resulting in the failure of a correct application of the pesticide to control the pest” (Hemingway, Field, and Vontas 2002).

In olive flies particularly, even though the OP-based insecticides are basically restricted nowadays, their heavy use during the previous years has resulted in olive fly populations, which confer high levels of resistance to these compounds (Tsakas and Krimbas 1970; Vontas et al. 2011). However, the last years, resistant populations to pyrethroid and spinosyns insecticides have also been reported. Although resistance cases referring to pyrethroid insecticides have been reported in olive fly populations since 2008, the precise mechanism of this type of resistance is still under investigation (Margaritopoulos et al. 2008). A recent study between a resistant to α-cypermethrin pyrethroid olive fly population and a susceptible one revealed new aspects concerning the metabolism of the pyrethroid insecticides (Pavlidi et al. 2018a). Lastly, even though spinosyns have been included in the olive fly control program since 2005, and although they are sporadically used, cases of resistant populations have also been reported (Kakani et al. 2010).

1.4.2. Insecticide resistance mechanisms

In pests of agricultural and public health importance, four major mechanisms responsible for the development of insecticide resistance have been reported until now, which are briefly analyzed below (Feyereisen 1995; Ffrench-Constant, Daborn, and Le Goff 2004; Bass and Field 2011; Khan et al. 2020).

Behavioral resistance is developed when the insects alter their typical behavior, when for example they recognize an insecticidal application and avoid it (Zalucki and Furlong 2017). **Penetration resistance** is defined as the mechanism in which the resistant insects absorb the insecticide more slowly than the susceptible ones, due to modifications in their outer cuticle barriers, which slow down the uptake of the chemicals into their bodies (Balabanidou, Grigoraki, and Vontas 2018). Reduced cuticular penetration of β-cypermethrin was reported in a resistant *B. dorsalis* strain (Lin et al. 2012).

Target site resistance is the decreased sensitivity of various target-site proteins [Nicotinic Acetylcholine Receptor-nAChRs (Homem et al. 2020), Modified Acetylcholinesterase-MACE (Cassanelli et al. 2005), Knock-down Resistance-KDR in sodium channel (Lynd et al. 2018), and Gamma-Aminobutyric Acid-GABA receptor (Zhang et al. 2016)] to insecticides, through structural modifications and/or point mutations, which prevent the insecticide binding or interacting at its site of action, resulting in a reduced effectiveness of the chemical compounds.

Target site insensitivity has been implicated as a mechanism of OP resistance in tephritid species (Vontas et al. 2011). Three point mutations in the AChE gene have been identified in fenitrothion-resistant *B. dorsalis flies*; the G488S and the I214V (Hawkes et al. 2005), and a third one, the Q643R, near the end of the peptide (but in an area not related to GPI anchoring) (J. Hsu et al. 2006). In *C. capitata*, the G328A mutation in the AChE (97% identical at gene level to *B. oleae*) was found responsible for the reduced AChE catalytic efficiency and the reduced sensitivity to inhibition by malaoxon (Magaña et al. 2008).

Especially in the olive flies, after three decades of intense application of OPs, three target-site mutations including two in the active site pocket (G488S and the I214V), and one located in the carboxyl-terminal domain of acetylcholinesterase gene (AChE) (Δ3Q), have been shown to be implicated in olive fly resistance to OPs (Stasinakis, Katsares, and Mavragani-Tsipidou 2001; Hawkes et al. 2005; Kakani and Mathiopoulos 2008; Francesco Nardi et al. 2006; Skouras et al. 2007).

Metabolic resistance is linked partly to the overexpression of various metabolic enzymes, which catalyze the insecticide breakdown and result in a broad-spectrum resistance status (Ranson et al. 2002). The enzymes that have been reported to be involved in this type of resistance belong in five gene families; the Carboxylesterases (CCEs) (Oakeshott et al. 2010), the glutathione-S-transferases (GSTs) (Enayati, Ranson, and Hemingway 2005), the UDP glycosyl-transferases (UGTs) (Hu et al. 2019), the cytochrome P450 monooxygenases (P450s) (Nauen et al. 2022) and the ATP-binding cassette (ABC) transporters (Merzendorfer 2014). Resistant insects may detoxify or degrade the chemical compounds faster than the susceptible ones, or prevent them from

reaching the target sites. Furthermore, they may possess higher concentrations or more effective forms of the enzyme(s) that convert the insecticides to non-toxic chemicals.

Metabolic detoxification has been implicated as a mechanism of OP resistance in several tephritid fruit flies (Vontas et al. 2011). Synergistic data strongly indicated the involvement of detoxification enzymes (CCEs and P450s) in resistant *B. dorsalis* populations (J. C. Hsu et al. 2011). Concerning the metabolic resistance of other tephritid species to pyrethroids, synergistic assays showed that PBO (used as specific CCE inhibitor) significantly enhanced the cypermethrin toxicity in *D. ciliatus*, indicating a potential role of P450s in pyrethroid detoxification (Maklakov et al. 2001).

Particularly in olive flies, the development of metabolic resistance emerged in the early 1970s, concerning two esterase genes (CCEs), suggesting a possible resistance of *B. oleae* to OPs (Tsakas and Krimbas 1970) and another case reported increased levels of P450 genes, related with pyrethroid resistance in *B. oleae* (Margaritopoulos et al. 2008).

Concerning more recent research updates, a study in 2013 produced, for the first time, a whole transcriptome dataset for *B. oleae,* using the pyrosequencing technology (Pavlidi et al. 2013). In the frame of that investigation, 55 P450, 43 GST-, 15 CCE- and 18 ABC transporter-genes were identified and phylogenetically classified. However, the lack of a good quality genome assembly at that time hindered further analysis of these genes, since a number of them were not correctly predicted. As a result, their role in the physiology and the fundamental biology of the olive fly was difficult to be studied. Nevertheless, the more recent release of an improved assembly of the olive fly genome, using linked-reads and long-read technologies, gave new prospects in this research field (Bayega et al. 2021). At around the same time, a new transcriptomic comparison between two resistant olive fly populations against a susceptible one, also revealed a large number of genes significantly differentially transcribed; among them, two P450s (named contig00436 and contig02103), both coding genes that belong to the CYP6 of P450s, were found interestingly up-regulated, suggesting a possible role in the metabolic processes of the pyrethroid insecticides (Pavlidi et al. 2018a).

1.5. Insect molecular biology technologies in support of improved understanding of the pest and development of novel control strategies

The recent developments in functional and sequencing technologies, generate the opportunity to study insecticide resistance, and other mechanisms which are targeted in pest control strategies (i.e. symbiotic interactions), in a versatile manner, and utilize the acquired knowledge towards the development of innovative pest control approaches, which will go beyond the traditional ones.

Concerning the omics technologies, while more insect genomes are being sequenced and annotated, several types of analysis (i.e. transcriptomics, proteomics, peptidomics, metabolomics, bioinformatics and others) provide a massive amount of data, which contribute in the interpretation of various biological aspects of insect organisms (Nelson 2018; Balabanidou et al. 2019; Bayega et al. 2021; Ingham et al. 2021; M. Kefi et al. 2021).

This information can be further functionally exploited with the use of molecular tools (Perry and Batterham 2018; J. G. Scott and Buchon 2019; Douris et al. 2020). Such tools, initially included the GAL4/UAS system, in which the genes of interest are expressed in a spatiotemporal controlled manner (Samantsidis et al. 2020). Other genetic tools, which either knock-down the gene expression levels (RNA interference, RNAi) (Mary Kefi et al. 2019), or knock-out and knock-in specific gene sequences (Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR/Cas9) (Sim et al. 2019), have lately been applied in tephritid species, and create new prospects for the functional characterization of candidate genes *in vivo* (Sim et al. 2019). These reverse genetics methods aim to further investigate the role of certain genes in several pathways (Scharf, Zhou, and Schwinghammer 2008; Homem and Davies 2018). Another tool which is being applied for the functional investigation of the role of genes of interest in certain processes (e.g. insecticide resistance), is the recombinant expression of the genes, using different expression systems (Nauen, Zimmer, and Vontas 2021). The heterologous gene expression in model-insects like in *Drosophila melanogaster,* under the GAL4/UAS system, which was previously mentioned, stable cell lines and bacterial cultures, followed by other assays (e.g. activity measurements against model substrates), offers multiple advantages and provides new data for the possible implication of the identified genes in resistance mechanisms (Tsakireli et al. 2019).

Lately, RNAi and CRISPR- based technologies are being investigated as a feasible and sustainable avenue for the management of various pest species (Jain et al. 2021; Adelman et al. 2017). Several studies, including the tephritid species *B. dorsalis* (Li, Zhang, and Zhang 2011; Zheng et al. 2015; Dong et al. 2016) and *B. tryoni* (Cruz, Tayler, and Whyard 2018), have demonstrated the feasibility of RNAi not only as a tool for the functional characterization of genes, but also as a means of pest control, through the silencing of essential for the insect genes. Furthermore, concerning the recent genome editing advancements in insects, for generating site specific genomic mutations, CRISPR technology has enabled the development of powerful strategies for insect control, such as gene drives (Gantz and Akbari 2018). Gene drive approaches aim to safely and effectively engineer and rapidly spread genetic modifications among populations of insects and other pest arthropods in the wild, either to reduce diseases, such as malaria (Hammond et al. 2016; Kyrou et al. 2018), or to control agricultural pests (Carrami et al. 2018; Scott et al. 2017). However, such approaches require sustained, open, and inclusive attention to potential environmental and social impacts, as well as regulatory and implementation challenges (Legros et al. 2021).

1.6. Overall aim of the study

The objective of this thesis is to provide new insights and characterize mechanisms implicated in insecticide resistance of the olive fly, *Bactrocera oleae,* the major pest of olive orchards worldwide, and in interactions with symbiotic bacteria, especially of the species *Candidatus* Erwinia dacicola (*Ca*. E. dacicola). A number of techniques, including gene editing (CRISPR/Cas9) and silencing technologies (RNAi), RNA sequencing, bioinformatics analysis, imaging techniques and classical bioassays were applied.

More specifically, the thesis is divided in 3 sections (apart from the general introduction and discussion chapters):

First section | The development and application of a biotechnology-based CRISPR/Cas9 tool in olive fly, *Bactrocera oleae*: a proof-of-concept approach.

The aim of this study was to develop and apply a highly precise genome editing tool for *Bactrocera oleae*, and particularly, a CRISPR/Cas9 system-based approach. We chose to target the *scarlet* gene in *B. oleae,* which provides an easy to screen eye color phenotype, in order to demonstrate that this technology is applicable to this non-model organism. The availability of such a genetic tool will enable a better understanding concerning the potent roles of various genes and mechanisms (i.e. critical symbiont interactions, insecticide resistance), towards the future application of novel pest control strategies.

Second section | Characterization of genes associated with insecticide resistance in *B. oleae*.

In the first part of this section we attempted the functional validation of the Δ3Q mutation in the acetylcholinesterase gene (AChE) of *B. oleae*. The deletion of three amino acids in the last exon of AChE (Δ3Q) has been associated with organophosphorus resistance in *B. oleae,* after field screens and *in vitro* experiments, since 2008. However, this hypothesis has not been supported with *in vivo* evidence yet. The aim of this study was to investigate this hypothesis *in vivo*, specifically by introducing the Δ3Q mutation with the CRISPR/Cas9 gene editing tool, which was developed in the previous section, under a susceptible genetic background of a laboratory-reared *B. oleae* strain and study the mutant phenotype upon insecticide application.

In the second part of this section, we searched for gene candidates, implicated in pyrethroid resistance of olive flies, using transcriptome sequencing on olive fly malpighian tubules (MTs). The aim of this study was to identify genes not immediately apparent in the already existing whole organism RNA sequencing data, due to possible masking effects, through gene expression comparisons in MTs, one of the proposed detoxification tissues in insects, dissected out of pyrethroid resistant and susceptible olive flies.

Third section | Unraveling the unique adaptation of the olive flies to olives, via the symbiotic relationship with bacteria of the species *Ca.* E. dacicola.

The general aim of the study was to investigate and define critical aspects of the unique symbiotic relationship, between the olive fly host and the bacterial symbionts, especially *Ca*. E. dacicola.

For this purpose, the abundance of the major bacterial symbiont throughout the development was examined, extended microscopy analysis for the investigation of the gut tissue which houses the symbiont partners during the larval stages, depending on the developmental stage, was performed, as well as through two pairwise comparisons critical aspects of this symbiosis at a gene level were identified. Gastric caeca from second and third instar larvae were analyzed, in order to identify genes potentially involved in the development transition of the larvae, and gastric caeca from second instar larvae developing in olives and in artificial diet, were analysed in order to identify genes of the host, potentially involved in the regulation of this symbiotic relationship, since wild-type animals contain huge numbers of the symbiont partner, while the laboratory-reared do not. A better understanding of this symbiosis-based interaction will contribute to the future development of dysbiosis-based approaches.

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Chapter 2

Development and application of the biotechnology-based CRISPR/Cas9 tool in olive fly, *Bactrocera oleae:* a proof-of-concept approach.

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Chapter 2 |

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2.1. Abstract

The olive fruit fly *Bactrocera oleae* (*B. oleae*) causes great damage in quality and quantity of the olive production worldwide. Pest management approaches have proved difficult for a variety of reasons, a fact that has brought about a need for alternative tools and approaches. Here we report for the first time in *B. oleae* the development of the CRISPR/Cas9 gene editing tool, using the well-known eye color marker gene *scarlet*. Two synthetic guide RNAs targeting the coding region of the *scarlet* gene were synthesized and shown to work efficiently *in vitro.* These reagents were then micro-injected along with purified Cas9 protein into early-stage embryos. Successful CRISPR- induced mutations of both copies of the *scarlet* gene showed a striking yellow eye phenotype, indicative of gene disruption. Multiple successful CRISPR events were confirmed by PCR and sequencing. The establishment of an efficient CRISPR/Cas9-based gene editing tool in *B. oleae* will enable the study of critical molecular mechanisms in olive fruit fly biology and physiology, including the analysis of insecticide resistance mechanisms and the discovery of novel insecticide targets, as well as facilitate the development of novel biotechnology-based pest control strategies.

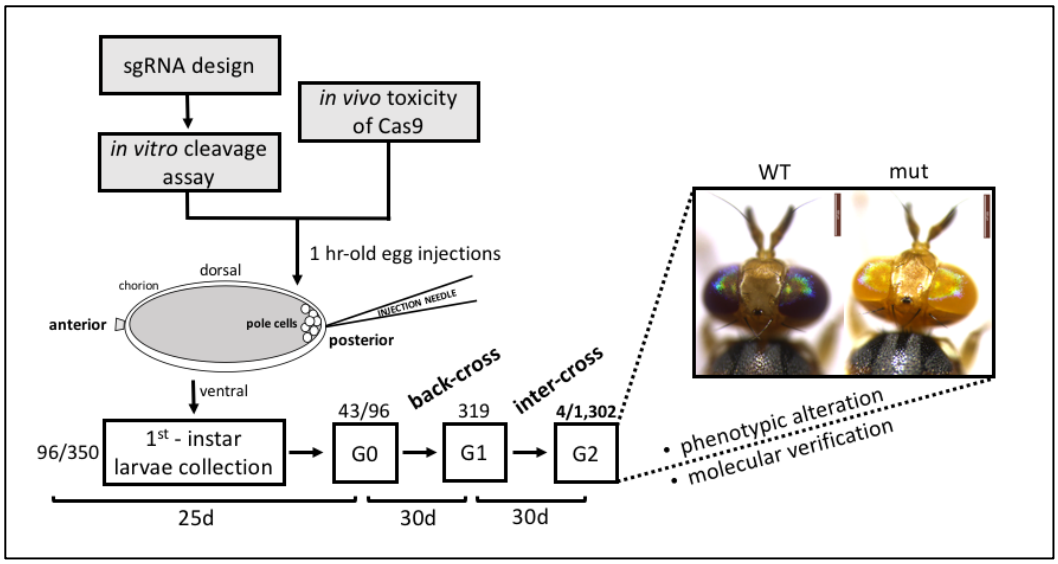

Figure 2.1. Graphical abstract of Chapter 2. Embryo micro-injections of Cas9/sgRNA complex, which targets the *scarlet* coding sequence, yielded multiple heritable genetic variants and the mutants showed a striking shift in eye color from dark-red to yellow.

2.2. Introduction

2.2.1. The CRISPR/Cas9 biotechnology tool

CRISPR/Cas9 (Clustered Regularly Interspaced Palindromic Repeats) tool was firstly discovered in prokaryotes, as part of their immune response to infection (Barrangou et al. 2007). Since 2012, it has proven to be a breakthrough research tool for gene editing, individual gene function investigation and disease model construction, towards potential therapeutics for human health (Wiedenheft, Sternberg, and Doudna 2012; Hsu, Lander, and Zhang 2014). CRISPR-Cas9 has been widely used to achieve precise gene modifications, by using single guide RNAs (sgRNAs) in order to direct the endonuclease Cas9 to specific genomic loci. The Cas9 protein-small RNA molecule (hybrid of trans-activating CRISPR RNA-tracrRNA and CRISPR RNA-crRNA) complex binds on the DNA target sequence next to the protospacer adjacent motif (PAM) through base-pairing, and a DNA-RNA hybrid is formed. The Cas9 enzyme then precisely cleaves the target dsDNA on both strands, resulting in a DNA double-strand break. The break can be repaired either by the non-homologous end joining (NHEJ) imperfect repair mechanism, which often creates random insertions or deletions in the DNA, or by the endogenous cellular machinery and homology-directed repair (HDR) mechanism, which requires a donor DNA template (Doudna and Charpentier 2014; Sternberg and Doudna 2015; Zhang, Wen, and Guo 2014) (**Figure 2.1**). CRISPR-Cas based systems are already being used to alleviate genetic disorders in animals (Barrangou and Doudna 2016) and they even enter clinical testing, while they hold a great potential for correcting genetic diseases and enhancing cell therapies, with promising preliminary results (Pickar-Oliver and Gersbach 2019). However, safety and efficacy issues need to be strongly monitored during such studies.

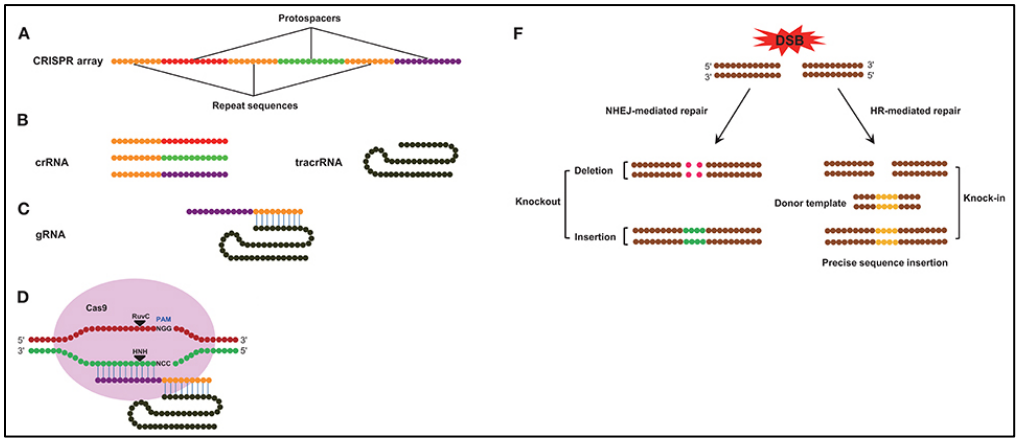

Figure 2.2. The mechanisms of CRISPR/Cas9 gene editing system. The Cas9 protein-guide RNA complex binds on the DNA target sequence next to the PAM sequence, and a DNA-RNA hybrid is formed. Cas9 precisely cleaves the target dsDNA on both strands, resulting in a DNA DSB. The break can be repaired either by the NHEJ imperfect repair mechanism, or by the HDR mechanism, which requires a donor DNA template (Figure adopted from Sun et al. 2017).

2.2.2. The CRISPR/Cas9 tool in model and non-model arthropod species

CRISPR/Cas9 gene editing system, as described before, serves as a relatively simple, easy to use and affordable molecular tool. This technology offers to insect scientists new opportunities in using genetics for the development of efficient alternative insect management approaches, applicable in species relevant to agriculture and public health. Especially in arthropods, this system can be used in order to explain resistance mechanisms that may cross species boundaries (Douris et al. 2016; J. Wang et al. 2016), as a screening tool for novel molecular targets, as well as to verify functional genes associated with physiological processes, such as embryonic development and host-symbiont interactions, and further provide novel strategies for future pest management (Sun et al. 2017).

CRISPR technology is already being extensively applied in model insects, such as *Drosophila melanogaster* (Huang, Liu, and Rong 2016; Bassett and Liu 2014), *Anopheles gambiae* (Dong et al. 2018) and *Tribolium castaneum* (Gilles, Schinko, and Averof 2015). It has also been applied in many non-model arthropod species as well, as a method for targeted mutagenesis (X. Li et al. 2015; Yu et al. 2019; Hiruta et al. 2018). Recently, the first gene editing tool, based on the CRISPR/Cas9 system, was reported for the non-insect spider mite *T. urticae* (chelicerate) (Dermauw et al. 2020).

In many of these studies, this has been accomplished by knocking out genes which show a visible marker phenotype (Wei et al. 2014). Particularly useful have been a subset of ATP-binding cassette (ABC) transporters, which are responsible for the translocation of various eye pigments into the proper eye compartment (M. Li et al. 2017; F. Li and Scott 2016). The white, brown and scarlet eye color genes in *Drosophila* encode ABC transporter proteins, which transport guanine or tryptophan respectively (Sullivan and Kitos 1976). Current models propose that the *white* and *brown* gene products interact to form a guanine specific transporter, while *white* and *scarlet* interact to form a tryptophan transporter. So, failure of scarlet function results in bright red eyes in *Drosophila* (Mackenzie et al. 1999). Conveniently, the disruption of these ABC transporter genes often causes an eye color alteration, which provides an easy to score phenotype.

This strategy has recently been used to demonstrate the utility of CRISPR in various tephritid species as well (Choo et al. 2018; Meccariello et al. 2017; Aumann, Schetelig, and Häcker 2018; Bai et al. 2019; Y. Wang et al. 2019; Zheng et al. 2019; S. Zhao et al. 2019; Sim et al. 2019), but it has not been extended to *B. oleae* so far. However, the efficiency of mutagenesis in such studies has been extremely low (**Figure 2.2**).

Although this genome editing technology is extensively being used, there still exist important limiting factors that affect the efficiency of the method in tephritid species, compared to cells and even other insect species; the eggs are smaller and more fragile and as a result they are easily damaged during the micro-injection procedures, they also need to be de-chorionated before injection and the hatching rate is strongly related to the culture conditions post injection as well.

Species	Citation	Gene	Injected embryos	G0 adults	G1 mutants	Phenotype
Bactrocera dorsalis	Sim et al., 2018	*white (exon 3)*	1,862	28	1	
		white (exon 7)	1,176	27	0	
	Bai et al. 2019	*white (exon 1)*	329	14	58	
		white (exon 3)	162	6	331	
	Zhao et al. 2019	*white (exon 1)*	302	55	4	
Bactrocera tryoni	Choo et al.,2018	*white (exons 2 and 3)*	187	9	7	
Anastrepha ludens	Sim et al., 2018	*white (exon 7)*	1,071	22	2	
Ceratitis capitata	Meccariello et al., 2017	*white (exon 3)*	927	13	1	
		(exon 7)	1,106	25	7	

Figure 2.3. The CRISPR/Cas9 gene editing system applied in tephritid species. Targeting the ABC transporter genes often results to an eye color alteration, which provides an easy to screen phenotype. Previous proof-of-concept experiments in tephritid species, targeting this gene family, report a very low mutagenesis rate.

2.2.3. Genetic manipulation attempts in *Bactrocera oleae*

Olive fly control currently is mainly based on the use of chemical (small molecule) insecticides. However, due to its high economic cost and environmental impact, as well as the increased levels of resistance to chemical insecticides that has occurred in many regions, there has been an urgent need for improved control methods. The development of novel tools for understanding the physiology and the molecular biology of targeted processes and mechanisms of the olive fly is an important prerequisite for that kind of approaches. Genetic transformation and gene editing technology represent such novel tools and the development of an efficient method for olive fly transgenesis stands as a prerequisite for effective future control strategies. Also the availability of the *B. oleae* whole genome sequence (Djambazian et al. 2018) may contribute to a better comprehension of the genetic and biochemical basis of the insect biology, aiming at a successful molecular manipulation of this insect species.

A few such pest control strategies have already been proposed, which basically rely on transgenic insects created in the laboratory and afterwards released in the field. For example, a germ-line transformation of *B. oleae* with a Minos vector carrying an enhanced green fluorescent protein (EGFP) gene (Koukidou et al. 2006) and with the use of a piggyBac vector marked with two fluorescent protein genes (Genç et al. 2016) have been already reported, with a few further applications, mostly towards a more effective SIT approach (Ant et al. 2012; Rempoulakis et al. 2014). However, taking under consideration the lack of precision inherent in transposable element mediated insertions, such studies cannot be used in order to target a specific genomic region.

2.3. Aim of the study

Under the recent advances in genome modification technology in a wide range of pest species, and the availability of the olive fly genome, the aim of this study was to develop a highly precise genome editing tool for *Bactrocera oleae*, and particularly, a CRISPR/Cas9 system-based approach. The availability of such a genetic tool will enable the functional validation of various genes and mechanisms (i.e. critical symbiont interactions, insecticide resistance) and will contribute in the future development of novel pest control applications.

2.4. Materials and methods

2.4.1. Rearing of *Bactrocera oleae*

The *B. oleae* strain which was used in this work originated from the Democritus Laboratories (National Centre of Scientific Research, Athens, Greece) fly stock. The strain was maintained under common rearing conditions at 25°C and 16:8 hours light: dark photoperiod. Adult females were allowed to oviposit on ceresin wax substrates and the newly hatched larvae were transferred to Petri dishes with artificial diet. The diet contained 550 ml distilled water, 20 ml extra virgin olive oil, 7.5 ml Tween-80, 0.5 g potassium sorbate, 2 g Nipagin, 20 g sugar, 75 g yeast hydrolysate, 30 g soy hydrolysate, 30 ml hydrochloric acid 2N and 75 g cellulose powder (Tzanakakis and Economopoulos 1967). The emerging larvae were transferred to Petri dishes containing a layer of sawdust, which serves as substrate for pupation. Adult diet consisted of 30 g yeast hydrolysate, 80 g sugar and 6.6 g egg yolk.

2.4.2. Target site selection

The *B. oleae scarlet* gene was identified by running a text search in the OrthoDB orthology database and was confirmed via similarity using protein BLAST. PCR and Sanger sequencing of *B. oleae Scarlet (BoSt)* exon one was performed on the *B. oleae* laboratory strain, in order to identify any possible SNPs segregating in the population. This sequence was then scanned for sgRNAs following a G-N19-NGG pattern outlined previously (Bassett and Liu 2014). All potential sgRNA targets were afterwards scanned against the *B. oleae* reference genome (NCBI accession number GCF_001188975.2) for potential off-targets, using CasOT command line software (Xiao et al. 2014); sequences were considered potential off-targets if they had less than two mismatches in the seed sequence and less than three mismatches in the distal sequence. Two sgRNAs were then chosen so as to generate an easily detectable deletion of approximately 150 bp in the *BoSt* gene (**Figure 2.6**).

2.4.3. sgRNA synthesis and RNP complex assembly

The template for the generation of each sgRNA was produced by a target-specific forward and a common reverse primer (Table 1,(Bassett and Liu 2014)). Phusion High-Fidelity DNA Polymerase (M0530S, NEB) was used in a 100 μl final reaction volume. PCR products were then gel extracted and purified (Macherey-Nagel, 740609), and DNA concentration and purity (OD, A260/A280 and

A260/A230) were measured on the Nanodrop ND-1000 Spectrophotometer, using 1 µl of DNA. The sgRNA synthesis was performed according to the instructions of Hiscribe T7 Kit (NEB, E2040S), using 300 ng of each target template followed by sodium acetate precipitation. RNA was on-column DNase digested, with DNAse I (RNase-Free DNase I Set Qiagen), in order to remove genomic DNA contamination. The injection mix was comprised of 0.6 µg/µl purified Cas9 protein (NEB, M0386T), 0.5 µg of each sgRNA and 1x injection buffer (0.1 mM sodium phosphate buffer pH 6.8, 5 mM KCl) in a final volume of 5 µl.

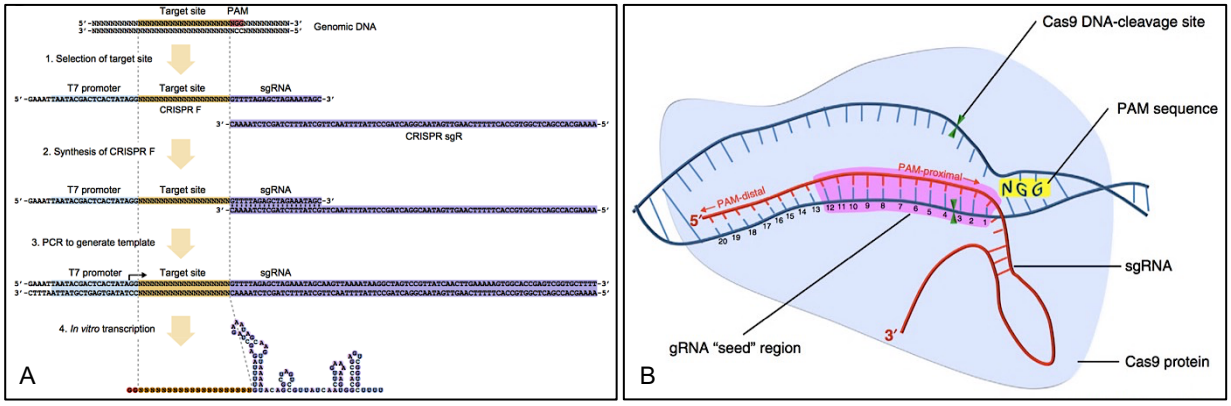

Figure 2.4. The main components of the CRISPR/Cas9 gene editing tool; sgRNAs and Cas9. (A) Generation of the DNA template for the *in vitro* transcription of the sgRNAs. DNA target site is indicated in orange, and PAM sequence in red. CRISPR Forward primer contains the T7 promoter (blue), the target site (orange) and overlaps with CRISPR Reverse (universal) primer (purple). (B) Cas9 cleavage of the DNA. The gRNA associates with Cas9 and together they associate with double-stranded DNA at the PAM sequence (NGG). The PAM proximal and distal regions of the guide RNA form an R-loop. Guide RNA nucleotides 1–12 form the seed region that makes critical associations with the target DNA sequence. Cas9 cleavage of the DNA (green arrows) occurs between the third and fourth nucleotides proximal to the PAM. (Figures adopted from A: Bassett and Liu 2014; B: Reid and O'Brochta 2016)

2.4.4. *In vitro* cleavage assay

The sgRNA efficacy was tested *in vitro*, using the commercially available Cas9 nuclease (NEB, M0386T). Briefly, a 518 bp DNA scarlet fragment containing the two sgRNA target sequences was amplified by PCR with specific primers (**Supplementary Table ST2.1**). The PCR product was purified using a PCR Product Purification Kit (Macherey-Nagel, 740609). The sgRNAs for the assay were produced by the T7 *in vitro transcription* (Minotech, 801-1) of 250 ngs pre-annealed oligos (previously described, **Supplementary Table ST2.1**), followed by a Dnase I treatment (NEB, M0303) and a phenol chloroform purification (Sambrook, Fritsch, and Maniatis 1989). sgRNA concentration was measured by Nanodrop and aliquoted in 1.2 pmole/µl preparations. 120 ng of the target PCR product were digested with 2 pmoles of each sgRNA-Cas9 complex, in a 10 µl final reaction volume, in 3.1 NEB buffer (50 mM Tris-HCl pH7.9,100 mM NaCl,10 mM $MgCl_2$, 100 µg/ml BSA), at 37°C for 2 hours (2.2 pmole of each sgRNA were pre-incubated with 2 pmoles of Cas9 nuclease, at a final volume of 4 µl, at 37°C for 20 minutes, prior to the final

addition of the buffer, target DNA and ddH20, in a final volume of 10 μl). The reaction was terminated by the addition of proteinase K (final concentration 500 μg/ml), followed by an incubation at 40°C for 20 minutes. Analysis of the enzymatic digestion was performed by electrophoresis on a 2% agarose gel.

2.4.5. Embryo microinjections

One-hour-old eggs were collected and de-chorionated in 1% sodium hypochlorite (pure solution 13%, Nr31149) for 1 minute, followed by repeated rinsing with ddH_2O. Embryos were then lined up in the same orientation on an apple agar plate and transferred with a double sided tape (Scotch) on a coverslip (VWR, 631-0146), with posterior ends pointing to injection site, before being allowed to partially desiccate in silica gel for 8 minutes and covered with Halocarbon Oil 700 (Sigma-Aldrich, 9002-83-9). The coverslip was then placed on a microscope slide and the micro-injections were performed under a Leica microscope (Leitz, GMBH) with a Leica micro-manipulator. Borosilicate glass capillaries (Harvard apparatus, GC100-7.5) were pulled in a needle puller (Shutter Instrument Flaming/Brown Micropipette Puller P-97) to produce sharp edges at the injection tips. The needle was filled with a micro-loading tip (Eppendorf, E2731410) with 1 μl of pre-loaded sgRNA-Cas9 mix, which was injected into the posterior end of the aligned embryos. Less than 90 minutes old t eggs, that have not reached pole cell formation, were used for injections.

Fluorescent CY3 plasmid (MIRUS Label IT, MIR7904) was injected at a concentration of 250 ng/μl as a delivery control. The injected embryos were allowed to develop for 3 hours and were examined under a fluorescent microscope. In order to investigate the toxicity of Cas9 protein on embryonic development and survival, we injected Cas9 at concentrations of 187, 350 and 750 ng/μl (**Supplementary Table ST2.2**). The final injection mix was comprised of 600 ng/μl Cas9 protein (NEB), 500 ng/μl of each sgRNA and 1x Injection Buffer. The freshly prepared mixture was incubated at 37°C for 15 minutes for the pre-assembly of the RNP complexes and kept on ice prior to injections. Microscope slides with injected embryos were placed in Petri dishes containing moist tissue paper and wrapped with Parafilm and were placed at 25°C for 72 hours, in order to allow larval hatching. 1st instar hatched larvae were then transferred in larval food for the remaining of the larval development.

2.4.6. DNA isolation and Genotyping by DNA Sanger Sequencing

The genomic DNA was isolated from whole flies using the CTAB (Cetyl-trimethyl-ammonium bromide) extraction protocol. Genotyping was performed using 1 μl of each DNA sample in a PCR using standard DNA Taq Polymerase (MINOTECH, 203-2) with a 56°C annealing temperature and 30s extension time. Gel purified PCR products were sent for sequencing (Cemia; Larissa) and the resulting sequences were aligned in Ugene (Okonechnikov et al. 2012).

2.5. Results

2.5.1. *In vitro* testing of sgRNAs

In order to disrupt the *B. oleae Scarlet* (BoSt) gene, two sgRNAs were designed to target exon 1, creating an approximately 190 base pair (bp) deletion (**Figure 2.6A**). To evaluate the efficacy of the two sgRNAs in guiding Cas9-induced gene editing at their target site, we first tested these sgRNAs *in vitro*. The Cas9 digest of the *BoSt* locus showed that incubating sgRNA-1/Cas9 or sgRNA-2/Cas9 with the target resulted in the production of two DNA bands with the expected size and a faint one of the initial full-length gene fragment (sgRNA-1: 247, 271, 518; sgRNA-2: 93, 425, 518 bp) (**Figure 2.6B**). In addition, incubating both sgRNAs with the target, resulted in the production of the three expected products (93, 178 and 247 bp) and a faint 271 bp band, suggesting that sgRNA-1 cutting efficiency is higher than that of sgRNA-2. No DNA cleavage was detected when the PCR fragment was incubated with either Cas9, sgRNA-1, or sgRNA-2 alone. Together, these results indicate that both (together or individually) sgRNA-1 and sgRNA-2 can effectively guide Cas9 protein to cut DNA at their respective target sites.

2.5.2. Embryo microinjections and toxicity testing of Cas9 protein

To assess the delivery of sgRNA/Cas9 ribonucleoprotein (RNP) complex into olive fly embryos, we first tested microinjection of a fluorescent Cyanine dye (CY3) plasmid into 1-hour-old fertilized eggs. A fluorescent signal was clearly detected in the injected embryos at the posterior pole, where the injection was performed, whereas the non-injected embryos showed no fluorescence signal (**Figure 2.7**). Signal in anterior poles of both injected and non-injected embryos was due to auto-fluorescence. These results suggest that CRISPR reagents can also be successfully injected into olive fly embryos. Furthermore, of the three Cas9 concentrations tested (187, 375, 750 ng/µl), none showed any significant increase in viability rate, compared to injection buffer (**Supplementary Table ST2.2**), so we proceeded with the highest one (750 ng/µl). The survival of embryos which were injected with sgRNAs was slightly less, but comparable to that observed with injection buffer (**Supplementary Table 2.3**).

2.5.3. Detection of Yellow eye mutants in the G2 generation

A total of 350 *B. oleae* eggs were injected (**Supplementary Table ST2.3**) with the two sgRNAs, targeting exon 1 of *BoSt* gene. From those, 96 hatched into larvae, 51 turned into pupae, and 43 survived adult eclosion (21 females and 22 males, G0 adults). These G0 adults were backcrossed to non-injected males and females, originating from the same laboratory strain, producing 161 and 158 G1 offspring respectively (319 totally). All G0 and G1 adults displayed wild-type eye color. The 161 and 158 G1 adults were mass mated (two separate cages) and produced 850 (415 females and 435 males) and 452 (223 females and 228 males) G2 offspring, respectively. Of those 1,302 G2 adults, only four showed the yellow eye phenotype: two females, one male and one dead pupa. After crossing each yellow-eye female with the yellow-eye male, all G3 offspring displayed the yellow-eye phenotype.

2.5.4. Characterization of the induced mutations

Genotyping of the four yellow-eyed G2 mutants was performed in order to characterize the CRISPR-induced mutations. Visualization of the PCR products on an agarose gel reveled a three-band pattern in each fly; each band was excised and sent for sequencing independently (**Figure 2.8**). The lowest band (330bp) showed a deletion of approximately 190bp, corresponding to the fragment in between the two sgRNA cut sites. The highest band (510bp) aligned almost perfectly with the wild-type sequence, except for a single bp deletion, causing a frameshift mutation directly adjacent to the 3' sgRNA cut site. The middle band, displayed a sequence which only partially aligned to the reference gene; this was assumed to be a PCR artifact and it was discarded.

In order to assess the mutation frequency, 100 G1 individuals were genotyped for the 193 bp deletion. Only one was found to possess the deletion, suggesting that the frequency of this mutation was very low (1%; **Supplementary Table ST2.3**). However, this screening method does not include the frequency of the single nucleotide deletion (the 510bp highest band) which was detected in the yellow-eyed flies. The specificity of our CRISPR events was also assessed by sequencing the predicted off-target sites in the yellow-eyed flies. The three potential off targets for the first sgRNA (NW_013581217.1: 2026365-2026388, NW_013581218.1: 1140233-1140256, NW_013581297.1: 125145-125168) and the one for the second (NW_013581551.1:155611-155634) were found to contain no polymorphisms, indicating that there were no off-target effects (data not shown).

2.6. Discussion

The extension of genetic technology into non-model species has been proceeding rapidly. However, each new species must be evaluated for its tractability to these new methods. Here, we present the first report of CRISPR/Cas9 in *B. oleae,* by targeting the visible eye marker *Scarlet.* In the current study we report the first successful demonstration of the CRISPR/Cas9 gene editing tool, by targeting the *scarlet* eye gene in *B. oleae.* Through this, we demonstrate that this technology is applicable to this non-model organism, and can be used for the functional characterization of mechanisms and the development of alternative genetic tools and strategies, in order to efficiently control this destructive tephritid species.

In more detail, in this study we have established an approach to deliver sgRNA/Cas9 RNP complexes into *B. oleae* embryos by microinjection. The efficiency of CRISPR induced mutations in this study was quite low. Only a small number (4/1,302) of G2 progeny showed the mutant yellow-eye phenotype. All of these 8 (4 individuals x 2 chromosomes) mutated sites appeared to arise from only 2 alleles; one 193bp deletion and one single nucleotide deletion. Furthermore, the percentage of G1 individuals carrying the deletion was estimated at 1% (**Supplementary Table ST2.3**). Although this estimate is likely low, due to the inability of the PCR to detect small frameshift mutations, the low frequency of the detectable deletion is still far lower than the rates which have been reported in some other species (Cui, Sun, and Yu 2017). In Drosophila, rates can reach as high as 71% (Champer et al. 2019). However, the efficiency observed here is comparable to other reports of tephritid species (Sim et al. 2019; Choo et al. 2018). In addition,

the fact that no somatic cell mutagenesis was achieved and mosaicism effect was not detected in G0 and G1 generations, as noted in other tephritid (Bai et al. 2019) and non tephritid species (Khan, Reichelt, and Heckel 2017), also constituted a critical limiting factor in the screening procedures that followed; the requirement of molecular genotyping methods adds one more element of complexity in the experiment.

Another CRISPR event in *B. oleae* (Meccariello et al. 2020), which didn't yield in heritable progeny and several other studies in closely related to *B. oleae* tephritid species have been reported. These include *Ceratitis capitata, Bactrocera dorsalis and Bactrocera tryoni* (Choo et al. 2018; Meccariello et al. 2017; Aumann, Schetelig, and Häcker 2018; Bai et al. 2019; S. Zhao et al. 2019). In particular, eye color has been used as a visible marker to establish CRISPR as a proof of concept target. This trend is also present in other non-dipteran species such as the Lepidopteran *Helicoverpa armigera* (Khan, Reichelt, and Heckel 2017) and the Hemipteran *Nilaparvata lugens* (Xue et al. 2018). However, the disruption of the same gene sometimes yields quite different shifts in eye color. While a detailed characterization of eye pigmentation is beyond the scope of this study, it is interesting to note that the dark-red-brown to yellow switch observed in *B. oleae* is different from the red to bright-red switch observed upon disruption of the *D. melanogaster* homolog (Mackenzie et al. 1999). The yellow *B. oleae* eyes also confirm a previous association of scarlet with "lemon" (yellow) eyes (J. T. Zhao et al. 2003).

Future work will likely be dedicated in improving efficiency through a more efficient delivery of the reagents to the developing embryos. Already, by the time that purified Cas9 protein has become commercially available, the direct injection of a cocktail of sgRNA and Cas9 into developing embryos, has increased the effectiveness of the method (Kistler, Vosshall, and Matthews 2015). Cas9 protein provides various advantages over Cas9 mRNA, such as lower viscosity injection mixtures, lower sensitivity to degradation, and immediate cleavage of the target DNA target after the complex is introduced (Reid and O'Brochta 2016). In addition, the transgenic expression of Cas9 *in vivo,* substantially has improved the efficiency of CRISPR in *D. melanogaster* and gave insights for such developments in other non-model insect species (Port et al. 2014).

As it concerns the available methods for detecting the presence of mutated genes and offspring, the enzymatic detection of heteroduplex PCR products, the depletion of a restriction site, high resolution melt curve analysis, and Sanger sequencing are the most common. All of these strategies have been shown to be effective, although they differ in their sensitivity and ability to produce quantitative data. Already, the development of a T7 Endonucleasse I (T7EI) enzymatic assay, which can accurately recognize insertions and deletions ≥ 2 bases generated by non-homologous end joining (NHEJ) activity, combined with amplicon sequencing, has led to an important increase of the resolution of detection method (Choo et al. 2020). Finally, developing methods to genotype individual insects without killing or impairing their ability to reproduce is a continuing issue to be managed (Reid and O'Brochta 2016).

The expansion of CRISPR/Cas9 system-based approaches in non-model pest species, could substantially facilitate the understanding of the insect physiology and the molecular mechanisms that regulate biological processes, such as insecticide resistance. The technique has been used

to assess the impact of canonical mechanisms of cytochrome P450s (H. Wang et al. 2018), and target site mutations (Zuo et al. 2017; J. Wang et al. 2017) related to resistance, in non-model lepidopteran species. CRISPR tool has also been used to establish the mode of action of enigmatic compounds such as chitin synthase inhibitors (Douris et al. 2016) and *Bacillus thuringiensis* derived toxins (J. Wang et al. 2017; H. Wang et al. 2018). This is especially useful in cases where a given allele is difficult or impossible to be characterized *in vitro,* such as the acetylcholinesterase Δ3Q mutation (Kakani and Mathiopoulos 2008) which has been reported in olive flies. The addition of CRISPR to the toolkit of *B. oleae* genetics thus provides a powerful reverse genetics tool, in order to understand insecticide resistance mechanisms in this species.

CRISPR is also a prerequisite for several components of the most promising non-pesticide-based control strategies. For example, efficient gene drive-based control relies on the introduction of a CRISPR expression cassette, which replicates itself, via homology directed repair (Gantz et al. 2015) (Hammond et al. 2016; Kyrou et al. 2018). The recent identification of a sex determination regulator in Mediterranean fruit flies (Meccariello et al. 2019), suggests an optimal target for this approach and combined with CRISPR/Cas9 system, provides a path for the development of a gene drive strategy in *B. oleae*.

CRISPR gene editing tool could also be used for the disruption and the functional characterization of genes implicated in the interactions between the olive flies and their symbiotic bacteria, which confer the necessary substrate for the larval development in the infested unripe olives (Estes et al. 2009). Already, several studies have disrupted the symbiotic relationship between host-plants and microorganisms, with the use of the CRISPR/Cas9 genome modification system, shedding insights on agricultural field (Ludwików et al. 2016; Ji et al. 2019; L. Wang et al. 2019). Furthermore, in a very recent study, CRISPR/Cas9 gene editing system was used in order to investigate the role of the Outer Membrane Protein A *(ompA)* gene of the *Cedecea neteri* symbiont of *Aedes aegypti* mosquitoes, in the host-microbe interactions; mutated bacteria showed an impaired ability to form biofilms during host gut colonization, resulting in a significantly reduced infection prevalence of the host compared to the control (Hegde et al. 2019).

Consequently, the successful establishment of an efficient CRISPR/Cas9 genome modification tool for the olive flies, provides the basis for the study of *B. oleae* genetics and for a further development of novel and alternative control strategies against this destructive agricultural pest.

2.7. Figures

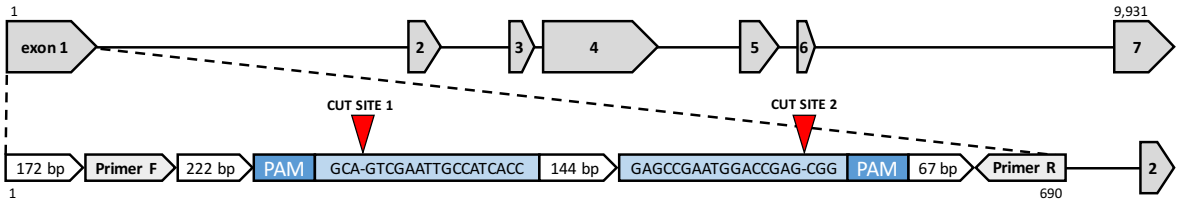

Figure 2.5. Schematic diagram of the gene sequences and sgRNA target sites in the *B.oleae Scarlet* (*BoSt*) gene. The olive fly *Scarlet* gene (LOC106621460) is approximately 10 Kbp and consists of 7 exons. In the scheme they are highlighted: the position and the sequences of the two designed sgRNAs (light blue), the protospacer adjacent motifs (PAM, blue) and the two cutting sites (red triangles). The two sgRNAs were chosen so as to generate an easily detectable deletion of approximately 190 bp.

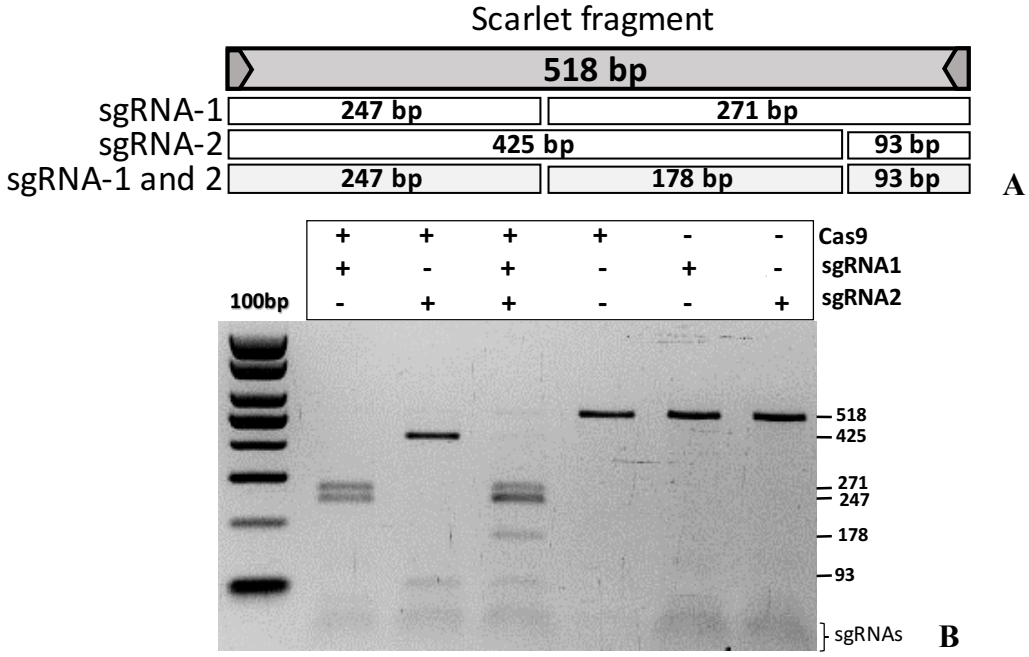

Figure 2.6. *In vitro* cleavage assay
(A) Schematic diagram showing the expected *Scarlet* gene fragments, after the *in vitro* cleavage assay, where the two sgRNAs were incubated with the Cas9 nuclease in different combinations. (B) Scarlet fragment was incubated with sgRNA/Cas9 complexes and then analysed in a 2% agarose gel. The presence or absence of each component in the reactions is marked by either "+" or "-" respectively. Small lines on the right of the gel indicate the sizes in bp of the full length target DNA and the fragments produced by sgRNA mediated cleavage. This result indicates that both (together or individually) sgRNA-1 and sgRNA-2 can effectively guide Cas9 protein to cut DNA at their respective target sites.

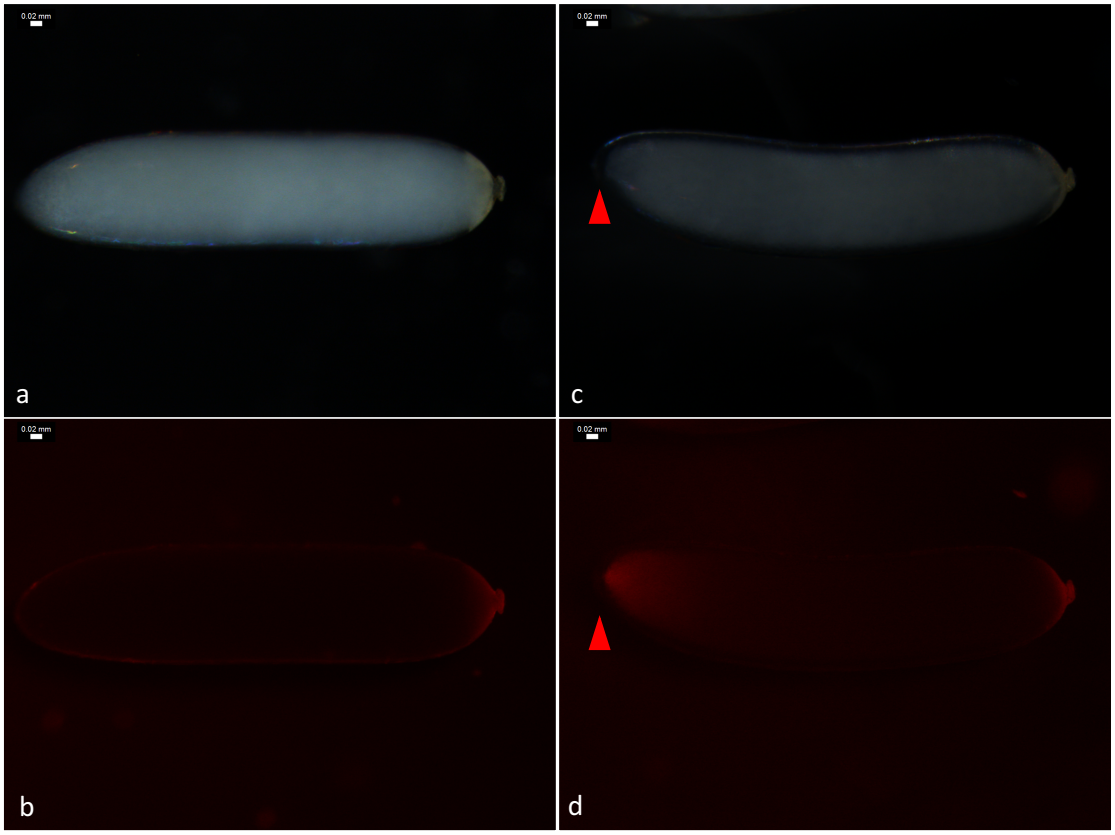

Figure 2.7. Microinjection of CY3 plasmid delivery control into olive fly embryos. The injected and non-injected embryos were monitored under a fluorescence microscope. (a, c) Brightfield microscopy pictures of non-injected (a) and injected (c) embryos. Red arrow indicates the spot of injection (posterior end). (b, d) Fluorescence microscopy pictures of non-injected (b) and injected (d) embryos. Red arrow indicates a fluorescent signal at the posterior end of the embryo (spot of injection). These results suggest that CRISPR reagents can also be successfully injected into olive fly embryos.

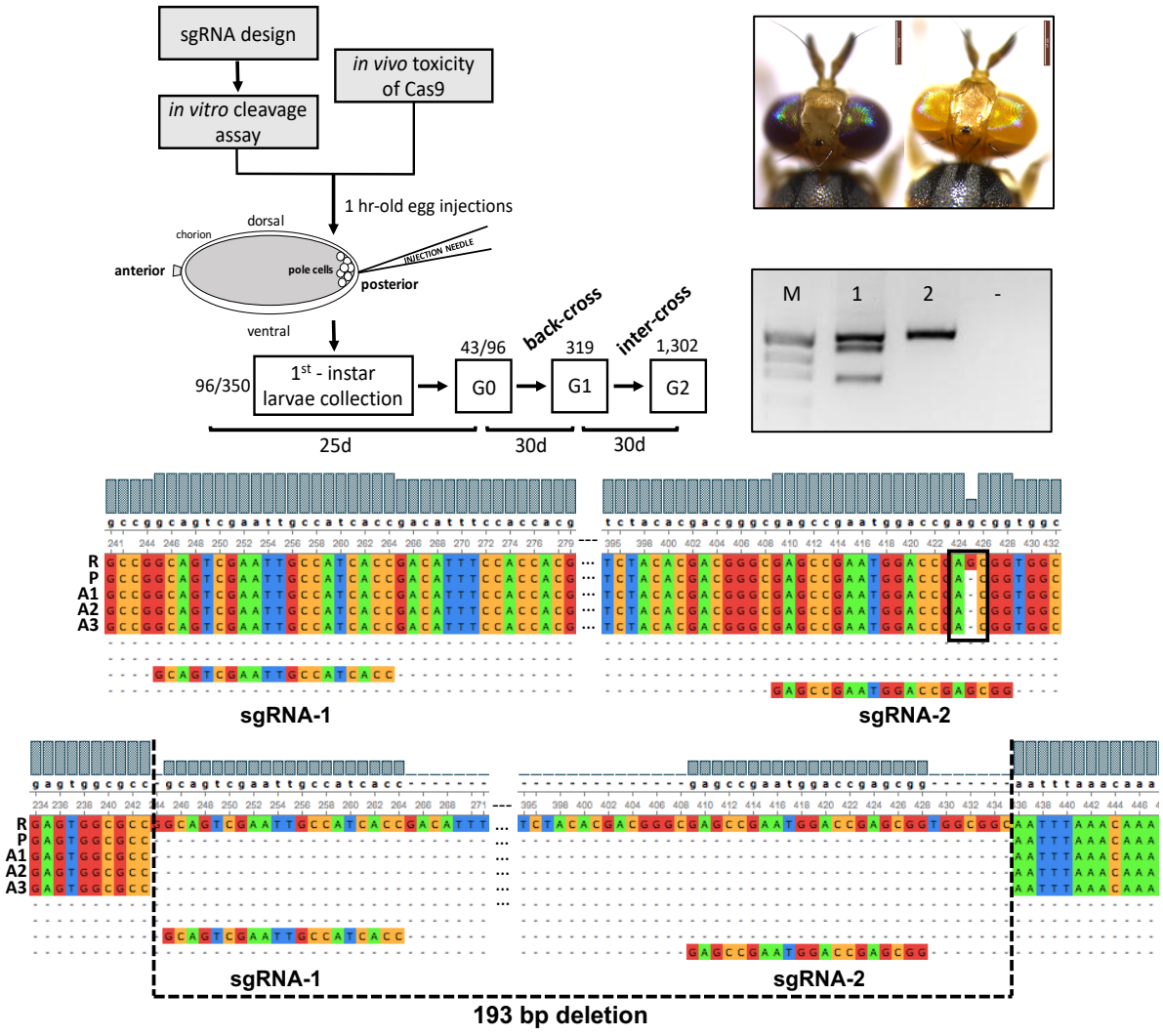

Figure 2.8. CRISPR/Cas9-mediated gene editing of the *Bactrocera oleae Scarlet (BoSt)* gene. (a) Schematic diagram of the workflow for the microinjections, mating schemes and offspring numbers. (b) Comparison of *B. oleae* wild-type eye color and CRISPR-/Cas-induced yellow-eye mutant phenotype. (c) Visualization of the PCR products on the agarose gel, which reveals a three-band pattern in each yellow-eye fly. M: marker, 1: mutant, 2: wild type fly (d) Sequencing alignments of PCR products (lowest band:1 and highest band:2) of *scarlet* fragment from wild-type (R: Reference) and mutant (P: Pupa, A1-3: Adults 1-3) flies. (1) The lowest band (330bp) shows a deletion of 193bp, corresponding to the DNA fragment between the two sgRNA cut sites, for all four mutants. (2) The highest band (510bp) aligned almost perfectly with the R sequence, except for a single bp deletion (Guanine-G: black box), causing a frameshift mutation, directly adjacent to the 3' sgRNA cut site.

2.8. Supplementary Material

Supplementary Table ST2.1. Primers used in the study.

Description	Primer	Sequence
Actin	Actin_F	CGGTATCCACGAAACCACAT
	Actin_R	ATTGTTGATGGAGCCAAAGC
Scarlet	Scarlet_F	TCAATGGAGCAAACCCGCA
	Scarlet_R	CTGGAACCCATTAGCGCCAT
sgRNA production	Long_st_5	GAAATTAATACGACTCACTATAGG**AGCCGAATGGACCGAGCGG**GTTTTAGAGCTAGAAATAGC
	Long_st_3	GAAATTAATACGACTCACTATAGG**TGATGGCAATTCGACTGC**GTTTTAGAGCTAGAAATAGC
	Uni_R	CAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCCACGAAAA
Off-targets	3'offt_F	GCGGACGCGATAATGACTAC
	3'offt_R	CCATTAGCACGCACTGAACC
	5'offt_F	CGGGCATATTCGATGAGGCA
	5'offt_R	ATTCACCATCGGCACTCGTT

Supplementary Table ST2.2. Summary of injections with different concentrations of Cas9 protein.

Injection	Viability rate
Non-injected	76 %
Injection Buffer	40 %
187 ng/µl Cas9	48.3 %
375 ng/µl Cas9	50 %
750 ng/µl Cas9	53.5 %

Supplementary Table ST2.3. Embryonic development rates after microinjections.

	Injected eggs	Hatched larvae	Pupae	G0 adults	G1 adults	Mutation frequency in G1	G2 adults	Scarlet G2 adults
Injection Buffer	180	60	34	32	300	-	-	-
Scarlet	350	96	51	43 21♀/22♂	319 161/158	0.01	1,301	4

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Chapter 3

Characterization of genes associated with insecticide resistance in *B. oleae.*

Chapter 3 |

Title: Characterization of genes associated with insecticide resistance in *B. oleae.*

Part 3.1

Functional validation of the Δ3Q mutation in acetylcholinesterase (AChE), previously associated with organophosphorus resistance.

3.1.1. Abstract

Olive fly pest management in Greece has been mostly relied on the use of chemical (small-molecule) insecticides. The long overuse of organophosphorus-based (OP) insecticides has resulted in the development of insecticide resistance. OPs target the acetylcholinesterase enzyme (AChE) and suppress its function, which is the hydrolysis of the neurotransmitter acetylcholine, in order to prevent neuro-toxicity and subsequent death. Particularly in olive flies, the resistance to OPs has been associated until now with two point-mutations and one deletion in AChE gene. This short deletion of three glutamines (Δ3Q), from a stretch of five glutamines, has been reported in the last exon of AChE, outside of the active site of the gene, in contrast to the other two substitutions (Ile214Val and Gly488Ser), which reside in the active site of the enzyme, affecting its allosteric structure. Δ3Q is suggested to result in a better anchoring of AChE on the cell membrane, and as a result, the availability of the molecules that hydrolyze ACh and interact with the insecticide is being increased, having as a consequence the insect survival at higher doses of insecticide, conferring resistance to these compounds. However, this hypothesis has not been supported with *in vivo* evidence yet.

In order to investigate *in vivo* the effect of Δ3Q mutation, in association with organophosphate resistance, we used the CRISPR/Cas9 gene editing system which was developed before. A strategy was implemented in which purified and commercially available Cas9 protein along with multiple sgRNAs and a synthetic donor ssODN DNA template (IDT) were introduced into early-stage embryos with microinjections, in order to knock-in the Δ3Q modification, by homologous recombination (HDR). The results showed that although *in vitro,* five out of the total seven sgRNAs effectively direct the Cas9 to the desired sites, in order to cleave the DNA and potently integrate the donor template (including the Δ3Q instead of the WT 5Q in the end of AChE), the corresponding result was not accomplished *in vivo*; approximately 2,174 olive fly embryos were injected and sequenced in groups. Sequencing results did not show any DNA cleavage, suggesting an insufficient Cas9 RNP uptake by the oocytes, resulting in a micro-injection protocol with very low efficiency. We conclude that the knock-in method requires further improvements, in order to successfully introduce mutations and study them functionally with the CRISPR/Cas9 genome modification tool.

3.1.2. Introduction

The main part of the olive fly control in Greece has been based on chemical (small-molecule) insecticides. For decades, organophosphorus-based (OP) insecticides were intensively applied, particularly dimethoate and fenthion, a practice which has led to the development of insecticide resistance to these compounds (Vontas et al. 2011). The latest years, the pyrethroid alpha-cypermethrin and the macrocyclic lactone spinosad are being used for the control of *B. oleae*. However, even though the olive fly populations in Greece confer moderate levels of resistance, compared to other pest species, it is not well-defined whether the field evolved resistance reduces the pesticide efficacy and has practical consequences to the control management (Kampouraki et al. 2018).

OPs target the acetylcholinesterase enzyme (*ace*, AChE) and suppress its function. AChE is a key-enzyme in the central nervous system of the insects, which hydrolyzes the acetylcholine (ACh) neurotransmitter, in order to terminate the nerve impulse in the cholinergic synapses (**Figure 3.1.1.A**). OP insecticides inhibit AChE, by covalently phosphorylating the serine residue within the active site of the enzyme and thus preventing it from its main role. As a consequence, ACh is not hydrolysed, its concentration in the synaptic cleft is being increased, resulting in an overstimulation of the insect's nervous system, which leads it to death (Aldridge 1950) (**Figure 3.1.1.B**).

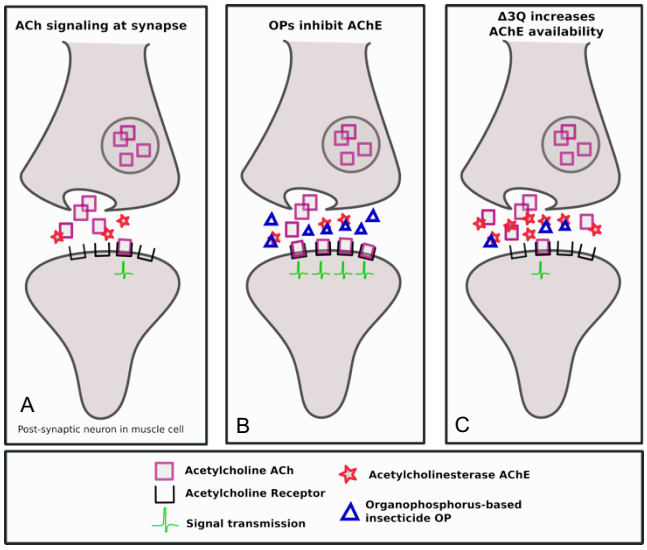


Figure 3.1.1. The role of AChE in the central nervous system of the insects. (A) The primary role of AChE is to hydrolyze ACh in the synaptic cleft and terminate the neuronal transmission. (B) OP and CB insecticides inhibit AChE, and as a consequence, ACh is not hydrolyzed, it is accumulated in the synaptic cleft and the insect is leaded to death, due to nerve over-stimulation. (C) The hypothesis is that Δ3Q deletion contributes in a better anchoring of AChE on the cell membrane, and as a result, the availability of the molecules that hydrolyze ACh and interact with the insecticide is increased, and the insect survives higher doses of insecticide, conferring resistance to these compounds.

Most insects have two different *ace* loci encoding 2 distinct AChE genes (1 and 2). Cyclorrhaphan clade flies (*D. melanogaster, B. oleae*) have only a single AchE2 locus. As it concerns the AChE evolution in Dipteran, the main synaptic AChE in mosquitoes is AChE1 and in *Drosophila* it is the AChE2. By this it is proposed that since *Drosophila* lacks an AChE1 ortholog, AChE2 has taken over the function as the main synaptic AChE (Huchard et al. 2006) and the extra copy of AChE1 was lost probably because it was replaced by the alternative transcript of the remaining one (Rhee et al. 2013). Furthermore, the AChE gene in *Drosophila* has two isoforms, one of which is membrane-anchored, whereas the other one is soluble; however, these two isoforms differ significantly in size, due to the last exon (>50kDa or 500 amino acids). AChE2 of *Bactrocera oleae*

has a gene length of approximately 140 Kbp and consists of long introns and ten exons (the first exon is non-coding). In addition, two isoforms of the gene exist, which they only differ at the final exon. Like *Drosophila*, the first one AChE2-1 encodes 673 amino acids (aa) (protein ID: XP_036217470.1), and AChE2-2 encodes 650 aa (protein ID: XP_036217476.1) (**Supplementary Figure SF3.1.1.**).

OP resistance in olive flies has been associated until now with two point-mutations and one deletion in AChE (Hawkes et al. 2005). The two point-mutations in AChE that have been reported to confer resistance, are both located in the catalytic site of the enzyme and consist of single-base substitutions at crucial sites within the enzyme's active site. Such modifications affect the orientation of the amino acids and the hydrolysis kinetics of the enzyme, resulting in a decreased rate of neurotransmitter hydrolysis (Kozaki et al. 2001; Fournier et al. 1992). More specifically, an isoleucine to valine (Ile214Val) (also detected in *D. melanogaster* and *M. domestica*) and a glycine to serine (Gly488Ser) base substitutions have been reported in AChE exons three and six respectively (Hawkes et al. 2005). The latter one has been associated with a 35-40% reduction of AChE catalytic efficiency (JG Vontas et al. 2002). Furthermore, a double mutation haplotype (the majority of the samples, where OPs are extensively used for *B. oleae* control, are homozygous for both mutations (Hawkes et al. 2005)), confers up to a 16-fold decrease in insecticide sensitivity (JG Vontas et al. 2002).

However, more recently, a short deletion of three glutamines (Δ3Q, CAA-CAA), from a stretch of five glutamines (CAA-CAA-CAG-CAA-CAA), has been detected in the tenth exon of AChE, outside of the active site of the gene. Δ3Q has been associated with resistance to OPs, linked with a novel mechanism this time, different from the steric alteration of the active site residues of the enzyme (Kakani and Mathiopoulos 2008; Kakani et al. 2014). More specifically, AChE is composed of a single molecular form, that is a glycosylated dimer attached to the cell membrane, via a glycolipid GPI anchor; expression studies with the mutated AChE in cells showed that the catalytic domain of the enzyme is not affected, but its activity is being increased, suggesting an improved anchoring of the mutant enzyme in the synaptic cleft (Kakani et al. 2011). Subsequently, the hypothesis, which has been supported with bioinformatic algorithms (big-PI Predictor), is that more AChE molecules are being anchored in the membrane, due to the easier cleavage of the shorter molecule, which happens normally and is substituted by a GPI anchor. AChE thus participates efficiently both in the metabolism of ACh and in the interaction with the insecticide, offering to the insect an increased tolerance towards the OP insecticides, since the ACh in not accumulated in the synaptic cleft and the insect survives under higher doses of insecticide (**Figure 3.1.1.C**).

Experimentally, biochemical assays have shown that the Δ3Q mutant, slightly differs, as it concerns its enzyme activity (only 10% up), against the WT-control, most likely due to an increased efficiency in the processing and the maturation of the mutant precursors into active GPI-anchored enzymes (Kakani et al. 2011). It's worth noting that homozygous Δ3Q individuals have not been reported in any of the field populations that have been studied, implying that Δ3Q mutation, always identified in combination with the I214V and G488S points mutations, has a larger fitness cost than those two (Kakani et al. 2014). However, this phenomenon has to be

tested *in vivo* as well, since *in vitro* assays usually do not import all the functions of a eukaryotic organism, such as post-translational modifications like the Δ3Q case study.

CRISPR/Cas9 genome editing system stands as a convenient tool for the integration of such gene modifications, via the HDR (Homology Directed Repair) mechanism and their further investigation. This direct and highly precise pathway can repair double-stand brakes (DSB) of the DNA, using as a scaffold a donor DNA template with homology to the damaged DNA, already manipulated to integrate specific alterations to the gene (**Figure 3.1.2**).

CRISPR/Cas9 transformative technology has been already applied in previous studies and successfully assessed the editing of genes virtually in tephritid species and particularly through the HDR pathway. Specifically in *C. capitata*, it was achieved a 57-90% knock-in rate in the G1 generation, upon injection of a CRISPR/Cas9 RNP complex, assembled with different sgRNAs and a short single-stranded oligodeoxynucleotide donor, to convert an enhanced green fluorescent protein into a blue one, towards the establishment of a new SIT-like approach (Aumann, Schetelig, and Häcker 2018). Accordingly, a more recent study has reported the efficient recreation of a point mutation, initially found in *D. melanogaster,* in *B. tryoni,* demonstrating that CRISPR/Cas9 technology can be used to trial conditional mutations for the ultimate aim of generating genetic sexing agricultural pest strains for SIT (Choo et al. 2020).

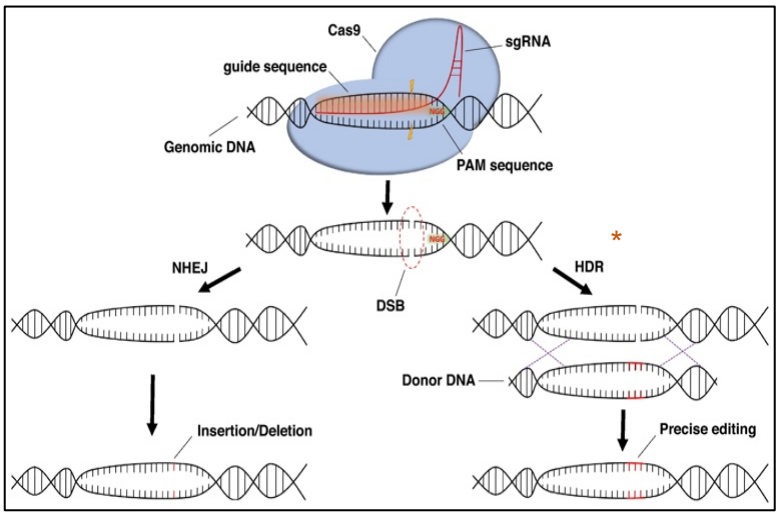


Figure 3.1.2. The CRISPR/Cas9 genome modification technology. The Cas9 protein-sgRNA complex binds the DNA target sequence next to the protospacer adjacent motif (PAM) and a DNA-RNA hybrid is formed. Cas9 then precisely cleaves the target dsDNA on both strands, resulting in a DSB; this break can be repaired either by the non-homologous end joining (NHEJ) imperfect repair mechanism, which often creates random insertions or deletions in the DNA, or by the endogenous cellular machinery and the HDR mechanism, which requires a donor DNA template (Homem and Davies 2018)**.**

Several molecular diagnostic tests have already been developed and are being widely applied for the detection of potent mutations in the *ace* gene, associated with resistance at higher OP doses (including the two point mutations and the proposed Δ3Q deletion) (Kakani et al. 2014; Nobre, Gomes, and Rei 2019), aiming at monitoring the frequency of them in natural *B. oleae* populations. As a consequence, any solid *in vivo* functional evidence that proves the implication of Δ3Q in OP resistance, will further support a more efficient pest management strategy.

3.1.3. Aim of the study

The deletion of three amino acids in the last exon of AChE (Δ3Q) has been associated with organophosphorus resistance in *B. oleae,* upon field screenings and *in vitro* experiments. However, this hypothesis has not been supported with *in vivo* evidence yet. The aim of our study was to investigate this hypothesis *in vivo*, and more specifically by introducing the Δ3Q mutation, with the CRISPR/Cas9 gene editing tool developed in Chapter 2, under a susceptible genetic background of a laboratory-reared *B. oleae* strain and study the mutant phenotype upon insecticide application.

3.1.4. Materials and Methods

3.1.4.1. Insects

The *B. oleae* strain which was used in this study originated from the Democritus Laboratories fly stock and is maintained under controlled conditions in IMBB, as previously described in depth (section **2.4.1. Rearing of *Bactrocera oleae*).**

3.1.4.2. Target site selection

The *B. oleae ace* gene (AChE) was identified by running a text search in the OrthoDB orthology database and was confirmed via similarity using protein BLASTp. AChE length is approximately 140 Kbp and consists of 10 exons (the first one is non-coding). Δ3Q mutation is a short deletion of three glutamines (CAG-CAA-CAA) in a stretch of five (CAA-CAA-CAG-CAA-CAA, wild type 5Q), which is present in the last exon of only AchE2_1 (exon 9 from now on, first exon is not included), in the C-terminal peptide (Kakani and Mathiopoulos 2008).

PCR and Sanger sequencing of *B. oleae ace* exon 9 (where Δ3Q mutation is reported) was performed on the Democritus laboratory strain, in order to identify if there were any SNPs within this region in the lab population. Afterwards, this sequence was scanned for any potent single-guide RNA sequences (sgRNAs), following a G-N19-NGG pattern outlined previously (Bassett and Liu 2014) and Chop-Chop online tool (Labun et al. 2019). All potential sgRNA targets were afterwards scanned against the *B. oleae* reference genome (NCBI accession number: LOC106625586) for potential off-target hits, using the CasOT command line software (Xiao et al. 2014), and no additional exact hits were detected. Seven sgRNAs were designed, each one with a protospacer adjacent motif (PAM) cut site, around the 5Q target (**Supplementary Figure SF3.1.2)**.

3.1.4.3. sgRNA synthesis and CRISPR/Cas9 components targeting the *ace* locus in *B. oleae*

The template for the generation of each sgRNA was produced by a target-specific forward and a common reverse primer (**Supplementary Figure SF3.1.1.** (Bassett and Liu 2014)). Same procedure as previously was followed for the synthesis of the sgRNAs to target AChE (section **2.4.3. sgRNA synthesis and RNP complex assembly**). In order to introduce the Δ3Q mutation

into the *B. oleae* AChE gene, a 130 nucleotide single-stranded oligo DNA donor template (ssODN), containing the 9 base deletion (Δ3Q), was designed and ordered (Integrated DNA Technologies, IDT). The PAM sequences of the sgRNAs were mutated in the oligo and the MwoI restriction site, located next to the target site, was abolished for the downstream diagnostics (**Supplementary Table ST3.1.1**). The ssODN sequence was ordered as a 10nmol Ultramer Oligo and resuspended in DEPC water to a final concentration of 2μg/μl upon arrival.

3.1.4.4. *In vitro* cleavage assay

The sgRNA efficacy was firstly tested *in vitro* for all seven sgRNAs, using the commercially available Cas9 nuclease. Briefly, a 367 bp DNA fragment containing the AChE exon 9 and all the seven sgRNA target sequences was amplified by PCR with specific primers (Supplementary Figure 3). 92 ng of the target PCR product were digested with 2 pmoles of each sgRNA-Cas9 complex (1:5.5 ratio of substrate: complex). Same procedure as previously was followed (section **2.4.4. *In vitro* cleavage assay**). Analysis of the enzymatic digestion was performed by electrophoresis on a 3.5% agarose gel.

3.1.4.5. Design of a screening method with a restriction enzyme

A screening procedure was designed and set up, with the use of MwoI restriction enzyme (R0573S, NEB). MwoI cuts twice the WT AChE exon 9 (5Q), providing three DNA fragments (106bp, 134bp, 120bp) and the when Δ3Q is present (meaning that the oligo has been integrated) MwoI is cutting once; as a consequence, two DNA fragments are created (240bp and 120bp), due to the depletion of the first cut site, upon donor integration.

Briefly, in order to test the efficacy of the restriction enzyme assay, a 2-hour digestion at 60°C was performed, with 0.5μl of the enzyme, 5μl of purified DNA fragment and 2μl of CutSmart™ Buffer in a 10μl final reaction volume, according to the manufacturer's instructions. Analysis of the enzymatic digestion was performed by electrophoresis on a 3.5% agarose gel.

3.1.4.6. Embryo microinjections

The CRISPR/Cas9 ribonucleoprotein (RNP) complex, consisting of the sgRNAs and the commercially available Cas9 nuclease (NEB, M0386T) (incubated at 37°C for 15 minutes to form the complex prior to injections), along with the ssODN HDR template were injected in the posterior poles of one-hour-old de-chorionated embryos, following the established embryo micro-injection protocol (section **2.4.5. Embryo microinjections**). The injection mixes were comprised of 0.6 μg/μl purified Cas9 protein, 0.5 μg of sgRNA, 0.5 μg of ssODN and 1x injection buffer (0.1 mM sodium phosphate buffer pH 6.8, 5 mM KCl) in a final volume of 5 μl. Microscope slides with injected embryos were placed in Petri dishes containing moist tissue paper and were wrapped with Parafilm and placed at 25°C for 72 hours in order to allow larval hatching. 1st instar hatched larvae were then transferred in larval food for the remaining of the larval development. The microinjections to early-embryos were performed by Ioannis Livadaras, technician of the IMBB.

3.1.4.7. DNA isolation and Genotyping by DNA Sanger Sequencing

The genomic DNA was isolated from whole flies using the CTAB extraction protocol, as described previously (section **2.4.6. DNA isolation and Genotyping with DNA Sanger Sequencing**). Genotyping was performed using 1 μl of each DNA sample in a PCR reaction, using the standard EnzyQuest Taq Polymerase, with a 30s annealing step at 54°C and 30s extension time at 54°C. Gel purified PCR products were sent off for Sanger sequencing (Genewiz) and the resulting sequences were visualized and aligned with the reference *B. oleae* genome sequence, by the assembling-to-reference tool of UGENE toolkit (Okonechnikov et al. 2012).

3.1.5. Results

3.1.5.1. Designing the CRISPR/Cas9 components to target the *ace* locus in *B. oleae*

A strategy was implemented, in which the Cas9 protein in complex with multiple sgRNAs and a donor DNA template for homologous recombination were introduced into early stage embryos with microinjections, in order to obtain a knock-in of the Δ3Q mutation. A 360 bp region of the ninth exon of AChE was sequenced in ten *B. oleae* individuals of the Democritus laboratory strain, in order to determine if there were any single nucleotide polymorphisms (SNPs) within this gene region. Seven guide RNA sequences (sgRNAs) were designed and produced with protospacer adjacent motif (PAM) cut sites a few bases upstream or downstream of the Δ3Q locus (**Supplementary Figure SF3.1.2**). A BLASTn search of the guide sgRNAs against the *B. oleae* reference genome revealed no likely off-target matches.

3.1.5.2. *In vitro* testing of sgRNAs

The investigation of the cleavage efficacy of the seven constructed sgRNAs, by guiding the Cas9-induced gene editing at their target site, was assessed through an *in vitro* cleavage assay with purified Cas9 nuclease. The Cas9 digest of the *ace* locus showed that incubating (together and individually) sgRNA #1/Cas9 or sgRNA #2/Cas9 with the target, did not result in the production of two DNA bands with the expected size; only the initial full-length fragment was detected (**Supplementary Figure SF3.1.3B**). However, incubating sgRNAs #3-#7 individually with the target, resulted in the production of the two expected products (approximately 250 and 110 bp). Furthermore, it seems that sgRNAs #5 and #6 showed the highest efficiencies, as measured by the disappearance of the wild type uncut band. No DNA cleavage was detected when the PCR fragment was incubated with either Cas9, or the sgRNAs alone. These results indicate that the five out of seven designed sgRNAs can effectively guide Cas9 protein to cut DNA at their respective target sites, with a higher efficiency observed in two of them (#5 and #6). (**Supplementary Figure SF3.1.3.C**).

3.1.5.3. Introduction of the Δ3Q mutation into *B. oleae ace*

In order to introduce the Δ3Q mutation into the *B. oleae* genome, a 130 nt single-stranded oligo donor template (ssODN) containing the (CAACAACAGCAACAA ➔ CAACAA) nine base-pair

deletion was designed and ordered (**Supplementary Table ST3.1.1**). The CRISPR/Cas9 RNP complex consisting of the sgRNA complex and the Cas9 enzyme was injected into embryos along with the ssODN HDR template. A restriction assay with MwoI was evaluated in the laboratory-reared population (LAB), showing an effective three-band pattern at the WT- non-mutated AChE fragment (**Supplementary Figure SF3.1.3A**).

3.1.5.4. Micro-injections and crosses

The CRISPR/Cas9 RNP complex was microinjected into approximately 2,174 embryos in total (microinjections were performed according to the details described in **2.4.5. Embryo microinjections**). The results showed that although *in vitro* five out of seven sgRNAs (**Supplementary Figure SF3.1.3B,C**) direct the Cas9 to the designed cleavage sites for the integration of the donor DNA template, the intended DNA cleavage and the integration of Δ3Q were not accomplished *in vivo*, as this was confirmed with sequencing.

Five synthesized sgRNAs (#3-7) were injected in one-hour-old dechorionated embryos which were allowed to develop for 3 days inside the petri dish. First instar hatched larvae (fourth-sixth day) and dead non-hatched embryos were used for DNA isolation, PCR amplification and Sanger sequencing in pools, in order to detect any possible DNA cleavage. Furthermore, sgRNAs #5 and #6 were injected, combined in the same injection mix, and the larvae were allowed to develop. First instar hatched larvae were transferred in solid larval food, for the remaining of the larval development. When they turned into G0 adults, they were backcrossed to non-injected males and females, originating from the same strain, in a 1:3 ratio, and they were allowed to mate and lay eggs (26 small cages with injected females and 30 cages with injected males). After the emergence of the G1 offspring, G0 parents were sacrificed and sequenced in pools. The injection rates are collected in (**Supplementary Table ST3.1.2**).

The purpose of these early stage screenings was to investigate whether a potent DNA cleavage is accomplished or not and is detectable in G0 generation, aiming to proceed in a targeted manner.

3.1.6. Discussion - Conclusions - Future approaches

In order to investigate *in vivo* the effect of Δ3Q mutation, which is in association with organophosphate resistance in olive flies (Kakani et al. 2011), we applied the CRISPR/Cas9 genome modification technology (developed previously in Chapter 2 and published at (Koidou et al. 2020)). Aim of our study was to introduce the Δ3Q mutation (deletion of the nine bases) in the last exon of the acetylcholinesterase gene, under the genetic background of a susceptible olive fly strain, reared under laboratory conditions, and study the phenotype upon exposure to OPs. The ultimate goal of this investigation was to provide *in vivo* data for this proposed novel mechanism of resistance.

For this purpose, a strategy was implemented in which purified and commercially available Cas9 protein along with multiple sgRNAs and a synthetic donor ssODN DNA template (IDT) were introduced into early embryos with microinjections, in order to knock-in the Δ3Q modification, by

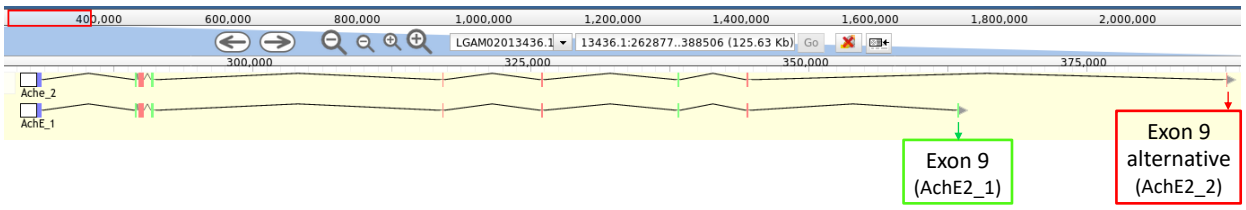
homologous recombination (HDR mechanism). The components of the *in vivo* experiment were evaluated firstly *in vitro*. The success of the method was designed to be explored in the next generations by PCR, followed by an enzyme digestion (MwoI) and sequencing techniques. The plan was to generate homozygotes (at G2 generation) after back-crossing twice with the susceptible laboratory strain and subject these mutants in toxicity tests (bioassays), in order to detect any possible contribution of this mutation in the resistance phenotype and further characterize it.

The results showed that although *in vitro* five out of the total seven sgRNAs direct the Cas9 to the desired sites in order to cleave the DNA and integrate the donor template (including Δ3Q instead of WT 5Q in the end of AChE), the corresponding result was not accomplished *in vivo*. Specifically, a total of approximately 2,174 olive fly embryos were injected and sequenced in groups. Sequencing results did not show any DNA cleavage. In addition, 660 G0 adults were back-crossed with non-injected individuals. These G0 flies were sequenced in groups too, but still no DNA modification was detected.

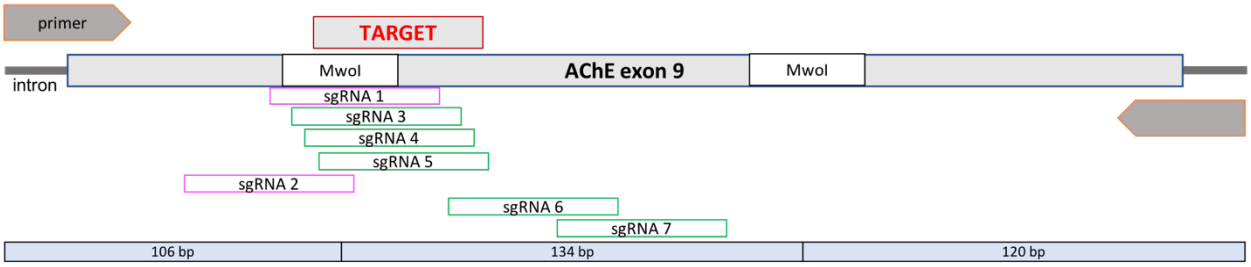
According to the results of our previous work (Koidou et al. 2020), and as discussed in Chapter 2, the rate of somatic cell mutagenesis in olive fly appears to be very low, close to zero (no mosaic individuals were detected in G0 and G1 generations). The fact that no somatic cell mutagenesis was accomplished and mosaicism effect was not detected in G0 and G1 generations, contrary to other tephritid (Bai et al. 2019) or non tephritid species (Khan, Reichelt, and Heckel 2017), also constituted a critical limiting factor in the screening procedures that followed; the requirement of molecular genotyping methods for the detection of integrated mutations adds one more element of complexity. Furthermore, the phenotypic alteration from brown to yellow eyes was observed in the G2 generation, in the homozygous state of the CRISPR event, in only 4 out of the 1,302 G2 adults (which had developed from 350 injected G0 embryos), suggesting an insufficient Cas9 RNP uptake by the oocytes, resulting in a micro-injection method with very low efficiency. However, the efficiency in olive flies is comparable to other reports of tephritid species, previously described in Chapter 2 (Sim et al. 2019; Choo et al. 2018).

In order to successfully introduce mutations and study them functionally with the CRISPR/Cas9 genome modification tool, the knock-in method requires further improvements; the micro-injection efficiency, the detection method for screening the incorporated mutations and the sequencing methodologies are fields that require further improvement. Already, the development of a T7 Endonucleasse I (T7EI) enzymatic assay, which accurately recognizes insertions and deletions ≥ 2 bases, which are generated by non-homologous end joining (NHEJ) activity, combined with amplicon sequencing, has led to an important increase in the resolution of the detection method (Choo et al. 2020). Furthermore, the development of new methods to genotype insects individually, without killing or impairing their ability to reproduce, is a continuing issue (Reid and O'Brochta 2016). Finally, the transgenic expression of Cas9 *in vivo*, as well as the alternative delivery of the Cas9 RNP complex to the germline, by injecting the oocytes of adult females, instead of embryos, substantially have improved the efficiency of CRISPR technology and have given insights for such developments in other non-model insect species (Port et al. 2014; Chaverra-Rodriguez et al. 2018).

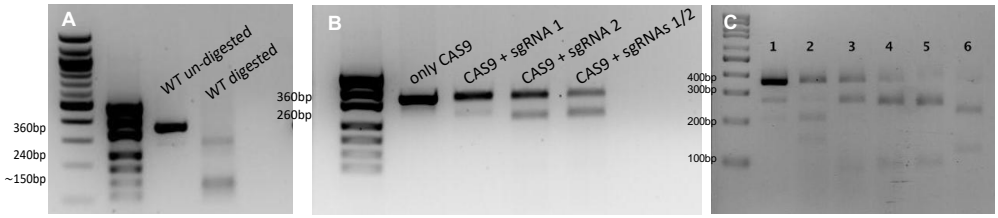
3.1.7. Supplementary Material



Supplementary Figure SF3.1.1. The AChE gene in *Bactrocera oleae*. The olive fly AChE-2 has a gene length of ~140 Kbp and consists of long introns and ten exons (the first one is non-coding). Two isoforms of AChE exist, which they only differ at the last, like the *Drosophila one*; the first one AChE2-1 encodes 673 amino acids (aa) (protein ID: XP_036217470.1) and the other AChE2-2 encodes 650 aa (protein ID: XP_036217476.1). AChE2-1 (which contains the 5Q glutamine stretch) is ~143bp.



Supplementary Figure SF3.1.2. Schematic diagram of the gene sequences and the sgRNA target sites, in the *B. oleae* AChE gene. In the scheme they are highlighted: the 5Q DNA target (red), the position and the sequences of the seven designed sgRNAs (pink and green boxes), the two MwoI restriction cut sites (white) and the two AChE primers (orange arrows) designed to hybridize intronic sequence, before and after the ace exon 9 and generate an ~360 bp DNA fragment.



Supplementary Figure SF3.1.3. Testing the efficacy of the CRISPR/Cas9-KI components with *in vitro* assays. (A) MwoI cuts twice the WT *ace* exon 9 (5Q), providing three DNA fragments (106bp, 134bp and 120bp) (although it is a 3% agarose gel, it is difficult to distinguish the three bands, because of the small base pair difference in between them); upon Δ3Q integration, MwoI will cut once, creating two DNA fragments (240bp and 120bp), easy to be distinguished from the un-cut WT bases (approx. 120bp). (B, C) AChE fragment was incubated with sgRNA/Cas9 complexes and analyzed in a 2% agarose gel. (B) This result indicates that both (together or individually) sgRNA-1 and sgRNA-2 cannot effectively guide Cas9 protein to cut DNA at their respective target sites, since full AChE product still exists and the expected cleaved DNA (120 and 240 bp) fragments are not observed. (C) This result indicates that all five sgRNAs can guide Cas9 protein to cut DNA at their respective target sites and the expected cleaved DNA fragments are observed (250 and 110 bp); 1: ace, only Cas9, no sgRNAs, 2: ace, Cas9, sgRNA-7, 3: ace, Cas9, sgRNA-4, 4: ace, Cas9, sgRNA-5, 5: ace, Cas9, sgRNA-6, 6: ace, Cas9, sgRNA-3).

Supplementary Table ST3.1.1. Primers that were used in the study.	
Bo_ace_outofe9_F1	GTTTCCAGTCGTTTTCGCCAT
Bo_ace_outofe9_R1	TGTGTGCGTGTGTTTGTATCTG
Bo_ace_e9_Long_5 sgRNA#1	GAAATTAATACGACTCACTATAG**TGCAGGGAGGAAGCGGATGA**GTTTTAGAGCTA GAAATAGC
Bo_ace_e9_Long_S sgRNA#2	GAAATTAATACGACTCACTATAG**GTGTTGTTGCTGTTGTTGCA**GTTTTAGAGCTAG AAATAGC
Bo_ace_e9_Long_1n sgRNA#3	GAAATTAATACGACTCACTATAG**GCTGTTGTTGCAGGGAGGAAG**GTTTTAGAGCT AGAAATAGC
Bo_ace_e9_Long_2n sgRNA#4	GAAATTAATACGACTCACTATAG**GTTGTTGCTGTTGTTGCAGGG**GTTTTAGAGCTA GAAATAGC
Bo_ace_e9_Long_3n sgRNA#5	GAAATTAATACGACTCACTATAG**GAGTGTTGTTGCTGTTGTTGC**GTTTTAGAGCTA GAAATAGC
Bo_ace_e9_Long_4n sgRNA#6	GAAATTAATACGACTCACTATAG**GAACACTTGCTACTGCAACAA**GTTTTAGAGCTA GAAATAGC
Bo_ace_e9_Long_1 sgRNA#7	GAAATTAATACGACTCACTATAG**GAGCATCGTGGCGTTCATGT**GTTTTAGAGCTAG AAATAGC
UNI_R	CAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTT CACCGTGGCTCAGCCACGAAAA
WT *ace* (5Q)	AAATAACCACCAACTCTCTCCATCTTTTTTTCAGCGCAATGTGAAGTCAAAACATCA TCCGCTTCCTCCCTG**CAACAACAGCAACAA**CACTTGCTACTGCAACAAAGGAGCA TCTGGCGTTCATGTTGACGCTGTCAC
ssODN from IDT (Δ3Q mutation)	AAATAACCACCAACTCTCTCCATCTTTTTTTCAGCGCAATGTGAAGTCAAAACATCA TCCGCTTCCTCCCTG**CAACAA**CACTTGCTACTGCAACAAAGGAGCATCGTGGCGT TCATGTTGACGCTGTCAC
MwoI	5’ … GCNNNNN---NNGC … 3’ 3’ … CGNN---NNNNNCG … 5’

Supplementary Table ST3.1.2.
Summary of injections with five sgRNAs.

sgRNAs	**Injected eggs**	**G0 larvae for seq**		
3	390	133		
4	380	146		
5	140	33		
6	210	38		
7	140	27		
3,4,5,6,7	260	132		
		G0 larvae	**G0 pupae**	**G0 adults**
5, 6	654	256	161	150
total	2,174			

3.1.8. References

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Chapter 3 |

Title: Characterization of genes associated with insecticide resistance in *B. oleae.*

Part 3.2

Looking for candidate genes implicated in pyrethroid resistance, using transcriptome sequencing on olive fly malpighian tubules, one of the major detoxification tissues in insects.

3.2.1. Abstract

The detoxification of insecticides and other harmful substances has been proposed to take place in the malpighian tubules of insects, which are blind ending tubes, with excretive and osmoregulative role, and arise at the junction of the midgut and the hindgut. In an attempt for a better understanding of the detoxification of insecticides in olive flies, malpighian tubules were dissected out of a susceptible and one resistant to the pyrethroid insecticide α-cypermethrin population. Sequencing of the extracted RNA was performed using the Illumina platform, in three biological replicates for each one of the two populations. Sequencing reads were then aligned back to the olive fly reference genome sequence, gene expression levels were calculated and the up- and down-regulated genes, both in resistant and susceptible populations were identified. As expected, many genes that are well known to be implicated in insecticide resistance were identified, such as cytochrome P450s (CYPs), glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs). Moreover, several other genes were also identified, but their role in insecticide resistance mechanisms remains to be elucidated via further investigation. The over-expression of two P450 genes (CYP4P6 and CYP6G28) that were highlighted in the RNA sequencing data, was quantitatively validated with RT-qPCR analysis and the functional validation of one of them, through gene silencing (RNAi technology), revealed a promising phenotype, upon insecticide treatment. The results of this work may contribute to a better understanding of the mechanisms conferring insecticide resistance to α-cypermethrin in olive flies. However, further functional characterization studies are required. Therefore, such knowledge may assist the development of effective strategies for controlling this destructive pest and protecting the olive trees, the cultivation of which is of great regional importance.

3.2.2. Introduction

Although significant research has been performed on pesticide metabolism and resistance across many insect species, little is known about the exact tissues in which these metabolic enzymes are localized and expressed. The consensus is that the fat body and the midgut are the major sites of insecticide detoxification (Kliot and Ghanim 2012). However, apart from their main role in innate immunity mechanisms of the insect and recently as a secondary pool of vertically transmitted bacteria (Faria and Sucena 2013), the insect malpighian tubules (MTs) have also been proposed to participate in the breakdown and the renal clearance of toxic substances, such as the insecticides (Beyenbach, Skaer, and Dow 2010; Dow and Davies 2006).

MTs develop in the early insect embryo and are functional before the larva begins to feed. The initially short MTs elongate during morphogenesis, through cell rearrangements and are located at the junction of the midgut and the hindgut. As the tubules elongate, a population of mesenchymal cells becomes apicobasally polarized; these are the stellate cells (sc), so-called for the star-like shape they adopt in the adult tissue (Beyenbach, Skaer, and Dow 2010). The olive flies have two MTs which are divided in two branches each and they further consist of two macroscopic cell types: large principal cells (pc) that account for most of the mass of the tubule and small, thin stellate cells (**Figure 3.2.1** depicts the MTs of *Drosophila melanogaster (*Dipteran), which resemble in many aspects with the olive fly ones). MTs in Dipteran species expand in size once established, in order to satisfy the growing demands on osmotic and ionic regulation, as well as the renal clearance. Furthermore, they function as autonomous immune sensing organs, which produce antimicrobial peptides in response to microbial infections (Wang et al. 2004).

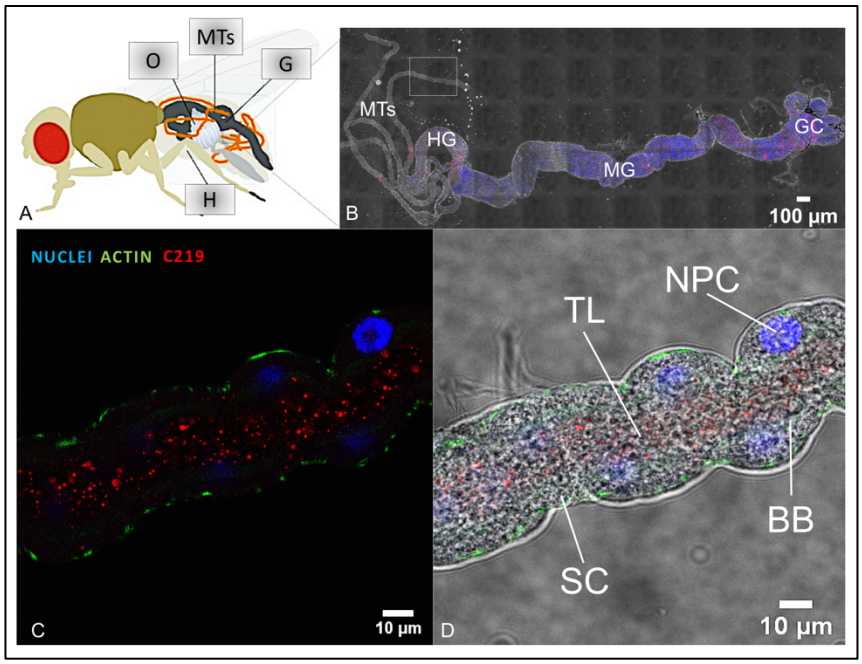


Figure 3.2.1. The Malpighian Tubules (MTs) of *D. melanogaster*. (A) Remake of a schematic representation of MTs in Drosophila, from (Faria and Sucena 2013); O: ovary, G: gut, H: hemolymph. (B-D) Confocal scanning microscopy images of a dissected part of the gut of Myo genotype in *D. melanogaster* (Denecke et al. 2022). Blue color marks nuclei (DAPI), green actin filaments (phalloidin) and red C219 P-gp (ABC transporter). White box indicates the zoom panels with higher magnification, scale bars; (B) 100µM, (C-D) 10µM; HG: hindgut, MG: midgut, GC: gastric caeca, SC: stellate cell, TL: tubule lumen, NPC: nuclear of principal cell, BB: brush border.

Genes and functions previously unrelated to MTs are being discovered through genomic, transcriptomic, proteomic, and metabolomic studies (Beyenbach, Skaer, and Dow 2010). The high abundancy of many organic soluble transporters in these datasets suggests the MTs as a powerful liver-like detoxification organ and at the same time a kidney-like secretion system, dedicated to the removal of the foreign organic cations and anions out of the circulation of the insect (Beyenbach, Skaer, and Dow 2010). Particularly, previous studies have shown an enrichment of a GST (CG17522) and a P450 gene (Cyp6a18) in *Drosophila* MTs and a significant enrichment and localization of four P450 genes (CYP6M3, CYP6Z1, CYP6Z2 and CYP6Z3) in the MTs of a resistant mosquito strain (Wang et al. 2004; Ingham et al. 2014). Furthermore, when constructing *Drosophila* transgenic lines driving overexpression or RNAi against *cyp6g1*

(cytochrome P450 gene) in MTs, the survival of the flies was negatively affected, upon DDT exposure (Dichlorodiphenyltrichloroethane, organochloride insecticide) (Yang et al. 2007).

However, until now, all transcriptome studies between insecticide-resistant and susceptible olive fly populations have assessed gene expression over the entire organism (Pavlidi et al. 2013; 2018). This method may result in the elimination of gene candidates, due to possible masking effects; even huge variations in expression levels between a resistant and susceptible strain may not be apparent, if the expression of a gene is restricted to an organ, which contributes just a tiny amount of mRNA to the total RNA pool, or differential expression occurs in only one tissue. As a consequence, dissecting specific organs and sequencing exclusively their transcriptomes, offers the opportunity to identify novel insecticide resistance-related genes, in *B. oleae*.

3.2.3. Aim of the study

The objective of this study was to compare gene expression in malpighian tubules (MTs), one potent major organ linked to xenobiotic detoxification in insects, between an insecticide resistant and a susceptible olive fly strain and identify gene candidates, not immediately apparent in the already existing whole organism RNA sequencing data.

3.2.4. Materials and Methods

3.2.4.1. Insects

The study was carried out mainly with field-derived olive flies. More specific, olives from two regions on the island of Crete were collected; Panagia (35.115503, 25.338225, Kastelli Pediados, Heraklio), and Evaggelismos (35.186293, 25.307123, Kastelli Pediados, Heraklio). The olives were kept at room temperature (RT), and the emerging adults were used in the downstream experiments.

The Hybrid *Bactrocera oleae* strain which was used in this study as the control reference, was originated after multiple crosses between male adults of the Democritus Laboratory Strain and female flies collected from infested orchards in Crete. This hybrid strain, as well as the pure Democritus laboratory strain, were maintained under standard rearing conditions at 25°C and a 16:8 h light: dark photoperiod, as described in depth in **2.4.1. Rearing of *Bactrocera oleae.***

3.2.4.2. Malpighian Tubule Dissections and RNA extraction

RNA samples were prepared with malpighian tubule (MT) sets dissected out of 3-5-day old adult female olive flies (**Supplementary Figure SF3.2.1**). Two strains, a resistant (Panagia) and a susceptible one (Hybrid) were used in the analysis, three biological replicates were prepared per each strain and each biological replicate consisted of 7-10 MT sets. The resistance levels of Panagia strain to α-cypermethrin were investigated by Natassa Kampouraki (PhD, Prof. J. Vontas Laboratory, 2019), prior to the MT dissections, with contact bioassays at a diagnostic dose,

compared to the levels of the Laboratory-reared and Hybrid susceptible strains, that are maintained under standard laboratory conditions (**Supplementary Table ST3.2.1**). Data were analyzed with Polo-Plus (LeOra Software) using Log Dose probit to determine the lethal concentration required to kill 50% of a population (LC50) and establish 95% confidence interval levels (CL). The resistance ratio (RR) of Panagia population compared to the control Lab strain was approximately 80.6-fold, whereas against the control hybrid strain it was 26.65-fold. These results suggested that this Panagia population (2019) is a resistant olive fly population.

MTs were dissected out of the adult flies on ice, in PBS 1X and collected in DNAse free Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted using the TRI Reagent® (Molecular Research Center, Inc., TR 118), according to the manufacturer's instructions and RNA pellets were re-suspended in RNase-free DEPC H_2O. Afterwards, the samples were DNase treated using DNAse I (Ambion™ DNase I (RNase-free), Cat. No AM2222) in order to remove any contaminating DNA. RNA concentration and purity (OD, A260/A280 and A260/A230) were measured in Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) using 1 µl of RNA. The ratio between the absorbance at 260 nm and 280 nm was used to evaluate purity; we assumed ratios between 1.8 and 2.0 to be pure and especially for the RNAseq analysis between 1.9 and 2.0. RNA integrity was checked on a 1.5% agarose gel.

3.2.4.3. Transcriptome sequencing

Approximately 1.5µg of each purified RNA sample, from each one of the three biological replicates for Hybrid and Panagia strains were sent to Macrogen, Inc. (Korea) for mRNA paired-end library construction, with the Illumina Truseq stranded mRNA sample preparation kit, following the manufacturer's instructions. Each library was sequenced on the Illumina platform with the paired-end method and a read length of 100 bp.

3.2.4.4. Bioinformatics analysis

The bioinformatics analysis was conducted by Dr. Panos Ioannidis.

Reads were first quality-trimmed using trimmomatic (Bolger, Lohse, and Usadel 2014), in order to remove sequencing adapters and low-quality bases. Trimmed reads were then mapped on the publicly available *B. oleae* reference genome ((Bayega et al. 2021); GCF_001188975.3) using the Hisat2 short read aligner (Kim et al. 2019), and read counts for each of the predicted genes in the official gene set were calculated with featureCounts (Liao, Smyth, and Shi 2014) at the gene level. EdgeR (Robinson et al. 2010) was used to find genes that were significantly (FDR <1e-03) differentially expressed with a fold change > 4 ($\log_2$FC > 2).

Gene ontology (GO) term functional enrichment analysis was performed using gProfiler (Raudvere et al. 2019). However, since *B. oleae* is not included in the gProfiler database, the GO term analysis was conducted using *D. melanogaster* as a proxy. More specifically, for each *B.*

oleae gene of interest, its most similar *D. melanogaster* gene was found using BLAST (Camacho et al. 2009). These *D. melanogaster* genes were subsequently searched for enriched functions in gProfiler. Determining whether *B. oleae* genes were full-length was done using BLAST. More specifically, each *B. oleae* predicted protein was searched against the Uniref50 protein database, with an e-value cut-off of 1e-25. Self-hits were first excluded and by using custom Perl scripts we searched for hits covering >90% of the *B. oleae* protein (query), as well as the database protein (subject). This approach ensured that the *B. oleae* protein had an end-to-end match with a protein from another species.

3.2.4.5. Quantitative Real-Time PCR (qRT-PCR) validation

RNA (0.5 mg) from each biological replicate was reverse transcribed using Oligo dT (MINOTECH) and Reverse Transcriptase (MINOTECH RT), according to manufacturer's instructions. Briefly, a two-step protocol was followed, including a 10-minute incubation step at 65°C and a 2-minute incubation step on ice, of the initial mixture (RNA, oligo-dT, dNTPs and DEPC H_20). Afterwards, a second mixture (buffer, 0.1M DTT, RNAse inhibitors and Reverse Transcriptase enzyme) was added in the initial one and the reactions were incubated at 42°C for one hour, followed by a 15-minute incubation step at 70°C. Finally, RNAse H was added in each reaction and they were incubated at 37°C for 20 minutes. The newly synthesized cDNAs were stored in -20°C until further use.

A qPCR assay was designed and developed for the quantification of expression of two CYP genes of interest, which were shown to be interestingly up-regulated in the MT-specific dataset; **CYP4P6** (accession no. LOC106621406) and **CYP6G28** (accession no. LOC106618199) and primers were designed based on their nucleotide sequences. The expression levels were normalized against two validated reference genes, beta-*actin* (accession no. GAKB01001968.1) and *S7* (40S ribosomal protein, accession no GAKB01005984.1) (Pavlidi et al. 2018). Gene-specific primers were designed using the NCBI Primer Blast Tool and OligoAnalyzer (Integrated DNA Technologies) and are detailed in **Supplementary Table ST3.3.3**. Concerning the two target genes, one primer of each set spanned two exons, in order to avoid DNA amplification (Kefi et al. 2018). Standard curves were constructed with Panagia and Hybrid cDNA, in 1, 1:5, 1:25 and 1:125 dilutions. All amplification efficiencies of designed primers were within acceptable range (90-120%) (Bustin et al. 2009).

Reactions were performed on a CFX ConnectTM Real-Time PCR Detection System (#1855201, Bio-Rad). Each qRT-PCR reaction consisted of 5 μl SYBR Green 2X (BioRad SsoAdvanced Universal SYBR Green Supermix 2X), 1 μl of undiluted cDNA, and 10 mM of each gene specific primer pair, in 10 μl final reaction volume. qPCR was performed with the following thermal conditions: 3 minutes at 95°C, 40 cycles of 10 seconds at 95°C and 45 seconds at 60°C, followed by a melting curve step (95°C for 10 seconds, 65°C for 5 seconds and 95°C for 0.5 seconds), in order to check the presence of a unique PCR product. Each qPCR experiment consisted of three independent biological replicates, with two technical replicates for each, and each run always included a non-template control. Amplification specificity of the products was displayed through the production and detection of a single peak in the melting-curve analysis. Results of the PCR

were analyzed by the Bio-Rad CFX ManagerTM 3.1 software. Fold change was calculated using three biological replicates, three technical replicates and normalized with two housekeeping genes, actin and S7, using the 2 -ΔΔCT method (Pfaffl 2001). The statistical significance of the gene expression values was calculated using t-test (R statistical language); A value with $p<0.001$ was considered to be statistically significant.

3.2.4.6. dsRNA design and synthesis for RNA interference (RNAi) experiment

Several studies have demonstrated the feasibility of RNAi as a tool for the functional characterization of potential gene targets, in order to better understand their functions (Jain et al. 2020). In brief, a short double-stranded RNA molecule (dsRNA) is administrated in the insect elicits a response in the cell mediated by the enzyme Dicer, which cleaves the dsRNA in fragments of 21-23 base pairs (siRNA). siRNAs are loaded onto a multiprotein RNA Induced Silencing Complex (RISC) and while the one strand (the passenger) is discarded and degraded, the guide strand remains within RISC as template, in the silencing reaction. Afterwards, the guide strand assembles into a functional siRNA-RISC complex, which contains the siRNA bound to the Ago protein. The target mRNAs are recognized by base pairing and are bound by the siRNA-RISC complex and subsequently the mRNA degradation is induced, resulting in a down-regulation of the gene target. The target mRNA is then dissociated from the siRNA, and the siRNA-RISC complex is released to process further mRNA targets (**Figure 3.2.2**).

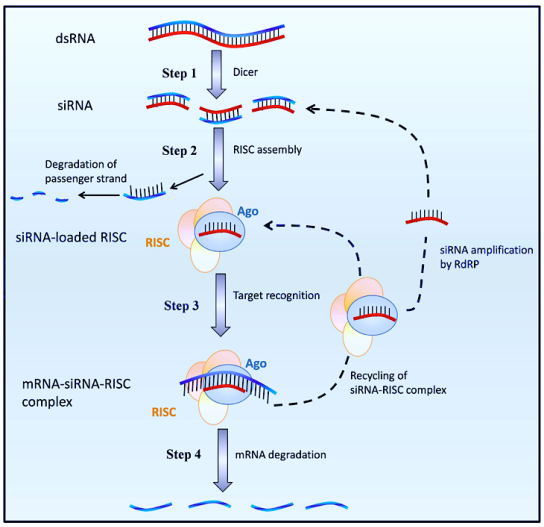


Figure 3.2.2. Schematic representation of RNA interference (RNAi) technology: Since RNAi does not depend on a difficult germ-line transformation technology, it soon became a widely applied gene silencing method. The most common ways to deliver the dsRNAs in the cells are by micro-injections and feeding assays (Kampouraki et al. 2018b; Li, Zhang, and Zhang 2011). Dicer cleaves the administrated dsRNA into short fragments (siRNAs) and antisense strands of siRNA bind to the RISC complex, transport it to the target mRNA and degrade it, hindering the translation of it. Schematic from (Cuccato et al. 2011).

We used established protocols for dsRNA injection (Scharf, Zhou, and Schwinghammer 2008; Kampouraki et al. 2018a) and monitored the mRNA levels following the dsRNA administration, in an attempt to associate the gene silencing with a phenotypic alteration (increased mortality) of a resistant field population of olive flies, upon insecticide application.

RNA (0.495 mg) from a Panagia resistant RNA replicate was reverse transcribed using Oligo dT (EnzyQuest) and Reverse Transcriptase enzyme (EnzyQuest RT), according to manufacturer's instructions as it was previously described in a two-step protocol (**3.2.4.5 Quantitative Real-Time**

PCR (qRT-PCR) validation). 1μl of the undiluted cDNA was used as template in a PCR reaction per target gene, with Phusion polymerase (Thermo Scientific™, F530S), with a 67°C annealing step for 30 seconds and a one minute at 72°C extension step, for 30 cycles (primers in **Supplementary Table ST3.3.2** and protocol in **Supplementary Table ST3.3.5**). Full gene PCR products (approx. 1,500bp) were purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, 740609.250) and the concentration and the purity were measured in Nanodrop.

1 μl of per purified PCR product (4P6: 65.4 ng/μl, 6G28: 48.9 ng/μl) was used as template in a PCR reaction with Kapa polymerase (Kapa Biosystems, KK1014) and the three T7 long primer sets (one set per gene; LacZ control gene is also included) with a 50°C annealing step for 30 seconds and an extension step at 72°C for 42 seconds, for 35 cycles. Both dsRNAs (4P6 and 6G28 genes) were designed to amplify a DNA fragment of 347 and 483 bp respectively, at the left side of each gene, close to the 5' end (primers in **Supplementary Table ST3.2.2**, targets in **Supplementary Table ST3.2.3** and protocol in **Supplementary Table ST3.2.5**). PCR products were also purified and measured in Nanodrop.

100 ng from each purified DNA template was used in a T7 *In vitro* Transcription reaction (MEGAscript™ T7 Transcription Kit, Invitrogen, AM1334), according to the manufacturer's instructions (protocol in **Supplementary Table ST3.2.5**). The reactions were incubated overnight (~16 hours) at 37°C. The newly prepared dsRNAs were purified with phenol-chloroform-isoamyl alcohol (25:24:1 mixture), 3M sodium acetate and chloroform, followed by a precipitation step with 100% ice cold ethanol and a final washing step with 70% ethanol. The products were eluted in 21 μl DEPC H_2O. The 1:10 diluted dsRNAs were measured in Nanodrop and were run in a 1.2% agarose gel, in order to check their purity and integrity before injections. Undiluted dsRNAs were stored in -20°C until use.

3.2.4.7. RNAi nano-injections

Each dsRNA was administered in 3-5 day old female and male adult flies, via nano-injections (Huang et al. 2015). Briefly, the insects were anesthetized on ice for approximately 60 seconds in groups and placed on small glass platforms. Each individual, was injected with approximately 1 μg of dsRNA per gene (69 nl insert per injection, triple injection was performed using a 5μg/μl dsRNA stock), between the first and the second abdominal parts. The injections were performed with a Nanoject II Auto-Nanoliter Injector (Drummond Scientific, Broomall, PA, USA). Injected flies with lacZ dsRNA were used as control reference. The injected adults were kept in plastic cages at 25°C and 16:8 hours light: dark photoperiod, under standard rearing conditions (30% sugar liquid diet, solid diet and water) and mortality was checked 24 hours post-injection (p.i.).

3.2.4.8. Laboratory bioassays

The ds-injected adult flies were subjected to toxicity bioassays 72 hours p.i., with the type II pyrethroid insecticide alpha-cypermethrin (Sigma-Aldrich, 36128-100MG), using the topical application method (Kampouraki et al. 2018b). Insecticide solutions in acetone were applied on

the dorsal thorax, using a 10 μl Hamilton syringe (900 Series Microliter Syringes, HAMILTON). Insects were anaesthetized with a short exposure to carbon dioxide prior to insecticide application.

Different concentrations of alpha-cypermethrin were used (range 3-90 ppm) for the dose response bioassay; 10-15 adult insects were tested per dose and 5 doses were applied (including the control with pure acetone). For the single discrimination dose bioassays, insects were exposed to an insecticide concentration between LC20 (30ppm) and LC30 (40ppm) (according to the dose response bioassay data). Approximately equal numbers of male and female flies were tested in each dose. The insecticide treated adults were kept in small plastic cages at 25°C and 16:8 hours light:dark photoperiod, under standard rearing conditions (30% sugar liquid diet and water) and the mortality was calculated 48 hours post-exposure.

The results of the bioassays were estimated with probit analysis (Russell, Robertson, and Savin 1977) using PoloPC (LeOra Software, Berkeley, CA, USA); determination of the lethal concentration of the insecticide which is required to kill the 50% of the population (LC50) and establish 95% confidence intervals (CL). Resistance factors (RF) were estimated using LC50 levels as RF = RR = LC50 of the field sample/ LC50 of the susceptible strain.

3.2.5. Results

The purpose of this study, taking advantage of the recent evolution in RNA sequencing technologies and analysis methods, was to investigate the expression patterns of key gene families associated with insecticide resistance, with a particular focus on the P450 detox genes, in MTs, where part of the detoxification of insecticides and other harmful substances usually is noted to take place. For this reason, in the study we included olive fly samples from an a-cypermethrin resistant field population and a susceptible-laboratory-maintained strain, in order to identify genes whose tissue-specific enrichment might be linked to the resistant phenotype.

3.2.5.1. Differentially expressed (DE) genes

In this study we have sequenced the transcriptome of the malpighian tubules of olive flies that are resistant to α-cypermethrin, a pyrethroid insecticide, compared to the susceptible laboratory-reared population. We used the Illumina platform and generated a total of 378 million reads from a resistant (Panagia) and a susceptible lab population (Hybrid). A principal components analysis (PCA) using the expression levels of all expressed genes showed that the replicates of the two populations clustered separately from each other (**Figure 3.2.3**), indicating that their pair-wise comparisons can lead to valid results.

A total of 227 genes were differentially transcribed (FDR <0.001, $\log_2$FC >2), of which 123 were up-regulated in the resistant Panagia population, whereas another 103 were down-regulated (**Figure 3.2.4**). The most up-regulated genes include several apolipoprotein D genes, one dipeptidase, one oxidoreductase, an apnoia-like gene, a tyramine/octopamine receptor gene, and

several uncharacterized proteins. In contrast, the down-regulated genes, on the other hand, include genes such as two trypsins, one maltase, and a few uncharacterized proteins.

Furthermore, several over-transcribed detoxification genes were identified. More specifically, among the 123 genes that were found up-regulated, six cytochrome P450s (CYPs) from the CYP3 and CYP4 clans, one glutathione S-transferase (GST) and three UDP-glycosyltransferases (UGTs) were included. These gene families are frequently involved in metabolic resistance mechanisms, which are related to the detoxification of xenobiotic compounds and thus, it was expected to be up-regulated in the malpighian tubules of the resistant population. Among them, two P450s were shown to be highly over-expressed in resistant MTs, and were further functionally investigated; apart from their high expression levels' validation with qRT-PCR analysis, the silencing of one of them, CYP4P6, with the RNAi method, even though this CYP gene does not belong to the detox P450 clan 3, showed a promising phenotype. Increased mortality rates were reported, compared to the control, upon α-cypermethrin application.

3.2.5.1.1. Cytochrome P450 genes (CYPs)

As many as six CYPs were up-regulated in the resistant to α-cypermethrin Panagia population (**Supplementary Table ST3.3.2**). CYP4P6 (similar to CYP4P1 in *D. melanogaster*) is the most up-regulated CYP ($log_2FC = 6.55$) in this tissue specific dataset and it is also a full-length gene. This CYP is very well and very highly transcribed in the resistant strain, while it is virtually absent from the susceptible one. Furthermore, CYP6G28, a homolog of the *D. melanogaster* CYP6G1, is also significantly up-regulated in the resistant strain. However, its expression is not as good as that of CYP4P6, since certain coding sequences appear as not transcribed. Additionally, the automatically predicted CYP6G28 gene is also a fusion of two CYP6G1-like genes and manual curation was necessary in order to fix these gene models.

qPCR was used to validate the up-regulation of these two genes in the resistant strain. The levels of CYP4P6 and CYP6G28 were confirmed to be significantly ($p<0.001$) up-regulated in the resistant strain, compared to the susceptible one (**Figure 3.2.5).** More specifically, the up-regulation of CYP4P6 was estimated at 17.99 folds-up and for CYP6G28 at 1.57 folds-up. Generally, these findings show that especially CYP4P6 is indeed up-regulated in Panagia, as determined by the RNA sequencing data, although the RNAseq estimate was much higher (>64-fold). Their potential role in the olive fly α-cypermethrin resistance was further investigated with functional validation experiments, using RNAi technology and toxicity bioassay tests.

The remaining four up-regulated CYPs include CYP6G6 and CYP6G27 (both similar to CYP6G2 from *D. melanogaster*), as well as CYP6A70 and another CYP fragment (LOC106624818) (both similar to CYP6A13 from *D. melanogaster*); CYP6G6 is an interesting case since it has been previously associated to resistance against pyrethroid insecticides (Pavlidi et al. 2018). Closer examination of the genomic locus containing CYP6G6 showed that there are, in fact, three copies of this gene (6G6, 6G24, and 6G27). CYP6G6 and CYP6G27 are significantly up-regulated in the pyrethroid-resistant MTs studied here. These two CYPs are 91% similar to each other on the amino acid level, while the third CYP (CYP6G24) is much more divergent (~55% similar). These

three CYP6G2-like genes are fused in the official NCBI gene set into a single chimeric gene, with CYP6G6 and CYP6G27 being only 2.3Kbp apart and CYP6G24 being much further away, at ~17Kbp. Only CYP6G6 appears to be correctly transcribed in the resistant MTs and could, thus, be implicated in resistance to α-cypermethrin.

Finally, CYP6A70 was correctly predicted and also its transcription followed the predicted gene structure. The CYP6A70 fragment (LOC106624818) is missing the majority of the gene sequence and closer investigation of the corresponding genomic locus indicates the existence of genome miss-assemblies.

3.2.5.1.2. GST- and UGT- genes

As many as four phase-II detoxification enzymes were significantly up-regulated in the resistant population; one glutathione S-transferase (GST) and three UDP-glycosyl-transferases (UGTs) (**Supplementary Table ST3.2.2**). The GST gene (LOC106615161) is mostly similar to GST 1-1 from various tephritid species and having a length of 215 amino acids it appears to be full-length. Moreover, its transcription seems to be good, since it is uniform and follows the gene structure. It should also be noted that this gene is transcribed in both the resistant and the susceptible populations, albeit at lower levels in the latter. As for the three UGTs, two of them appear to be full-length (LOC118679823, and LOC106623379), while the third one (LOC118682404) is fragmented, apparently having a N-term truncation. The two full-length UGTs, however, are well-transcribed and their up-regulation in the resistant strain is high, at almost 210-fold for LOC118679823 and 32-fold for LOC106623379 (**Supplementary Table ST3.2.2**).

3.2.5.1.3. Non-detox genes

Genes with similarity to apolipoprotein D (apoD) were the most up-regulated genes in Panagia resistant population, all of them having a $\log_2$FC >5. There was a total of 12 apoD-like genes in the predicted gene set of the olive fly, ten of which are significantly up-regulated (data not shown). Based on BLAST searches, most of these genes are fragments with similarity to the *lazarillo* genes of *D. melanogaster*. The transcription for most of them is good, but only two appear to be nearly full-length: LOC106619033 and LOC118681230. Six of these genes are found in two genomic clusters, one containing four genes (LOC118681230, LOC118681227, LOC118681226, LOC118681228) and another one containing two genes (LOC106615586 and LOC118680168). LOC106619033 is the most highly expressed of all apoD-like genes and it is located at the end of genomic scaffold.

3.2.5.2. Functional characterization of highlighted MT- specific genes

Two P450 genes that were highlighted in the MT-specific RNA sequencing data were chosen to be further investigated, namely CYP4P6 and CYP6G28. After their qPCR validation, we further proceeded with an attempt to functionally characterize them. Three dsRNAs were designed and constructed (including dsRNA targeting the LacZ gene as a control reference), targeting the 5'

end of each gene, in order to silence it, with the use of the RNAi technology; thereafter the injected flies were subjected to α-cypermethrin bioassays, in order to check any possible implication of these genes in the detoxification of this pyrethroid compound.

The olive flies that were used in this study derived from infested olives collected from Evaggelismos region (Heraklio, Crete). The resistance levels of this population to α-cypermethrin were checked with a dose-response contact bioassay (**Supplementary Table ST3.2.6**) and the insecticide resistance level of the population was classified according to the criteria reported in (Torres-Vila et al. 2002); susceptibility (RF = 1), low resistance (RF = 2–10), moderate resistance (RF = 11–30), high resistance (RF = 31–100), and very high resistance (RF = > 100); Evaggelismos population exhibited very high levels of resistance compared to the laboratory-reared susceptible strain (with a RR= 146.23, 95% CI, 94.77 - 225.40) and subsequently it was used in the downstream experiments.

Three to five days old female and male flies were nano-injected with dsRNA that targeted each gene (CYP4P6: 115 flies, CYP626: 112 flies, LacZ: 100 flies). Mortality rates were calculated 24 hours p.i.; 15% mortality was observed in dsCYP4P6-injected group, 49% in the dsCYP628 and 13% in the dsLacZ injections. (The high mortality in dsCYP6G28 injected flies was probably due to methodology weaknesses). Furthermore, 24 and 48 hours p.i., 30 injected flies with each injected dsRNA (targeting each gene) were anesthetized, the malpighian tubules were dissected out, snap frozen in liquid N_2 and stored at -80°C until RNA extraction and cDNA synthesis. Three biological replicates, consisted of MTs dissected from ten injected flies each, were prepared for each one of the three genes. Protocols were previously described in depth (section **3.2.4.5. Quantitative Real-Time PCR (qRT-PCR) validation**). The silencing efficiency of the dsRNAs was checked at two timepoints for each target gene; 24 hours p.i. the knock-down in dsCYP4P6 injections resulted in a 30% (0.7) down-regulation of the target gene (CYP4P6), against both housekeeping genes. As it concerns the CYP6G28 knock-down, the dsRNA injections resulted in a 5% (0.95) down-regulation of the target gene, against the two housekeeping genes (**Figure 3.2.6.A**). 48 hours p.i. there was very limited silencing effect for both genes (data not shown). According to these data, it was decided to proceed only with CYP4P6 gene, since no silencing effect was observed in CYP6G28 gene with the currently available dsRNAs.

A contact bioassay with an LC25 single dose (35ppm α-cypermethrin) 72 hours p.i. was conducted, in order to find out if the silencing of the CYP4P6 gene confers increased levels of mortality, upon exposure to the pyrethroid insecticide α-cypermethrin, compared to the control (which was injected with dsLacZ). Final mortality rates were calculated 72 hours post bioassay and 21% mortality was reported in dsCYP4P6-injected group, while 4% in the dsLacZ one (**Figure 3.2.6.B**), suggesting a possible role of CYP4P6 in detoxification of α-cypermethrin. The whole experimental procedure (RNAi injections, RT-qPCR and bioassays) was repeated four times in total, however, in the current PhD thesis, only one of them is presented; higher than the accepted mortality rates were reported in the control samples in the other repetitions, and due to the season-dependent availability of this pest, the result could not be tested any further. Additional repetitions of the experiment should be conducted when adult field population is available, before a statistically significant result is indicated.

3.2.6. Discussion - Conclusions - Future approaches

In the present study we sequenced and analyzed the transcriptomes of olive fly MTs, in an attempt to investigate a possible role of this tissue in the detoxification of xenobiotic compounds. The MTs are proposed to be one of the organs where detoxification partially takes place in the insect body (Beyenbach, Skaer, and Dow 2010; Dow and Davies 2006), with the main detoxification tissues being the fat body and the midgut (Kliot and Ghanim 2012).

To dissect the insecticide metabolic pathway related to pyrethroid resistance in olive flies, further information on the sites of expression is required. This MT-specific dataset, revealed many potential gene candidates related to insecticide resistance, while few of them had also been detected in the past whole organism comparisons (Pavlidi et al. 2018). Overall, quite a number of detoxification genes were up-regulated in the resistant Panagia population (MT dataset) and upon their functional characterization, they can potently be linked to the observed resistant phenotype of this population. The most promising genes, based on their transcription pattern and quality, consist of two P450s; CYP4P6 and CYP6G28. Additionally, a UGT5-like gene (LOC118679823) was also very highly up-regulated in the Panagia population. Besides the well-known detoxification enzymes, virtually all apoD genes were up-regulated in the resistant flies. Even though this gene is implicated in stress response (Sanchez et al. 2006; Walker et al. 2006) and lipid transport in neurons (Yin et al. 2021) in *Drosophila*, it is not yet known if and how its overall function can be associated to insecticide resistance.

Previous studies have proved the implication of these two highlighted (from the current MT-specific datasets) P450 genes, CYP4P6 and CYP6G28, in insecticide resistance mechanisms of *Drosophila*. CYP6G28 (the homolog of CYP6G1 in *Drosophila*, accession no. LOC106618199) has been identified as a major factor in DDT resistance in *D. melanogaster* populations and since 2002 it has been spread globally (Le Goff and Hilliou 2017). However, it has been recently associated with resistance to the neonicotinoid imidacloprid (Fusetto et al. 2017). Furthermore, CYP4P6 (the homolog of CYP4P1 in *Drosophila*, accession no. LOC106621406) has been found to be involved similarly in a DDT resistant fly strain (Chahine and O'Donnell 2011; Seong, Coates, and Pittendrigh 2019). Using RT-qPCR analysis we validated the expression levels for these two detoxification genes (**Figure 3.2.5**). CYP4P6 was approximately 18 times more highly expressed in the resistant Panagia strain, which is in consistency to the RNAseq data, although the RNAseq estimate was much higher (>64-fold). However, the over-expression of CYP6G28 was not quite confirmed by qPCR (only 1.8 folds up). Furthermore, we attempted to further characterize the CYP4P6 gene by knocking it down, using the RNAi technology; interestingly, a 30% down-regulation of the CYP4P6 gene (compared to the control levels of the gene in non-injected individuals) conferred a 21% mortality to the olive fly group, upon α-cypermethrin application, compared to the control (4%) (**Figure 3.2.6**). However, as previously mentioned, despite the promising phenotype conferred upon silencing of the CYP4P6 gene, foreshadowing a possible implication of it in pyrethroid resistance, the season-dependent limitation factor of this pest species did not allow the export of a complete and statistically significant conclusion, within the framework of this thesis.

A general enrichment in the transcription of several genes from the five major detoxification gene families was reported in the MT-specific RNA dataset of resistant olive flies, providing new insights for the detoxification of pyrethroid insecticides in this species. Following the encouraging preliminary results reported in this study (concerning the role of the CYP4P6 gene), which require further validation steps, the interesting gene cases can be further investigated with *in vitro* insecticide metabolism assays; after the expression in proper heterologous systems (i.e. *E. coli*, baculovirus, *D. melanogaster*), the recombinant enzymes could be further tested for their ability to metabolize α-cypermethrin (and other pyrethroid compounds) *in vitro*, using chromatographic and/or mass spectrometric techniques (e.g. HPLC/MS).

3.2.7. Figures

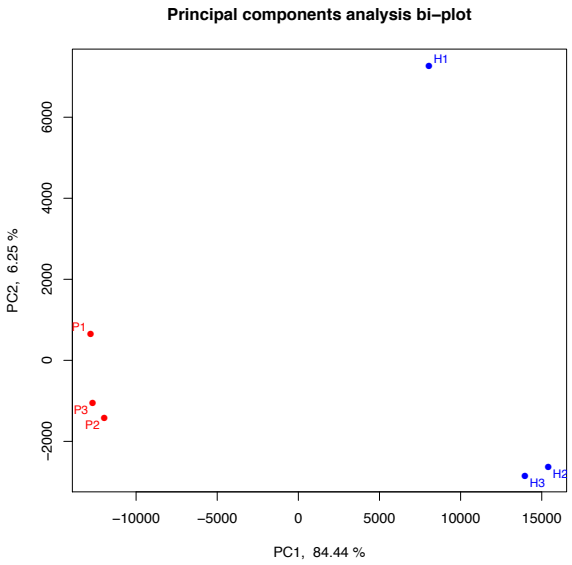


Figure 3.2.3

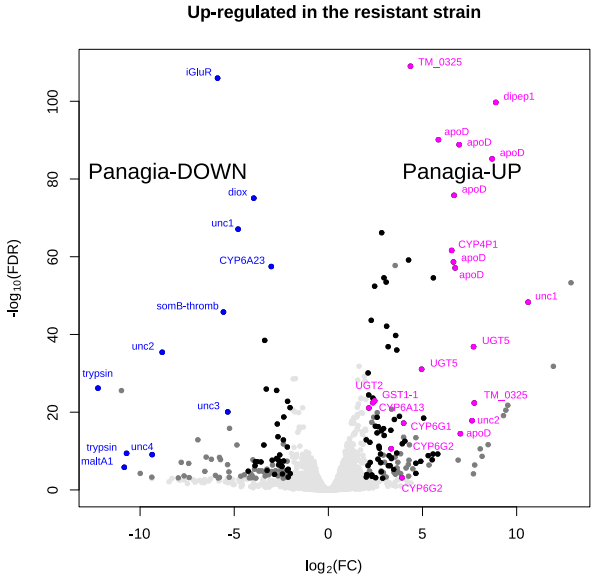


Figure 3.2.4

Figure 3.2.3. PCA plot based on the expression levels of all genes. The replicates of the resistant Panagia population (P1, P2, P3; in red) are clearly separated from the ones belonging to the susceptible Hybrid (laboratory-reared) strain (H1, H2, H3; in blue).

Figure 3.2.4. Summary of the differentially expressed genes. Each dot represents one gene; dark gray-statistically significant (FDR <0.001, logFC >2) differential expression (DE); black: DE genes that are also full-length (based on BLAST searches) and have expression levels above the background noise; light gray: non-DE genes. Some of the most important genes are highlighted in magenta (up-regulated in the resistant strain), or blue (down-regulated in the resistant strain). *Unc*: uncharacterized proteins; *UGT*: UDP-glucuronosyl-transferase; *apoD*: apolipoprotein D; *TM_0325*-uncharacterized oxidoreductase TM_0325-like; *dipep1*:dipeptidase 1; *CYP*: cytochrome P450; *GST*: glutathione S-transferase; *iGluR*: ionotropic glutamate receptor, kainate 2; *diox*: gamma-butyrobetaine dioxygenase; *somB-thromb*: somatomedin-B and thrombospondin type-1 domain-containing protein; *trypsin1*: trypsin theta-like; *trypsin2*: trypsin I-P1/hyaluronan-binding protein 2; *maltA1*: maltase A1.

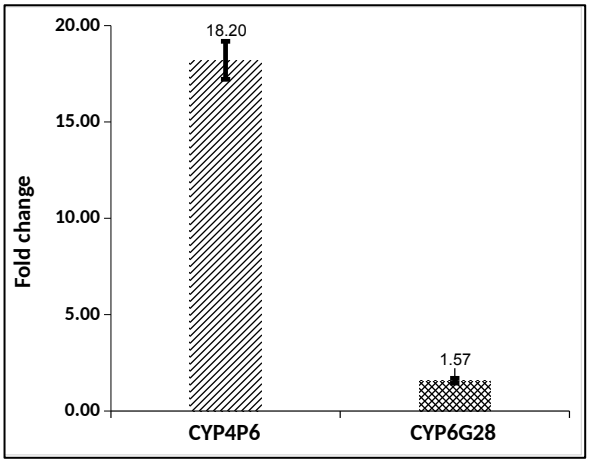


Figure 3.2.5. Up-regulation of CYP4P6 (left) and CYP6G28 (right) in a resistant population (Panagia) against a susceptible (Hybrid) *B. oleae* strain, using quantitative RT-PCR. CYP4P6 (the homolog of Drosophila CYP4P1, accession no. LOC106621406) shows a ~18-fold increased expression levels in the resistant population, which is in consistency to the RNAseq data. Over-expression of CYP6G28 (the homolog of Drosophila CYP6G1, accession no. LOC106618199) is not quite confirmed by the qPCR analysis. Each value is the average of three biological replicates. Error bars represent the accuracy of the mean (standard errors of the calculated mean, based on three biological replicates). The expression values were calculated statistically significant with $p<0.001$ using t-test (R statistical language).

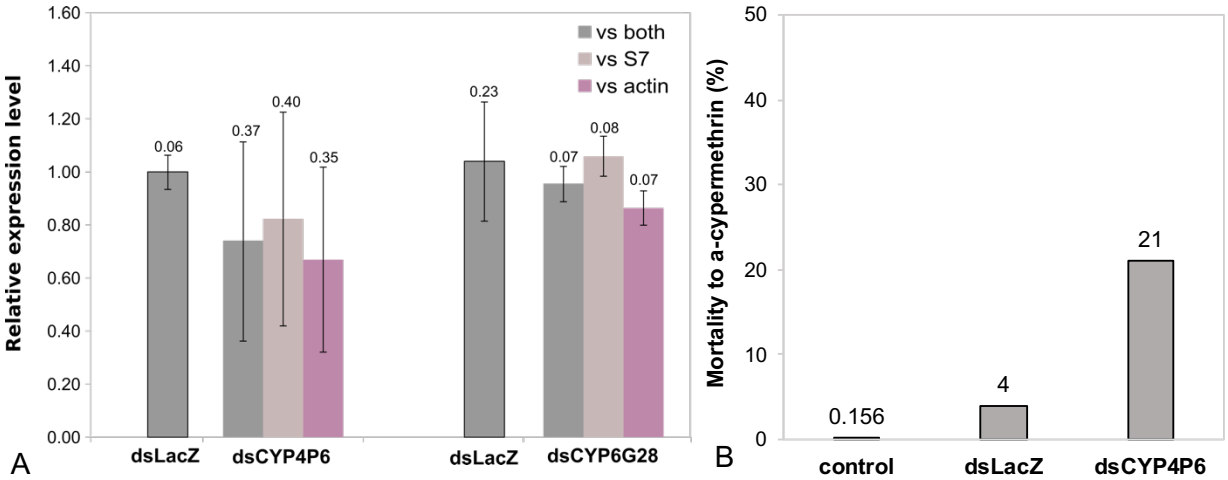
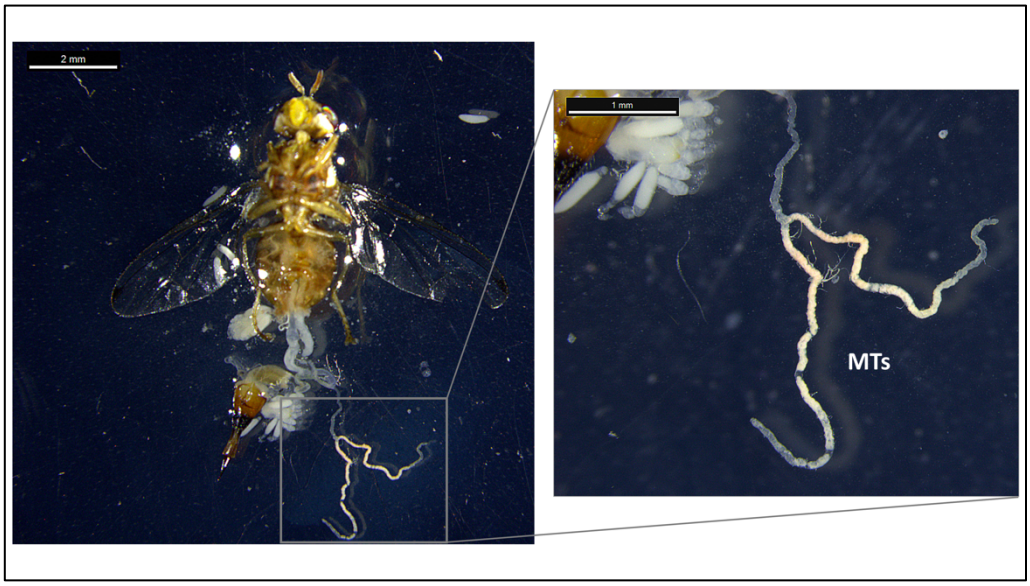


Figure 3.2.6. Impact of CYP4P6 silencing on exposure to α-cypermethrin (A) Silencing effect of CYP4P6 (left) and CYP6G28 (right) in Evaggelismos resistant olive fly population, after dsRNA injections, estimated with RT-qPCR. The silencing effect was estimated 24 hours p.i., using the Ct values of either the control genes (S7 and actin), or the average Ct values of both. Overall, the silencing effect is stronger on CYP4P6 (0.7 down-regulation compared to 1.0 of the dsLacZ control (mean values occur from the normalization against two housekeeping genes); the values are transformed in percentages %, i.e. 0.7 equals 30% downregulation of the gene, as mentioned in the results section), compared to CYP6G28 (0.95 equals 5% downregulation of the gene). Each value represents the average of three biological replicates. Error bars represent standard errors and the values are marked in the graph. (B) Mortality of Evaggelismos adults upon α-cypermethrin exposure (35ppm). Control, non-injected adults; dsLacZ, adults injected with dsLacZ; dsCYP4P6, adults injected with dsCYP4P6.

3.2.8. Supplementary Material



Supplementary Figure SF3.2.1. Malpighian Tubules (MT) dissected out of a female adult olive fly. The MTs of the olive fly are located at the junction of the midgut and the hindgut and consist of two cylindrical tubes, which are further divided each one to two "branches". Here the MTs are dissected in PBS 1X, under the stereoscope.

Supplementary Table ST3.2.1. Dose response of *Bactrocera oleae* field population (Panagia) and two laboratory susceptible strains (Lab & HYB) to alpha-cypermethrin, upon topical application bioassays.

Strain	N	LC50	95% CI	Slope	χ^2 (df)	RR (95% CI) vs Lab	RR (95% CI) vs Hybrid
LAB	137	0.417	0.24 - 0.66	2.206±0.324	11.49 (8)		
HYBRID	119	1.31	0.75-1.74	5.26 ± 1.27	13.3 (10)		
PANAGIA 2019	139	33.61	26.99 - 57.27	2.395±0.458	5.35 (5)	80.60 (50.18 - 129.32)	25.65 (16.85 - 39.04)

Bioassays and data analysis were conducted by Dr. Natassa Kampouraki (2019); N: total number of individuals tested (including control), CI: Confidence Interval, LC: lethal concentration, RR: resistant ratio= LC50 of resistant population /LC50 of susceptible population. A χ^2 test was used to assess how well the individual LC50 values observed in the bioassays were in consistency with the calculated linear regression lines.

Supplementary Table ST3.2.2: List of over-expressed detoxification enzymes in the malpighian tubules of the Panagia resistant population.

Geneid	log_2FC	FDR	Annotation	Completeness
LOC106621406	6.55	2.4E-62	CYP4P6-like	full-length
LOC106618199	3.99	6.6E-18	CYP6G28-like	full-length
LOC106618206-00001	3.91	7.4E-04	CYP6G2-like	full-length
LOC106618206-00002	3.33	2.4E-11	CYP6G2-like (contig02103)	full-length
LOC106624818	2.59	1.3E-20	CYP6A13-like	fragment
LOC106620249	2.15	8.3E-22	CYP6A13-like	full-length
LOC106615161	2.36	3.57E-23	GST 1-1	full-length
LOC118679823	7.71	1.38E-37	UGT5-like	full-length
LOC106623379	4.95	8.96E-32	UGT5-like	full-length
LOC118682404	2.46	1.14E-23	UGT2-like	full-length

Supplementary Table ST3.2.3. Primers that were used in the study.

qPCR primers (5' > 3')		**bp**
Bo_qCYP_4P6_F	AATGCTGACTCCTGCCTTCC	171
Bo_qCYP_4P6_R	CCCAATGCGGTCTCACAGAT	
Bo_qCYP_6G28_F	GTAGTGGACGAAGTGTTGCG	183
Bo_qCYP_6G28_R	TGGGTTGGACCAATACTCAGGA	
Bo_qactin_F	CGGTATCCACGAAACCACAT	159
Bo_qactin_R	ATTGTTGATGGAGCCAAAGC	
Bo_qs7_F	TTCGGTAGCAAGAAGGCTGT	
Bo_qs7_R	GGTAGGTTTGGGCAGGATTT	
Primers used for the dsRNA synthesis.		
Bo_CYP_4P6_F	ATGGTGTTCGGGACATTAATTA	1,545
Bo_CYP_4P6_R	CTATAACTTTAAACTCCTTTTTTCCA	
Bo_CYP_6G28_F	ATGCTGTCGGTAGATACTACTTG	1,560
Bo_CYP_6G28_R	TCACTGCCTCGCATTTCG	
T7_4P6_L_F	TAATACGACTCACTATAGGGAGCGGGTACGAACAGTTGAC	347
T7_4P6_L_R	TAATACGACTCACTATAGGGGGCAGGAGTCAGCATTTTTC	
T7_6G28_L_F	TAATACGACTCACTATAGGGGGCGTTGGTCTACATTTGGT	483
T7_6G28_L_R	TAATACGACTCACTATAGGGACGAAAGCGTCGTCAGTCTT	
T7_lacZ_F	TAATACGACTCACTATAGGGAGAAGGCAGGCTAATGACTATGGAG	
T7_lacZ_R	TAATACGACTCACTATAGGGAGAGAAGCCGGTATGTCACTCAGT	

Supplementary Table ST3.2.4. CYP4P6 and CYP6G28 gene sequences.

CYP4P6

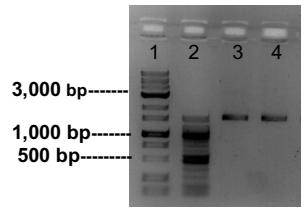
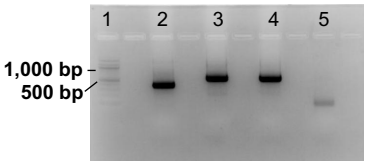
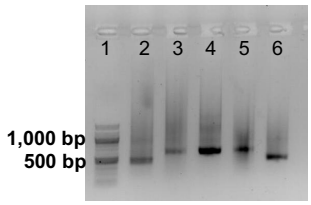
ATGGTGTTCGGGACATTAATTATTGGTGCGATCATAATCGCTTGCCTATACAAACTAAATAAGGATTATGTGGTGCTGACATT
ATTTACTAAGCGGGTACGAACAGTTGACTGCACTCCCTTGGAGAATTCAGTGGCGCTCCCCAAAGGACTCACAATATTTGG
CAATGCCTTCGATTTTGCATTAGCCTCTGAAGGCATGTTCGACTTCTTGCGAAAACTTGCTGCGGAAATGAAGCGCAGCTAT
CTACAATATGGACTATTCAGATCTTTCTACAACATAATCACCGCTGAGGATGCTGAGCTAGTGATGAATGATCAGAAAAATCT
GATTCACAAGGGTGTTATTTATAATTTTTTCGTGCCGTTCTTGAATAGAGGACTGCTGACGTCATCAGGCAAAAAATGGTACT
CTCGTAGAAAAATGCTGACTCCTGCCTTCCACTTTAAAATACTTACACAATTTGAAGAGATTTTTAAAGAAGAGAGCAAAAAA
TTTGTGGAGAGTTTGGAAGCTAGCGATTTAAGTTCTGTGACTTTAAACGAAATTATACCAAAATTTACACTCAACGCCATCTG
TGAGACCGCATTGGGTGTCAAATTGGATGAGCAAATGAATGCTGATCAATATCGTGGCAGCTTCAAAATGATCGAGGAAGT
ATTCTTGATGCGATGCCATAATCCGTTTTATATTATAGATTCCCTATATAAAGTATTTTTAGCACCTAAAATTAAGAAACACATT
TCAATTGTGCATTACTTCTCCAGTGAGATAATTAACAAGCGACGTCAAGCATTCGCGGAAGAACTAGAGCAGAGTGAAGACA
AGGAGGATAATCAAAGCTTCTATACGAAAAAACGCTACGCTATGTTGGACACACTCTTACGCGCCGAACGCGATGGACTCA
TTGATCATGTGGGTATATGTGAGGAAGTGAACACATTTATGTTTGAAGGCTACGACACCACATCGATGGCGCTCTTATTTTC
ACTTATGAACCTTTCACTATATCCAGAAATGCAGGAGCGTTGCTATCAAGAAATACTTGATTGCGTTGAAGATGACTTGAGC
CAATTGAATATTCAGCAGCTGTCCAAATTGCAGTATCTAGAGTGTTTCATTAAGGAGACGCTTCGTCTATACCCCTCAGTGC
CAGTTATAGCGCGTGAGGCTGCGAATGAAACTCGTCTCGCCAATAACCTAATATTGCCGAAAGGGGCTCAAGTAACCATAC
ATATAATAGATATACATCGAAGTGCTAAATACTATGAAAATCCGAATAAATTTGATCCGGAACGTTTTACAGCGGAAGCGTCA
GCCGGACGACATCCTTACGCGTACGTACCATTCAGCGCAGGACAAAGAAATTGTATCGGACAAAAATTTGCTATGCTGGAA
CTTAAGACGATTTTAGTTAACATCATAAAGACCTTTAAAATTTTAACGCTAATGAAGGATCAAGATATAAAGTTGGAATTCGG
CATGATTATAAGGACACCAAATATCATTAAAGTTAAATTGGAAAAAAGGAGTTTAAAGTTATAG

CYP6G28

TGCTGTCGGTAGATACTACTTGCCTCTTGGCGACCCTTTTGGCGTTGGTCTACATTTGGTGCCGCTATACATATGGCTACTG
GAAACGTAATAAGGTGCCCTATATGACGCCATTGCCACCGATTGGCAATATGGATGTTTTGTTTACAATGAGGAATAGTTTC
TATCTATATCTATCTGACGTTTATAAGGATGCGAAAATGTCAAAGGCCGCGGCAGTTGGCATTTATATACTCACTAAGCCGG
CATTAGTGTTACGTGAGCCGGAACTTATTAAATCTGTGCTAATCAAGGAGTTTCCGAAGTTCAGCAATCGTTCAGCTGGCTG
TGATCCACACGATGATGCTTTGGGCTCAAATAATATGTTTTTCATACGCAATCCACAATGGAGAGACTTGAGATCGAAAATA
TCGCCGACATTCACAACGGGAAAGATCAAACAAATGTATCCACTAATGACTGACATCAGCATAGAGTTAGAAACCCATTTGA
ATTCGTATGCAAAGACTGACGACGCTTTCGTAACCGAAGTTAAAGAGGTTTGTGCGCTCTTTACCACCGATATGATTGCTAC
CATCGCTTTTGGTGTGAAAGCTAATAGTCTTGTAAATCCGAATGGCGAATTCCGCACACAAGGACGAAAATTGCTAACCTTT
ACATTCGGTCGTGCCTTTAACTTCTTCGTCGCTTTTTTCTTTCCGAATTGGGTTACTACATTGCGCATTAAACTTTTTACGCC
GGAGTTCAGCTCATTTTTACGTGGCACGATCGATCATGTGATGGCGTTAAGAGAGAAGAGCAAAGCGAAGCGTAACGATCT
TATCGATGTGTTGGTAAGCCTGAAAGAAGAGGCTATTGCGAAAGGCGAGTACAATGCTCAGTTACAAGATGTGTTGACAGC
ACAAGCTGCGGTATTTCTATCAGCTGGTTTTGAGACGTCATCTGCGACGATGACTTTTGCGCTATACGAGCTATCGAAACGA
CCCGATTTGCAAGAACGTCTGCGCAATGAGATTTGTGAAGCTCTCCTAGCTGAGCAGGGTACAATTTCATATGAGACTATCA
ATAATCTACAATATCTGGGTATGGTAGTGGACGAAGTGTTGCGTTTATATCCAGTACTACCTTATCTCGATCGCGAACATCT
GCCGAAGAAAGGAGAAAATCAATTCGATCTTAAACCGTATTACGATTACACAGTGCCAATCGGTATGCCTATTTATATACCA
ATCTTCGGCATACAACGTGATCCTGAGTATTGGTCCAACCCAAACACTTTCAATCCTGAACGCTTCAGCGCCAAGAATAAGA
AAACTCATAAGCCTATGTCCTATTTTCCGTTCGGCAATGGACCACGTAACTGTATTGGTAGTCGGATCGGTTTGCTGCAAAC
GAAGATGGGTTTGGTGCATATATTGAAGAATCACTATGTGACCACTTGTGAGAAAACGCCGGCGGAAATCACTTTCGATCC
ACTGTCCATAGTTTTGAATTATAAGGGAGGCATTTACTTGAAATTTGTCAATGACAAACGCTATGAGCGAAATGCGAGGCAG
TGA

Gene sequences which were amplified by primers (amplifying the whole gene) are underlined, and yellow highlighted sequences are part of the T7 primers, which were designed for the construction of the dsRNAs for the RNAi experiment. (Sequence of the LacZ gene is not shown here).

Supplementary Table ST3.2.5. Protocols that were followed for the dsRNA synthesis for the RNAi injections.

Phusion reaction protocol (20μl final volume) 13.4 μl ddH_2O 4 μl 5X HF Buffer 0.4 μl 10mM dNTPs 1 μl 10 μM of each primer 0.2 μl Phusion pol	 Lanes; 1: 1kB DNA ladder RTU, 2: 100bp NEB ladder, 3: CYP4P6, 4: CYP6G28. The reaction generated the full gene sequences; approx. 1,500bp.
Kapa reaction protocol (25μl final volume) 18.2 μl ddH_2O 2.5 μl 10X Buffer A 1 μl 10mM dNTPs 1 μl 10 μM of each primer 0.3 μl Phusion pol	 Loaded lanes; 1: 100bp NEB ladder, 2: CYP4P6, 3: CYP6G28, 4: LacZ, 5: negative control. The reaction generated an approx. 500bp product.
Megascript T7 reaction protocol (20μl final volume) 7 μl DEPC H_2O 2 μl Buffer 100 ng DNA template 2 μl ATP, CTP, GTP, UTP 2 μl T7 enzyme	 1.2% agarose gel loaded with 1:10 diluted dsRNAs. Lanes; 1: 100bp NEB ladder, 2,3: CYP4P6, 4,5: CYP6G28, 6: LacZ;

Supplementary Table ST3.2.6. Dose response of *Bactrocera oleae* field population (Evaggelismos) against the laboratory susceptible strain (Lab), to alpha-cypermethrin, upon topical application bioassays.

Strain	N	LC50	95% CI	Slope	χ^2 (df)	RR (95% CI) vs Lab
LAB	137	0.417	0.24 - 0.66	2.206±0.324	11.49 (8)	
EVAGGELISMOS	119	60.979	44.60 - 83.025	3.719±0.856	3.57 (7)	146.23 (94.77 - 225.40)

N: total number of individuals tested (including control), CI: Confidence Interval, LC: lethal concentration, RR: resistant ratio= LC50 of resistant population /LC50 of susceptible population. A χ^2 test was used to assess how well the individual LC50 values observed in the bioassays were in consistency with the calculated linear regression lines.

3.2.9. References

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Chapter 4

Unraveling the unique adaptation of the olive flies to olives, via the symbiotic relationship with bacteria of the species *Candidatus* Erwinia dacicola.

This chapter was redrafted from Inga Siden-Kiamos, Venetia Koidou, Ioannis Livadaras, Evangelia Skoufa, Sevasti Papadogiorgaki, Stefanos Papadakis, George Chalepakis, Panagiotis Ioannidis, John Vontas (2022). Dynamic interactions between the symbiont *Candidatus* Erwinia dacicola and its olive fruit fly *host Bactrocera oleae*; Insect Biochemistry and Molecular Biology, 146, 103793, https://doi.org/10.1016/j.ibmb.2022.103793.

Chapter 4 |

Title: Unraveling the unique adaptation of the olive flies to olives, via the symbiotic relationship with bacteria of the species *Candidatus* Erwinia dacicola (*Ca.* E. dacicola).

4.1. Abstract

The olive fly, *B. oleae*, is the most important pest of olive orchards worldwide. The ability of olive flies to utilize olives for their development appears to be rare and unique in nature and has been associated with interactions with symbiotic bacteria. In the current study we reveal critical aspects of this unique symbiotic relationship between the olive fly and it bacterial partners, especially of the species *Ca.* E. dacicola. These new insights presented here may stand as the basis for the future development of novel olive fly control approaches which will target the symbiont, by using molecular and classical tools in smart applications.

The determination of the relative abundance of *Ca.* E. dacicola with real time quantitative PCR analysis during the life cycle of the olive fly revealed a significant fluctuation between different developmental stages; a higher abundance of the symbiont was detected during the second instar larval stage, a decrease during third instar to pupae transition, followed by an increase in the adult stage. With the use of confocal microscopy, the localization of the symbionts and the morphology of the gastric caeca, the part of the gut that they are localized, between different larval stages were studied. Second instar larvae were reported with distended gastric caeca, filled with a bacterial mass, while during the third instar stage they were much smaller and convoluted. We showed that the bacteria reside extracellularly in the gastric caeca and during the transition to late third instar stage, they are discharged into the midgut, accompanied by a change in the gastric caeca size and morphology.

Furthermore, a comparative transcriptomics analysis of gastric caeca dissected out of second and third instar larvae collected from the field, in comparison to samples obtained from a laboratory strain devoid of *Ca.* E. dacicola was carried out. Two pairwise comparisons were set; gastric caeca from second and third instar larvae, in order to identify genes potentially involved in this developmental transition and gastric caeca from second instar larvae developing in olives and in artificial diet, in order to identify olive fly genes potentially involved in the establishment and regulation of the symbiotic relationship, since wild-type animals contain huge numbers of the bacterial symbiont while the laboratory-reared do not. Significant changes in transcript expression were noted and genes associated with the developmental changes revealed by the microscopic analysis as well as responses to microorganisms were highlighted.

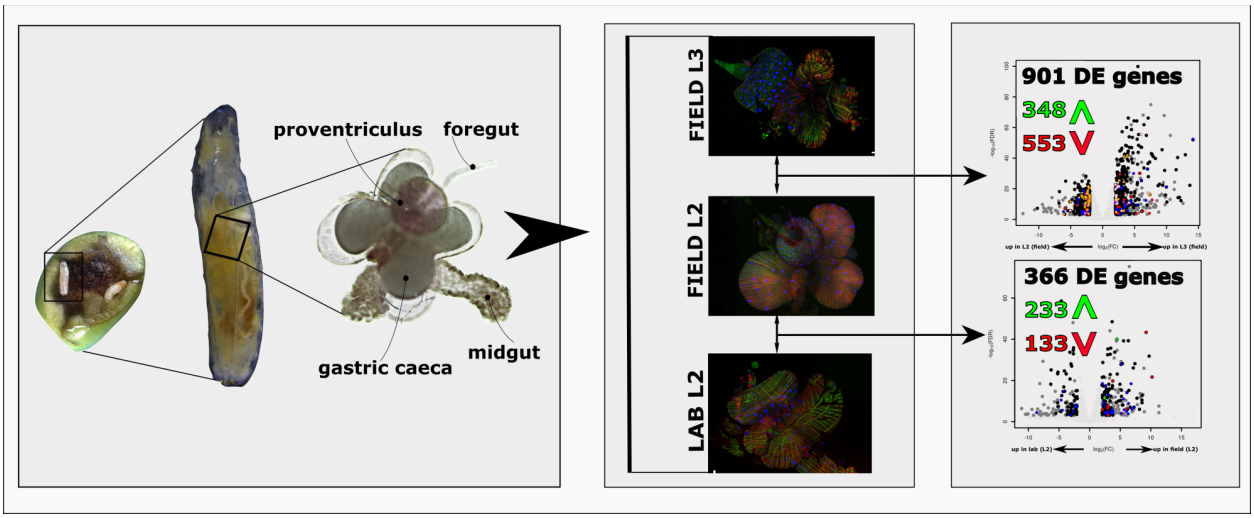


Figure 4.1. Graphical abstract of Chapter 4.

Statement of contributions in experimental procedures described in Chapter 4

(Abbreviations; ISK: Dr. Inga Siden-Kiamos, VK: Venetia Koidou, PI: Dr. Panos Ioannidis, IL: Ioannis Livadaras).

ISK: First stereoscopic observations**,** Transcriptomics analysis

VK: Rearing of insects, Antibiotic treatment, Tissue dissections, Fluorescent microscopy analysis, RNA isolation, qPCR analysis, Preparation of samples for RNA sequencing, Confocal scanning imaging analysis and sample preparation, pH assays

PI: Bioinformatics analysis, Data curation, Transcriptomics analysis

IL: Rearing of insects, Collection of samples for qPCR and RNAseq, Tissue dissections

4.2. Introduction

4.2.1. Symbiotic bacteria in the olive fly and the significance for the host

Unripe fruit is avoided by most fruit flies because of the high concentration of secondary metabolites which have anti-nutritive, antimicrobial, and toxic properties (Michael Ben-Yosef et al. 2015a). Olive flies are an exception to this norm, as they have the unique ability to develop in unripe (green) olives, presumably detoxifying hazardous secondary metabolites, ability which has been attributed to their symbionts (M. Ben-Yosef et al. 2014; Pavlidi et al. 2018).

"Performance experiments" have been widely used in order to investigate the nutritional significance of microorganisms to insect hosts (Huang, Jing, and Douglas 2015). The survival, larval growth, and adult fecundity of insects with and without the complement of microorganisms are being determined in these tests, through diets that contain or lack the nutrients of interest. If the performance of the microbial-free insects is hampered on a nutrient-deficient diet, in contrast to the insects still carrying microbes, it is quite likely that the insect gets those nutrients through

the bacterial partners. Both "performance experiments" and studies of the nutritional ecology of insects show that some, but not all, insects housing midgut bacteria, subsequently gain nutrients from this association (Billingsley and Lehane 1996).

As it concerns the olive flies, a vertically transmitted obligate bacterial symbiont discovered in different populations of olive flies from several countries (A. M. Estes et al. 2012; Savio et al. 2012; Sacchetti et al. 2008), *Ca.* E. dacicola appears to play a key role in the detoxification process (Capuzzo et al. 2005). Aposymbiotic olive fly larvae (i.e. larvae devoid of the bacterial symbiont) were unable to develop in unripe olives beyond the second instar larval stage, whereas they developed normally in ripe (black) olives and reached the adult stage, albeit the fitness cost (Michael Ben-Yosef et al. 2015a). The main toxic secondary metabolites in unripe olives for the insects, are the phenolic compounds, with oleuropein being the most abundant. Oleuropein concentrations are higher in green olives and decrease as the olive fruit ripens. These compounds promote the cross-linking of the foliar proteins, lowering their nutritional value, while also they act as antimicrobials, preventing plant tissue decomposition (Dobler, Petschenka, and Pankoke 2011). *Ca*. E. dacicola is proposed to play a role in increasing the bioavailability of cross-linked amino acids in unripe olives, allowing the insect to cope and survive under such a toxic environment (Michael Ben-Yosef et al. 2015a). Moreover, in olive fly larvae which were developing in unripe olives, several genes both of the host and *Ca*. E. dacicola were significantly up-regulated, including detoxification and digestive enzyme genes, suggesting that while olive fly larvae feed on unripe olives, *Ca*. E. dacicola amino-acid metabolism is stimulated (Pavlidi et al. 2018). The ability of the olive flies to develop in unripe olives combined with the unique symbiotic relationship with *Ca*. E. dacicola, strongly suggest that this bacterial species has an essential role in the life cycle of the host.

Apart from the vertically transmitted *Ca*. E. dacicola, olive flies are colonized with several other bacterial species as well, acquired from the environment and presumably residing in the gut (A. M. Estes et al. 2012; Campos et al. 2022). The first approaches, with the use of traditional microbiological approaches, had identified several other bacterial symbionts, belonging in the genera *Bacillus, Lactobacillus, Klebsiella, Micrococcus, Pseudomonas, Serratia* (Konstantopoulou et al. 2005). These species possibly contribute to the adult fly's fitness in a diet-dependent fashion (Michael Ben-Yosef et al. 2010; M. Ben-Yosef et al. 2014; Michael Ben-Yosef et al. 2015b), fact which yet has to be defined.

Taking advantage of the latest features of next generation sequencing, extra bacterial species are being identified and associated with olive flies, but further investigation on the exact mechanisms is needed (Bigiotti et al. 2021); *Pseudomonas putida* and *Asaia* sp. were detected in Italian (Sacchetti et al. 2008), *Acetobacter tropicalis* in Greek (Kounatidis et al. 2009), *Enterobacter* sp. in United States (A. Estes et al. 2018), and *Tatumella* sp. in Mediterranean olive fly populations (Blow et al. 2020). As a consequence, the olive fly carries a complex bacterial microbiome, composed with the major and obligate vertically transmitted endosymbiont *Ca.* E. dacicola, as well as other bacterial species, possibly horizontally transmitted during feeding, each contributing in their own unique manner in fly development (Campos et al. 2022).

4.2.2. Location of the bacteria in the olive fly gut and acquisition

Ca. E. dacicola has been identified in all wild populations investigated thus far, including populations from Greece (Kounatidis et al. 2009), Italy (Capuzzo et al. 2005), and the United States (A. Estes et al. 2018). These data suggest that this bacterium is an olive fly's obligate symbiont partner. *Ca*. E. dacicola is a Gram-negative γ-Proteobacterium species, belonging in the Enterobacteriaceae family (Capuzzo et al. 2005), which cannot be cultured outside of its insect host and is vertically transmitted to the next generation (maternal transmission to the offspring) (A. M. Estes et al. 2009). This endosymbiont is not present in laboratory-reared colonies, probably due to the antibiotics and preservatives required for the preparation of artificial diets (Sacchetti et al. 2008; A. M. Estes et al. 2012), except for one study in which the bacterium was detected in a few specimens of a lab hybrid population (Anne M. Estes et al. 2014).

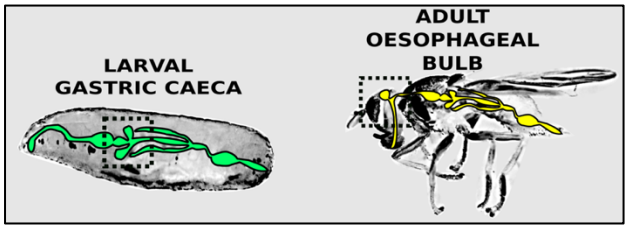


Figure 4.2. The tissues of the olive flies that house the symbiont partners. Symbiont partners are located in different organs in the olive fly, depending on its developmental stage. Schematic representation which shows the larval gastric caeca and the adult oesophageal bulb (Livadaras et al. 2021).

Similarly to many other insect bacterial symbionts, *Ca*. E. dacicola, resides in blind sacs located at the larval anterior midgut, called gastric caeca (Petri 1909). The proventriculus, which acts as a sieve, in order to regulate the entry of food into the midgut, is attached to the blunt-ending gastric caeca lobes. The gut and the gastric caeca are the main organs involved in the secretion of digestive enzymes, as well as in the food digestion and nutrient absorption (Herboso et al. 2011). Until now, TEM studies in tephritid larvae have suggested that the symbiotic bacteria reside intracellularly within the cellular membrane of the epithelial cells of the digestive tissue of larval gastric caeca (A. M. Estes et al. 2009). Contrary, in Coleopteran species, which have been documented since the early 1970s, the bacterial symbionts are reported extracellularly, in the lumen of different types of gastric caeca lobes (Lehane 1997) (**Figures 4.1 and 4.3**).

During adulthood, the bacteria have been reported in a cephalic organ, part of the digestive tract, but outside of the cellular membranes of the host cells (the oesophageal or pharyngeal bulb, **Figures 4.1 and 4.2**), which is connected to the pharynx. There the symbionts multiply rapidly and form masses that reach the midgut. Then, they migrate through the midgut and reach an evagination of the female hindgut at the ovipositor. During oviposition, the mother's contractile perianal glands (diverticula) become loaded with bacteria and convey symbionts to the eggs via egg smearing (A. M. Estes et al. 2009; Capuzzo et al. 2005).

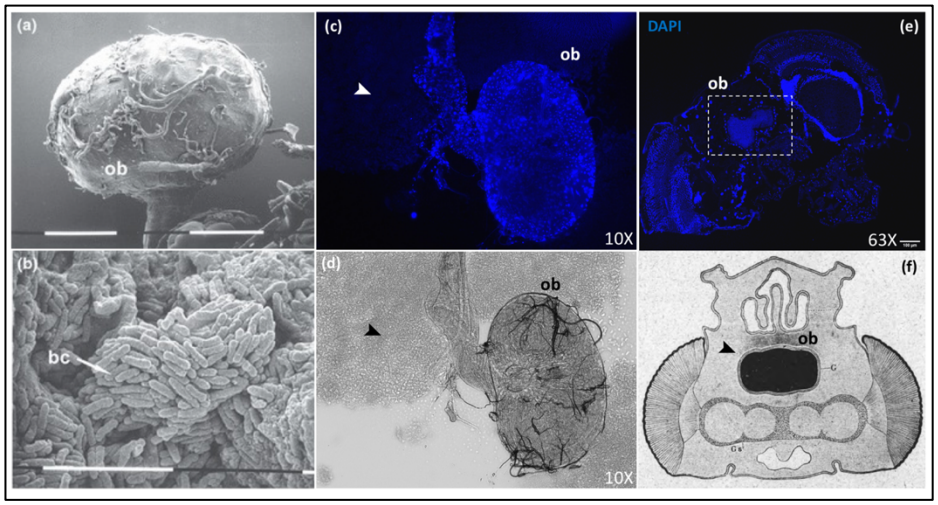


Figure 4.3. The oesophageal bulb of the olive flies. Adult olive flies house their symbiont partners in a cephalic organ, the oesophageal bulb (ob), which is located in the head of the fly. (a) SEM image showing the bean-like structure of the ob; Bar=100 lm (Sacchetti et al. 2008) (b) SEM image depicting the bacterial mass inside the ob; bc: bacterial colony, Bar = 10 lm (Sacchetti et al. 2008) (c) Fluorescence microscopy image of an ob whole mount, stained with nuclear Hoechst stain (blue) (V. Koidou unpublished data) (d) same as (c) whole mount of an ob exposed to visible light. (V. Koidou unpublished data); white and black arrowheads point the distinct bacterial mass which surrounds the bulb (e) 7μm cryo-section of adult wild-type female ob, stained with DAPI (blue) (V. Koidou unpublished data); white-dot square points out the localized bacterial mass in the ob, in the center of an olive fly head (f) Schematic transverse section of a female adult head (first documentation of L. Petri in 1909); black arrowhead points out the ob.

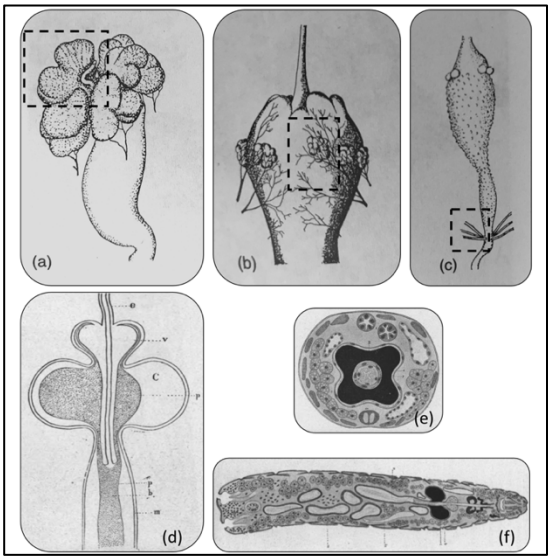


Figure 4.4. First schematic documentations of gastric caeca in Coleopteran species and olive flies. The gastric caeca consist of the part of the insect gut which connects the anterior part of the gut, the foregut, with the middle part of it, the midgut, and the tissue where the symbiont partners are located during the larval stages. (a-c) The different types of gastric caeca that have been reported in Coleopteran species (Billingsley and Lehane 1996); a: four large convoluted gastric caeca of *Stegobium paniceum*; (b) 8 “rosette”-shaped caeca of *Leptura rubra*; (c) 2 sets of paired sac-like caeca of *Cassida viridis*. (d-f) The gastric caeca of *B. oleae* were initially reported from L. Petri in 1909, and are characterized as four big and convoluted lobes, similarly to *Stegobium paniceum* (a).

4.3. Aim of the study

The olive fruit fly, *Bactrocera oleae (B. oleae)*, is the most important pest of olive orchards worldwide. The ability of olive flies to utilize unripe olives for their development appears to be rare and unique in nature and has been associated with interactions with symbiotic bacteria. The general aim of this study is to investigate and define critical aspects of this unique symbiotic relationship, between the olive fly and the bacterial partners, especially of the species *Candidatus* Erwinia dacicola (*Ca.* E. dacicola).

For this purpose, the abundance of the major bacterial symbiont throughout the development of the host was examined, extended microscopy analysis for the investigation of the tissue where the symbiont partners are located, depending on the developmental stage, was performed, as well as pairwise transcriptomics comparisons were set to define critical aspects of this symbiosis at a gene level. There were analyzed gastric caeca from second and third instar olive larvae, in order to identify genes potentially involved in the developmental transition and gastric caeca from second instar larvae developing in olives and in artificial diet, in order to identify genes of the host, potentially involved in the establishment and regulation of this symbiotic relationship, since wild-type animals contain huge numbers of the symbiont partner, while the laboratory-reared do not. A better understanding of this symbiotic relationship will contribute in the future development of dysbiosis-like approaches.

4.4. Materials and Methods

4.4.1. Insects

For the antibiotic treatment experiments field-derived olive flies were used. More specifically, black infested olives were collected from an orchard close to Avdou village (Heraklio, 35.1339, 25.2639) in December 2018. The olives were kept at 10°C, in order to delay the development of the larvae and synchronize the experiments, and the emerging adults were used in the downstream experiments.

The qPCR, imaging and RNAseq analysis were carried out with Democritus Laboratory Strain, Hybrid and field-derived olive flies. The Hybrid strain that was used as control, was originated after multiple crosses between male adults of the Democritus Laboratory strain and female flies collected from infested orchards in Crete. The Hybrid strain as well as the pure Democritus laboratory strain were maintained under standard rearing conditions at 25°C and a 16:8 hours light: dark photoperiod as described in depth previously (section **2.4.1. Rearing of *Bactrocera oleae***).

As it concerns the field fly populations, ripe (black) and unripe (green) olives infested by *B. oleae* were collected from infested orchards of Crete, at different timepoints and maintained in the laboratory under the conditions described above. More specifically, infested unripe and ripe olives were collected from olive orchards in Municipality of Heraklio, in Crete; ripe olives were collected near the village Avdou, Hersonissos (35.230662, 25.435690) (during January and February 2017,

January and February 2020); unripe olives were collected from FORTH campus (Heraklio, 35.305791, 25.072834) (during August and September 2018). The olives were kept at 24°C ± 1°C; first and second instar larvae were picked from the olive mesocarp and third instar larvae were collected when they had exited the olives. Adults were collected in McPhail traps with 2% ammonia as an attractant (food lure traps (Dimou et al. 2003)). Samples used for the quantitative PCR experiments were snap frozen in liquid nitrogen and stored at -80°C until further use.

For the RNA sequencing, the immunohistochemistry and the pH investigation experiments, second and third instar larvae were dissected out of ripe olives collected from the FORTH campus (35.305791, 25.072834).

4.4.2. Antibiotic treatment

The protocol and the experimental design were kindly provided by Dr. Miki Ben Yosef (Prof. Yuval Lab) and it was conducted with a few minor modifications (Michael Ben-Yosef, Jurkevitch, and Yuval 2008).

Adult female flies (3-5 day old) were maintained along with male flies in big plastic cages for ten days, under a 30% sugar diet, in order to mate. On the eleventh day, the female flies were transferred in a separate cage and they were maintained under a diet containing piperacillin antibiotic (Sigma Aldright, 66258-76-2) (final concentration = 200μg/ml diet) for ten days (**Supplementary Table ST4.1**). The liquid diet was provided to the flies through Wettex pieces, in sterile cut Eppendorf tubes and it was replaced with fresh mixture every 24 hours. This ensured that the antibiotics were active and prevented a resistant bacteria buildup in the diet (the food doses with and without antibiotic were kept in -20°C). On the twenty-first day of the experiment, males (half of the initial number of the females that were introduced in the separate cage) were introduced in the same cage, in order to re-mate. The flies were maintained under the standard rearing conditions, at 25°C and 16: 8 hours light: dark photoperiod, under the classical solid diet, as was previously described (section **2.4.1. Rearing of *Bactrocera oleae***). Two days after, 70% EtOH-sterilized olives were placed in the cage; treated and un-treated females (G0) were allowed to oviposit their eggs on them. After 5-7 days the olives were dissected, and the developing G1 larvae were examined. The experiment was repeated twice in big cages and twice using small plastic cages for small groups of treatment and mating, in order to distinguish any differences.

4.4.3. PCR and fluorescent microscopy analysis for the detection of *Ca.* E. dacicola during the antibiotic treatment

During the ten-day antibiotic treatment, treated and untreated females (G0) were sacrificed (day 0-untreated, day 5 and day 10) and oesophageal bulbs (obs) were dissected out of their heads (**Supplementary Figure SF4.1**), in order to detect any possible presence of *Ca.* E. dacicola with PCR analysis. Grinded obs (Nanodrop measured) were used as template in individual PCR reactions with Taq DNA polymerase (MINOTECH, 203-1) and primer sets for the amplification of *Ca.* E. dacicola recA and OmpA genes and *B. oleae* actin gene as reference. PCR reactions with

54°C, 55°C and 55°C annealing temperatures for the three genes respectively, for a 30 seconds-step and an extension step at 72°C for 30 seconds, for 35 cycles (primers in **Supplementary Table ST4.2**) were performed. PCR products were run on an 1.5% electrophoresis agarose gel. Moreover, during the antibiotic treatment period, obs were also examined for the presence of bacteria, with the use of fluorescent microscopy. Obs were dissected out of the female fly heads in PBS 1X (on day 0, day 5 and day 10) and were incubated in Hoechst 33342 DNA stain (Invitrogen™, H3570) (1:1,000 in PBS 1X) for 15 minutes. Tissues were then gently mounted in Vectashield (Vector Laboratories, H-1000-10) on microscope slides. Imaging was conducted using a Zeiss Axioplan 2 Upright Light/Fluorescence Microscope, equipped with the appropriate filters and housed in the Institute of Molecular Biology and Biotechnology (IMBB) Microscope Facility of Foundation for Research & Technology-Hellas (FORTH, Heraklio).

4.4.4. DNA extraction and qPCR analysis for the quantification of *Ca*. E. dacicola among developmental stages of olive flies

For the quantification of *Ca.* E. dacicola among developmental stages in ripe (black) and unripe (green) olives, genomic DNA was isolated from whole-body insects, using the Cetyl-tri-methyl-ammonium bromide (CTAB) extraction protocol, with some modifications (Doyle, 1990). Briefly, pools of five individuals were ground with plastic pestles in 50 μl of extraction buffer (2% CTAB, Sigma), 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 0.2% 2-mercaptoethanol). The suspension was incubated for 15 minutes at 65°C, treated with RNase A (Qiagen, Cat. No: 19101) for 1 hour at 37°C, extracted with chloroform-isoamyl alcohol and DNA was precipitated with isopropanol.

Each qPCR reaction consisted of 5 μl SYBR Green 2X (BioRad), 30 ng genomic DNA template and 10 mM of each gene primer (*Ca.* E. dacicola recA and *B. olea*e actin genes, **Supplementary Table ST4.2**). The qPCR analysis was run on a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad), under the following thermal conditions. The reactions were heated to 95°C for 10 minutes, followed by 40 cycles heated at 95°C for 10 seconds, 58°C for 20 seconds, 72°C for 30 seconds, including a melting curve step (60-94°C for 1 second/ 0.5°C). Each run also included a non-template control reaction. Each sample contained pools of five individuals and four technical replicates were carried out for each sample. Individuals included first, second and third instar larvae, pupae, young and old females and males, which had developed in ripe (black) and unripe (green) olives. All statistical analyses were carried out in the R programming language (R Core Team 2022) using the “dplyr” library (Wickham, Henry, and Müller 2021). The results were analysed by the Bio-Rad CFX ManagerTM 3.1 software. Fold change was calculated and normalized against the copy number of the *actin* gene of the olive fly, using the Pfaffl method (Pfaffl 2001).

4.4.5. Gastric caeca dissections, total RNA extractions and RNA sequencing sample preparation

Samples for RNA sequencing were prepared with gastric caeca dissected out of second and third instar Hybrid and field-derived (collected from FORTH) larvae (three conditions: second instar Hybrid, second instar field, third instar field; four biological replicates per condition) and each biological replicate consisted of 10-12 gastric caeca (**Supplementary Figure SF4.3**). Tissues were dissected in PBS 1X, collected in RNAlater (Sigma Aldrich, Cat. No R0901) and stored at 4°C until the sets were complete. Afterwards, tissues were transferred in new tubes, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted and prepared for the RNA sequencing as previously described (section **3.2.4.2. Malpighian Tubule Dissections and RNA extraction**). The samples were DNase treated using DNase I (Ambion™ DNase I (RNase-free), Cat. No AM2222) in order to remove any contaminating DNA. RNA concentration and purity (OD, A260/A280 and A260/A230) were measured in Nanodrop and integrity was checked on an 1.5% agarose gel (**Supplementary Figure SF4.4**).

Approximately 1.5μg of each purified RNA sample were sent to Macrogen, Inc (Korea). mRNA paired-end libraries were constructed with the Illumina Truseq stranded mRNA sample preparation kit, following the manufacturer's instructions. Each library was sequenced on the Illumina platform with the paired-end method and a read length of 100 bp. The sequencing reads are available from the Sequence Read Archive (SRA) under the BioProject accession PRJNA782538 link to SRA.

4.4.6. Immunohistochemistry

Gastric caeca from second and third instar wild-type (dissected out of collected olives from FORTH) and hybrid larvae were dissected in PBS 1X and placed on ice. Following dissections, the tissues were incubated in 4% Formaldehyde Solution (Methanol free, Thermo Scientific, 28906) for 60 minutes and then in 3% Blocking Solution (BS, 3% BSA, 0.1% Triton X-100 in PBS 1X) overnight at 4°C. Tissues were incubated with an antiserum directed against *Anopheles gambiae* E-cadherin (Siden-Kiamos et al. 2020) (1:200 in BS) overnight at 4°C. The third day the tissues were incubated with Alexa Fluor 555-conjugated anti-mouse IgG secondary antibody (Invitrogen) (1:1,500 in BS) for 1.5 hours at RT. After BS washes, actin filaments were stained with Alexa Fluor 488-conjugated phalloidin (1:50 in BS for 30 minutes, Invitrogen Thermo Scientific, A12379) and nuclei were stained with DAPI (1:100 in BS for 20 minutes, AppliChem, Cat. No A1001). Tissues were then gently rinsed with PBS 1X twice, prior to mounting in Vectashield (Vector Laboratories, H-1000-10) on SuperFrost+ slides. Imaging was conducted using a Leica SP8 laser scanning confocal microscope, equipped with the appropriate filters and housed in the Institute of Molecular Biology and Biotechnology (IMBB) Microscope Facility of Foundation for Research & Technology-Hellas (FORTH).

4.4.7. Investigation of the olive fly midgut pH

Gastric caeca from second and third instar wild-type (larvae dissected out of ripe olives collected from FORTH campus) and hybrid larvae were dissected in PBS 1X, fixed in 4% Formaldehyde Solution for 20 minutes and then placed in 0.1% w/v Bromocresol Purple pH indication dye solution for ten minutes (0.1% w/v dye powder diluted in sucrose 10% in distilled H_2O, Sigma-Aldrich, 115-40-2). Attempts to provide the dye to the larvae through ingestion were performed, but no coloring of the gastric caeca was observed, maybe because of the blind-ending nature of the tissue (data not shown). Hybrid (second and third instar) larvae were used as control. The colors observed in each part of the gut were compared with standard buffered solutions containing Bromocresol Purple (diluted in NaOH 0.1mol/L) (**Figure 4.12**). Stereoscopic images were captured in a Leica Microsystems M205 FA fluorescence stereo microscope using the brightfield filter of a Digital Color Camera Leica DFC310 FX.

4.4.8. Bioinformatics analysis

The bioinformatics analysis was conducted by Dr. Panos Ioannidis.

Reads were first quality-trimmed using trimmomatic v0.39 (Bolger, Lohse, and Usadel 2014) in order to remove sequencing adapters and low-quality bases. Trimmed reads were then mapped on the publicly available *B. oleae* reference genome (Bayega et al. 2021); GCF_001188975.3) using the Hisat2 v2.1.0 short read aligner (Kim et al. 2019). Read counts for each of the predicted genes in the official gene set were calculated with featureCounts v1.6.0 (Liao, Smyth, and Shi 2014) at the gene level, with parameters and using the gtf file that is available in the RefSeq FTP server. EdgeR v3.28.1 (Robinson et al. 2010) was used to find genes that were significantly (FDR <1e-03) differentially expressed and also had a fold change (FC) >4 ($\log_2$FC >2).

Gene ontology (GO) term functional enrichment analysis was performed using the web-based version of gProfiler (Raudvere et al. 2019). However, since *B. oleae* is not included in the gProfiler database, the GO term analysis was done using *Drosophila melanogaster* as a proxy. More specifically, for each *B. oleae* gene of interest, its most similar *D. melanogaster* gene was found using BLAST (Camacho et al. 2009). These *D. melanogaster* genes were subsequently searched for enriched functions in gProfiler.

Determining whether *B. oleae* genes were full-length was done using BLAST. Each *B. oleae* predicted protein was searched against the Uniref50 protein database, with an e-value cut-off of 1e-25. Self-hits were excluded and using custom Perl scripts we searched for hits covering >90% of the *B. oleae* protein (query), as well as the database protein (subject). This approach ensured that the *B. oleae* protein had an end-to-end match with a protein from another species. Excluding lowly expressed genes is important for focusing on genes that are transcribed at levels above the background noise. A histogram of gene expression values in all samples indicated that a read count of 50 was a reasonable threshold to distinguish lowly from highly expressed genes (data not shown).

To further analyze the gene expression, data text searches were carried out. We noted that automatic functional annotation of the genome was not always correct and therefore we carried out our own BLAST analyses against the Landmark and Swissprot databases. We compared the results of these three analyses and when two of the three different analyses were consistent, we accepted this as the correct annotation. In cases where there were three different annotations or the protein was uncharacterized in one or two annotations we carried out manual searches of the Conserved Domain database of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?) and/or BLAST against the Genbank protein non-redundant database. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

4.5. Results

4.5.1. Antibiotic treatment yielded in inconsistent results and bacterial elimination was not accomplished

During the ten-day antibiotic treatment, treated and untreated females (G0) were sacrificed and obs were dissected out of their heads (**Supplementary Figure SF4.1**), in order to detect any possible presence of *Ca.* E. dacicola with two primer pairs amplifying recA and OmpA genes in PCR analysis. Ob samples from three timepoints were prepared; day 0 (untreated), day 5 and day 10. The PCR results for the two *Ca.* E. dacicola genes showed a reduction of both genes, but not an elimination, in all treated samples (**Supplementary Figure SF4.2**). In all PCR reactions, equal quantity (150μg) of ob DNA was loaded.

However, fluorescent microscopy revealed an elimination of the bacterial cells in the obs of the treated G0 females. In more detail, in all obs dissected out of untreated female flies (day 0), a bacterial “cloud” was detected, either inside or surrounding the ob, suggesting a probable rupture of the outer membrane of the bulb, due to the coverslip weight. In contrast, in the majority of the ob dissected out of from day 5 and day 10 treatment timepoints, no bacterial cells were detected. 20 samples from each timepoint were prepared and examined. On the fifth day of the treatment, 14 out of 20 dissected bulbs were devoid of bacteria, whereas 6 bulbs still carried bacteria. On the tenth day of the treatment, 17 out of 20 dissected bulbs were devoid of bacteria, whereas 3 bulbs still carried bacteria (**Figure 4.5**).

5-7 days after the oviposition of G0 treated female flies, the olives were dissected and the developing G1 larvae were examined. We observed that very few larvae emerged from the treated G0 mothers and almost all of them showed a developmental arrest at first instar larval stage, in contrast to the untreated control, where exclusively second instar alive larvae were found, during the same time frame. In more detail, in big cage experiments, very few progeny larvae developed from the antibiotic treated G0 females (24% of the dissected olives were hosting a larva) and the majority of them were dissected out dead (97%). 3% of the larvae dissected out alive and were found with a normal body size at second instar stage. In the untreated control condition, 54% of the dissected olives were hosting a larva (61% of them were alive and at second instar larval stage). In individual cage experiments, the percentages were even lower; after the antibiotic

treatment of G0, 8% of the dissected olives were hosting a larva (100% were dead at first instar larval stage) while 23% of the control olives were hosting a larva and all of them (100%) were alive at second instar larval stage (**Figure 4.6** and **Supplementary Table ST4.3**).

According to the previously described results, after the current antibiotic treatment protocol, even though it resulted in a significant clearance of the bacterial populations in the majority of the specimens observed with fluorescent microscopy, the small numbers of the examined samples and the non-consistent outcome, combined with the inconsistent PCR analysis results, suggest a moderate effective bacterial elimination method. In addition, the high levels of mortality that the progeny of the antibiotic treated mothers reported, made this experimental design an in-appropriate and non-feasible method to conduct further experiments.

4.5.2. Abundance of *Ca.* E. dacicola varies during the fly's life cycle

To estimate the abundance of *Ca.* E. dacicola in natural populations of olive flies, infested olives were collected from two different olive orchards in Crete, which had not been treated with insecticides. Olives that were not yet ripe (green olives) as well as ripe olives (black) were separately collected. The samples were transferred to the laboratory and the insects were allowed to develop. Larvae of the first two instar larval stages were picked from the olive mesocarp. Third instar larvae were recovered after their exit from the olives (jumping), and they were allowed to form pupae and develop into adults. Five individuals of each stage were pooled and DNA was extracted for qPCR analysis with primers specific for the *Ca.* E. dacicola *recA* gene and with *B. oleae actin* as a reference. For ripe olives, four to twenty different pooled samples were analysed, while for unripe olives three to sixteen (**Figure 4.7A,B**). In total 395 individuals were collected and tested from unripe and 390 from ripe olives.

The results show that there is variation of the abundance of the major bacterial partner between different developmental stages. More specifically, the relative amount of *Ca.* E. dacicola varies during the life cycle of the hodt, with a higher abundance of the symbiont in the second instar larval stage in both populations, a decrease during third instar to pupae transition, followed by an increase in the adult stage (**Figure 4.7A,B**). The differences of the 20-day-old females developing in unripe olives compared to any other life stage of this group was statistically significant (**Figure 4.7A**). The same pattern was detected in samples recovered from both unripe and ripe olives. However, the relative abundance of the bacterial partner was much higher in ripe olives and in these samples the fluctuation was much more pronounced than in larvae developing in unripe olives (**Figure 4.7B**). We ran an ANOVA, followed by a Tukey HSD test and found that *Ca.* E. dacicola levels were significantly higher in two life stages; 20-day-old female samples recovered from unripe olives, and second instar larvae recovered from ripe olives.

4.5.3. *Ca.* E. dacicola is present in the lumen of the larval gastric caeca and discharged in the transition to third instar larval stage

We have investigated the presence of the bacterial partners (including *Ca.* E. dacicola) in gastric caeca which were dissected out of larvae developing in infested ripe and unripe olives, using low-

resolution stereoscopy (LRS) and confocal microscopy analysis (CMA). In LRS observations, the gastric caeca of second to third instar larvae, dissected from the olive mesocarp, were greatly distended and a white mass filling the lumen of them was reported, in samples recovered both from ripe and unripe olives (**Supplementary Figure SF4.3**) Larvae at third instar stage that had exited the mesocarp (jumping stage) were also analysed. In this case the swollen gastric caeca pattern was not reported (0/220 were swollen compared to 85/85 in second instar larvae). Instead, the caeca had a convoluted appearance. We next determined if the change in size and morphology is also observed in the Democritus laboratory strain, devoid of the bacterial partner *Ca.* E. dacicola. These flies exhibited the same tissue pattern, that of distended gastric caeca in second instar larval stage, while smaller and convoluted in the third. To obtain a more detailed view of the gastric caeca, samples were immunolabeled with an antibody directed against epithelial E-cadherin, stained with phalloidin to highlight muscle actin filaments and with DAPI to mark the cell nuclei (**Figures 4.8,9,10**). The same pattern was verified with CMA, which confirmed that the white mass which was detected in the stereoscopy analysis constituted the characteristic rod-shaped bacterial cells. The presence of the bacterial symbionts in the gastric caeca lumen was highlighted with the DNA stain; bacteria were only observed in second instar larvae recovered from both ripe and unripe olives (**Figure 4.8a-g,k-n and Figure 4.9a-g**), and not in third instar larval samples (**Figure 4.8h-j,o-r and Figure 4.9h-j**), data which was in consistency with the stereoscopic observations. CMA also revealed that the caeca consist of a limited number of cells, estimated at approximately 20, in both stages. A “tight cage” of muscles arranged in doublets and highlighted by phalloidin, was observed to surround the organ. The same pattern was observed in the gastric caeca of laboratory reared-hybrid larvae, but with no bacterial partner presence (**Figure 4.10**). During the second instar larval stage, the gastric caeca were reported with a distended morphology (**Figure 4.10a-c**) whereas in third instar they were much smaller and convoluted (**Figure 4.10d-j**), suggesting that this transformation pattern of the tissue happens independently of the bacterial presence.

Tissues (same part of the gut) from 3-day old adults were dissected out of laboratory-reared-hybrid flies and flies which had developed in ripe olives and they were also examined with confocal scanning microscopy (**Figure 4.11**). It was interestingly observed that the gastric caeca epithelial cells had been eradicated, probably histolyzed; this data is in consistency with the results from the qPCR analysis and supports the theory that during the adult stages, the flies house the symbiont partners in a different tissue, the oesophageal bulb, which is located in the cephalic organ of the fly.

4.5.4. Fluctuation of the gut pH during the development of the olive fly

In order to determine if pH changes during the transition from second to third instar larvae and whether such changes were related to the presence of bacteria, gastric caeca from olive-developing and laboratory-reared larvae were examined. Dissected gastric caeca were incubated with the pH indicator dye Bromocresol Purple (**Figure 4.12**). In the second instar samples (swollen gastric caeca), pH was reported basic (purple) (**Figure 4.12a-d**), estimated at approximately 9.0, comparing to solutions of known pH (**Figure 4.12i**). In the third instar samples (shrunk lobes) pH was weakly acidic to neutral, at pH 6,0-6.5 (red) (**Figure 4.12e-h**). This pattern was observed in

the gastric caeca of both field-derived and laboratory-reared larvae, independently of the presence or absence of the major bacterial partner *Ca.* E. dacicola. This observation is in consistency with a previous one from L. Petri in 1909, proposing that bacteria play a not so critical role in this muscle transformation.

4.5.5. Transcriptomics analysis of the gastric caeca

The transcriptomics analysis was conducted by Dr. Panos Ioannidis and Dr. Inga Siden-Kiamos.

In order to obtain further information concerning the gene expression pattern, specifically in the gastric caeca during larval development, as well as the differences in transcript levels between the gastric caeca housing the *Ca.* E. dacicola and those devoid of the symbiont, a transcriptomics analysis was undertaken. Gastric caeca together with the proventriculus were dissected out of second (field L2) and third instar larvae (field L3) developing in infested ripe-black olives, which were collected from the field. For comparison, the same tissues were dissected out of second instar larvae of the laboratory strain (HYB L2) devoid of the major symbiont partner *Ca.* E. dacicola. Four biological replicates of each type were analysed by Illumina sequencing.

RNA sequencing data were mapped on the publicly available reference genome of *B. oleae* (Bayega et al. 2021), which contains a total of 12,958 protein-coding genes. Principal component analysis, using gene expression levels was performed, as a standard quality control measure, before embarking on downstream analyses **(Supplementary Figure SF4.5**). All replicates of each sample were clearly mapped together with one another, and separately from the replicates of the other two samples, meaning that the comparisons between any of the samples are reliable.

Two pairwise comparisons were carried out in order to identify differentially expressed genes of particular importance. More specifically, two field samples (gastric caeca from L2 and L3 larvae) were firstly compared, in order to identify genes potentially involved in the developmental transition from L2 to L3 larval stage. The second comparison was set between the two second instar larval samples (field and HYB gastric caeca). The gastric caeca of the olive-developing insects contain huge numbers of the bacterial symbiont, while the HYB organs are largely devoid of it. Subsequently, the second comparison attempts to identify olive fly genes potentially involved in the establishment and the regulation of the symbiotic relationship with the major bacterial partner *Ca*. E. dacicola. The results were filtered, so that only the significantly differentially expressed (FDR <1e-03) with a fold change >4 (log2FC >2) genes were included. Furthermore, genes expressed at a low level were excluded from the analysis, in order to focus on genes with expression levels above background noise.

- **First comparison:** gastric caeca from L2 and L3 larvae revealed differentially regulated genes associated with functional changes in gastric caeca during development

The pairwise comparison of gastric caeca of field second instar (field L2) and late third instar larvae (field L3) revealed 901 differentially expressed genes, of which 348 were upregulated in

the L2 larvae (**Figure 4.13A, left part**), whereas 553 were upregulated in the L3 larvae (**Figure 4.13A, right part**).

To analyse the comparative transcriptomics dataset and relate the differential gene expression patterns with the results from the microscopy experiments, we combined the analysis of gene function based on the genome annotation, with manual BLAST searches against the Landmark and Swissprot databases. We found that the majority of the differentially regulated genes were of unknown or disparate functions. However, groups of genes encoding proteins with similar functions were recognized (**Figure 4.14** and **Supplementary table ST4.4**). Of the total 901 genes, 767 (85%) belonged in the first category, while 134 could be characterized with broadly related functions into four groups; genes encoding proteases, mitochondrial proteins, proteins involved in signalling pathways and in muscle function.

Proteases were classified in digestive enzymes (trypsins, chymotrypsins, lysosomal aspartic proteases), metalloproteases and proteases similar to those involved in development of *D. melanogaster*. The comparison between the gastric caeca from second and third instar larvae revealed differentiated transcription of proteases and protease inhibitors between the two larval stages. In the second instar samples, ten genes coding for digestive enzymes were over transcribed. Furthermore, in the third instar samples, fewer genes encoding digestive enzymes and eight metalloproteases were upregulated, compared to the second instar samples.

Moreover, gene encoding eight mitochondrial proteins were upregulated in the third instar sample, while 28 were upregulated in the second instar sample, compared to the third one. Additionally, genes encoding ATP synthase subunits were upregulated in the second instar larvae samples, compared to the third one. The expression of 23 genes involved in signalling pathways were upregulated in the third instar samples, including eleven kinases, six phosphatases, five hormone receptors and one gene similar to G protein coupled receptor. In the same samples, four genes were down-regulated, including a nuclear hormone receptor, two genes related to juvenile hormone and one myogenesis regulating glycosidase. Lastly, a gene encoding myosin heavy chain 95F was upregulated in the third instar field sample. Moreover, three genes encoding myosin isoforms (myosin heavy chain Q, myosin light chain alkali, myosin-VIIa) and two *troponin* genes were upregulated in the second instar sample compared to the third one.

- **Second comparison:** gastric caeca from L2 field samples and L2 hybrid larvae revealed differentially regulated genes associated with the symbiotic relationship with bacteria

A comparison of second instar samples (field L2 versus HYB L2) was carried out in order to discover any differential gene expression due to the presence of the major bacterial partner, since the HYB strain is devoid of *Ca*. E. dacicola. 366 genes were differentially expressed, of which 233 were upregulated in the olive-developing larvae (**Figure 4.13B, left part**) and 133 were upregulated in the hybrid larvae (**Figure 4.13B, right part**). Of the total 366 genes, 47 (13%) were categorized as putatively having a role in the interaction with the symbiont partner (**Supplementary table ST5.5**). These are grouped according to broadly related functions in proteases and peritrophins.

The comparison between the two second instar larval samples (field L2 versus HYB L2) revealed eleven upregulated genes encoding digestive enzymes in the olive-developing larvae (including trypsins, chymotrypsins, protease inhibitors and metalloproteases), compared to the HYB strain. In the latter, eight genes encoding proteases were upregulated, including five digestive proteases. Furthermore, and since the microscopy analysis revealed that the caeca matrix (void), which separates the bacterial mass from the gastric caeca epithelium, possess similarities to the structure of peritrophic matrix (composed of peritrophins and chitin and separating the gut lumen from the gut epithelium), peritrophin-like genes were investigated. The comparison between the second instar larval samples, revealed two upregulated genes encoding peritrophins (peritrophin-44-like and peritrophin-48) in the field sample compared to the HYB.

- **Third category:** Immunity and response to microorganisms-related genes

The transcriptomics datasets were scanned with terms related to immunity and microbial response. The innate immune system of insects recognizes microbes through pattern recognition receptors (PRR) of different classes (i.e. Toll-like receptors, peptidoglycan recognition proteins (PGRPs), scavenger receptors, integrins, C-type lectins). The effectors against bacteria and fungi include proteases, protease inhibitors, lysozymes, phenol-oxidases, anti-microbial peptides, and lectins. The most studied examples of innate immunity regulation and function include the Toll and IMD pathways (Ligoxygakis 2013). Particularly the Toll pathway is activated in response primarily to Gram-positive, but also to Gram-negative bacteria (i.e. *Ca*. E. dacicola). Comparing the expression of the second and third instar olive-developing samples, 13 genes related to these categories were upregulated in L3 samples (PGRP SC2, toll-like receptor 7, serine protease grass, cecropin-1-like, attacin-B, peste-like, phenoloxidase 2-like, C-type lectin 37Db-like, pellino, integrin beta-PS, MyD88, phenoloxidase-activating factor 1, defence protein l(2)34Fc)). The differential expression of all of these genes was also higher in L3 when compared to the HYB L2 samples. In all cases, the level of expression was roughly similar comparing the two L2 samples.

Seven genes were more highly transcribed in gastric caeca from second instar larvae, compared to the L3 samples (serine protease 7, phenoloxidase-activating factor 2, lectizyme, lysozyme 1, scavenger receptor class B, jonah 65Aiii, spaetzli). Six out of the seven genes were expressed at similar levels in the HYB L2 samples (except *Jonah 65Aiii)*.

Ten genes encoding proteins putatively involved in response to microorganisms were more highly transcribed in the olive-developing second instar larvae, compared to the HYB strain (C-type lectin, lysozyme P-like, invertebrate-type lysozyme 3, PGRP SA, jonah 65Aiii, jonah 65Aiv, croquemort-like, serine protease grass, integrin alpha-PS3-like). Nine out of the ten genes had roughly equal expression levels in the two field samples. No genes encoding immune or defence related proteins were upregulated in the HYB second instar larvae compared to the field samples.

4.6. Discussion

The olive fly, *B. oleae*, is the most important pest of the olive orchards worldwide. The ability of the olive flies to utilize olives for their development appears to be rare and unique in nature and has been associated with interactions with symbiotic bacteria. In the current study we reveal critical aspects of this unique symbiotic relationship between the olive fly and its bacterial partners, specifically of the species *Ca.* E. dacicola. These new insights presented here may stand as the basis for the future development of novel olive fly control approaches, which will be targeting the symbiont partners, by combining molecular and classical tools in smart applications.

Estimation *of Ca.* E. dacicola across developmental stages of olive fly

The determination of the relative abundance of *Ca.* E. dacicola during the life cycle of the olive fly, from the larval to the adult stage, comparing flies developing in unripe and ripe olives, was performed using real time quantitative PCR (qPCR) analysis (**Figure 4.7**). The data revealed that the bacterial titre is fluctuating between different developmental stages. More specifically, the relative amount of *Ca.* E. dacicola varies during the life cycle of the host, with a higher abundance of the symbiont detected during the second instar larval stage, in both populations (derived from un-ripe and ripe olives), followed by a decrease during the third instar larval stage to pupal transition and finally an increase during the adult stage (**Figure 4.11**). Although this pattern was observed in samples recovered from both un-ripe and ripe olives, the relative abundance of the bacterial copies was much higher and the fluctuation was much more pronounced in the individuals developing in ripe olives, compared to those developing in unripe olives. This finding was quite unexpected; previous studies have shown that the major bacterial partner *Ca.* E. dacicola is absolutely vital for the development of the olive flies in unripe olives (for the detoxification of high levels of phenolic compounds) and the larvae lacking this symbiont are not capable of developing in unripe olives, while they can develop in ripe olives (Michael Ben-Yosef et al. 2015b). Due to the generally more unfavorable environment of the green olives, we expected the bacteria to be more abundant there, in order to provide nutrients to the fly, defend against anti-microbial secondary chemicals and assist the larvae by degrading the intact plant tissue (A. M. Estes et al. 2012; Levinson and Levinson 1984). However, it is possible that the bacterial partner does not reach such high numbers as in ripe olives, due to the poor nutrimental environment. As a consequence, our finding supports the suggestion that the bacterial partner mainly provides a source of nutrients rather than carrying out the detoxification of the oleuropein in the unripe toxic olives. This suggestion is in consistency with a previous study, which ranked the amino acid biosynthesis first, concerning the roles of the symbiotic bacteria of *Cryptorhynchus lapathi*, upon proteomic and genomic analysis (Jing, Qi, and Wang 2020).

Furthermore, the abundance of *Ca.* E. dacicola was very low during pupation and remained low in young adults which had developed in ripe olives, a finding which is in agreement with recent studies (Campos et al. 2022; Hammer and Moran 2019). In adults originating from unripe fruit (green) there was observed a substantial relative increase of *Ca.* E. dacicola. Previous studies (A. M. Estes et al. 2012) show a similar relative higher abundance of the major bacterial partner in ovipositing females, and the authors hypothesize that this will possibly increase the probability of vertical transmission. Alternatively, a different work suggests that *Ca.* E. dacicola confers a

fitness benefit to the ovipositing females, by supplementing their diet at this very intensive nutritional stage (Michael Ben-Yosef et al. 2010). Similar results were seen when *C. capitata* was reared without its symbionts (Michael Ben-Yosef, Jurkevitch, and Yuval 2008).

Imaging analysis

With the use of confocal scanning microscopy, the localization of the the symbiont partners and the morphology of the gastric caeca, the gut part which houses them, were explored during different developmental stages. Second instar larvae were reported with distended gastric caeca, filled with a bacterial mass, while during the third instar larval stage they were much smaller and convoluted. The imaging analysis explained the decrease in the bacterial copies that we observed in the qPCR experiments, since a discharge of the bacteria from gastric caeca to the midgut was detected during the transition from second to third (jumping) instar stage of the larval development. In second instar stage, the bacteria were found extracellularly and present in the lumen of the gastric caeca, refuting a previous statement which proposed that the bacteria in olive larvae are intracellular (A. M. Estes et al. 2009). That notion was based on evidence from a TEM analysis of gastric caeca of third instar larvae, which as it is shown here, they are largely devoid of the major bacterial symbiont. Third instar larvae have directed the bacterial partners in the midgut and this explains why extracellular bacterial symbionts (*Ca.* E. dacicola) were not detected in that study. Interestingly, we observed the same tissue transformation pattern in the laboratory-reared strain; the distended gastric caeca observed during the second instar larval stage, transform to a convoluted form, during the jumping third instar larval stage, although this strain is devoid of the major bacterial symbiont. This finding suggests that symbiotic bacteria are not the catalytic factor of this tissue transformation, even though this has a clear impact on them. Our findings indicate that this transition likely takes place through an activation of the muscles surrounding the gastric caeca and this will be interesting to be further investigated in future studies.

Scanning Electron Microscopy (SEM) combined with Transmission electron microscopy (TEM) analysis (conducted by Dr. Inga Siden-Kiamos) confirmed the same tissue transformation pattern.

Concerning the imaging analysis of the adult tissues (same part of the gut), the gastric caeca epithelial cells had been eradicated, probably histolyzed at early adulthood (3 days old); data which is in consistency with the qPCR results (from ripe olives) and supports the notion that the flies house the symbiont bacterial partners in a different tissue during the adult stages, the oesophageal bulb, which is located in the cephalic organ of the fly.

Gut pH investigation

We also carried out pH experiments, in order to determine if the presence of the major bacterial partner is related to the pH of the gastric caeca. The results showed that the pH of the gastric caeca is different between the second and third instar larval stages, but this is not dependent on the presence of the symbiont (**Figures 4.8,9,10**). The reason for the difference between the two

stages is not yet clear, but the pH alteration that is noted in the transition from second to third instar larval stage could reflect differences in the nutrient requirements for digestion processes. One other explanation could possibly be related to the digestion of the bacteria in the second instar stage, being more efficient in a high pH environment. We interrogated V-ATPases and carbonic anhydrases in our transcriptomics data, since these proteins are known to be involved in the regulation of pH (Nepomuceno et al. 2017; Patrick et al. 2006; Overend et al. 2016). We found carbonic anhydrases to be differentially regulated, but no clear correlation to the changes in pH during the host development was evident.

Transcriptomics analysis

Furthermore, in order to obtain a clear picture of the transcriptional activity in response to bacterial infection, we initially planned to sequence the olive fly tissue that houses the symbiont partners, the gastric caeca of larvae. To this end, we collected a field olive fly population, which we treated with piperacillin antibiotic in order to remove the major symbiont *Ca.* E. dacicola. Specifically, we used a protocol that had already been developed and applied on olive flies (M. Ben-Yosef et al. 2014). Fluorescent microscopy analysis revealed an elimination of the bacterial cells in the majority of the oesophageal bulbs that were examined, but PCR analysis showed a reduction of the *Ca.* E. dacicola copies in the treated samples, but not an elimination. Taken together, the antibiotic treatment protocol which was applied, gave inconsistent results concerning the bacterial clearance and caused high levels of mortality to the progeny. Subsequently, RNA samples for sequencing analysis was not feasible to be prepared this way.

Instead, a transcriptomics analysis of gastric caeca dissected out of second and third instar larvae that were developing in olives (field samples) in comparison to samples obtained from a laboratory strain devoid of *Ca.* E. dacicola was carried out and significant changes in the levels of transcript expression were revealed. In detail, gastric caeca from second instar olive-developing larvae which carried the bacterial partners and from third instar larvae which had discharged the bacteria in the gut (as our imaging studies prove) were examined. Furthermore, in order to compare the response to bacterial partners, we analysed dissected gastric caeca from the hybrid strain, which is devoid of *Ca.* E. dacicola and the larvae develop on standard laboratory diet. Pairwise comparisons between gastric caeca of second and third instar olive-developing larvae, and gastric caeca of second instar larvae developing in olives and under standard artificial diet, revealed 901 and 366 differentially expressed genes respectively. The fact that the first comparison gave a bigger output was quite expected, since besides the different developmental stages, many differences occur due to the presence or absence of the major bacterial partner. A detailed analysis of the data was undertaken, focusing on certain groups of genes that are proposed to participate in the bacterial symbiosis, as well as in the developmental metamorposis of the tissue.

In the comparative analysis, genes encoding proteases constituted a prominent class among the differentially expressed genes. Proteases have a number of different roles in insects; they participate in the digestion of the food as well as in the development and the tissue remodelling (A. Jongsma and Beekwilder 2011). Taken together, the data from the transcriptomics analysis suggests a high digestive activity in the gastric caeca of second instar larvae developing in olives

(this is consistent with the findings from the TEM analysis, which revealed active digestion of the bacteria). Alternatively, in the gastric caeca of third instar larvae, several genes which encode metalloproteases with functions related to tissue remodelling were significantly upregulated, while transcripts for digestive enzymes were downregulated (Grizanova et al. 2021). The crucial developmental stage of the late third instar is also highlighted after an overexpression of several genes that encode proteins involved in signalling pathways and putatively have important tissue remodelling roles that take place during pupation stage. The differences in metabolic activity between second and third instar larvae is reflected in the overrepresentation of genes with mitochondrial-related functions that reported an upregulation in second instar larvae.

From our microscopy analysis contractions of the muscles probably cause the shrinking of the gastric caecatissue, that takes place before the third instar larval stage. Consistent with this, an increased upregulation of genes encoding proteins involved in muscle function, such as myosins and troponins, was detected in the gastric caeca from second instar larvae. The presence of *Ca*. E. dacicola in the gastric caeca of the second instar larvae and the discharge in the third instar stage, indicates that the immune system is fine-tuned between the different developmental stages. An indication towards this direction represents the fact that several genes which encode proteins of the Toll pathway were downregulated in the second instar larvae. While the Toll pathway is believed to be specific for Gram-positive bacteria in *D. melanogaster*, and *Ca*. E. dacicola is a Gram-negative one, its role in homeostasis of the larval gastric caeca is therefore not obvious.

Finally, the comparison of gastric caeca of olive-developing second instar larvae against larvae of the laboratory-reared (hybrid) strain, suggests that digestion of the bacteria in the gastric caeca of the field larvae requires a specific combination of trypsins and chymotrypsin. The hybrid strain is reared under a standard laboratory diet, which contains hydrolysate from yeast and soybeans, already digested by enzymes; therefore, these flies have no strict requirements for proteases, in order to obtain nutrients. A transcript for lysozyme which was upregulated in the field sample, could play a role in the interaction with bacteria, since these enzymes are directly involved in the digestion of the bacterial cell walls. In addition, genes that encode three Jonah-like proteins were also upregulated in the field second instar gastric caeca sample; these are serine proteases that have been suggested to be involved in response to foreign agents, but whether they play an active role in controlling the bacterial partners remains to be investigated.

Future aspects

The existence of this olive fly-*Ca*. E. dacicola interesting interacting relationship has proven to be critical for the survival and the development of the fly in the olive mesocarp. However, the main molecular factors underlying this symbiotic relationship are still unknown. It's crucial to figure out which genes, both of the insect and bacterial, are involved in these interactions and what role each one serves. For this reason, it is critical to have access to the genomes of both symbiotic partners, in order to analyze these interactions. Both of the olive fly (RefSeq accession: GCF 001188975.1) (Bayega et al. 2021) and the *Ca.* E. dacicola genomes (Blow et al. 2020; Anne M. Estes et al. 2018) have been sequenced, even though the latter is still fragmented, due to the use

of short reading platforms (Illumina). Several studies have attempted to use this knowledge to better understand the advantages that the bacterial population provides to its insect host (Pavlidi et al. 2017; A. Estes et al. 2018). However, these efforts are impeded by both the currently available incomplete datasets and the absence of relevant methods for functional validation of the gene candidates, until recently. The new insights presented in this study, concerning the olive fly development and its interactions with this vertically transmitted and obligate major symbiont, can be exploited for the future development of symbiosis-based pest management strategies concerning the olive fly control.

This non-associated with the major obligate symbiont (*Ca*. E. dacicola) transformation of the gut compartment of the olive fly host (the gastric caeca) which was reported in this study, has provided novel directions for the study of this symbiotic relationship. Apart from the deeper information it was obtained for this physiological tissue pattern that this insect possesses, the transformation of the gastric caeca during the larval and the adult olive fly stages, generated a large number of annotated sequences, available to the wider scientific community for further investigation. Such studies could include functional characterization experiments as an attempt for a better understanding of the functions and their putatively roles of these genes in symbiosis. Interesting genes that were reported to be implicated in this unique interaction can possibly be functionally validated with the use of the newly established protocols of reverse genetics approaches, the gene knock-down (RNAi) and the gene knock-out (CRISPR/Cas9) molecular tools.

Furthermore, the gene datasets of this tissue specific RNA sequencing analysis concerning the olive flies, will contribute in the development of alternative approaches for the olive fly control, targeting its obligate microbial partner (*Ca*. E. dacicola) (dysbiosis). The goal of targeting microbial partners is to control insect pests by eliminating the microorganisms required for a sustained insect growth, reproduction and survival (Arora and Douglas 2017). Such specific molecular targets, like the ones revealed in the present study, linked to the co-evolutionary interactions, provide a strong motivation for the development of methods that will target this bacteriocyte-like symbiosis (Douglas 2015), since treated insects won't be able to re-gain this association horizontally, from other insects or from the environment. The elimination of the vertically-transmitted symbiont *Ca*. E. dacicola may involve the use of specific symbiocides i.e. effectors, which perturb the resident microbial partner and their interactions with the insect, like conventional antibiotics or inorganic antimicrobial compounds and their mixtures (with emphasis on molecules used in agriculture). Copper and propolis have already been evaluated, with quite encouraging results, but with limited specificity of the application (Bigiotti et al. 2019). However, a large-scale application of antibiotics may be quite unsuitable, due to various withdraws, including the high costs and the broad spectrum of the majority of them, as well as the concerns about public health and the associated antibiotic resistance in environmental organisms. The development of alternative approaches with potential to perturbate the symbiotic basis may include small molecule antagonists, peptide/protein toxins or RNAi-mediated suppression of insect genes, which underpin these associations (Arora and Douglas 2017). Such approaches, in terms of applied research, will combine big data analysis with cutting-edge functional validation methodologies, towards the creation of "smart" biopesticides (symbiocides) (Arora and Douglas 2017). Taken together, the release of all these potential gene targets may facilitate the design

and the implementation of novel tools in smart applications, aiming the disruption of the symbiotic relationship between *B. oleae* and *Ca.* E. dacicola, or other bacterial partners, through a bait trap system or directly applicable, with antimicrobial compounds and/or dsRNA, which will target key gene partners of this symbiosis.

The “basic research question” for understanding the mechanism allowing the olive flies to utilize olives for their existence, may have applications in the long term, via targeting certain genes and pathways, which might inhibit the development of the olive flies in unripe olives. The recent advent of the CRISPR/Cas9 genome modification tool (Koidou et al. 2020) and the advances in sequencing methods for *B. oleae*, can provide the opportunity to address the molecular basis of symbiosis in a systemic manner, as well as to apply the acquired knowledge for the development of novel pest control strategies that will go beyond the traditional approaches.

4.7. Figures

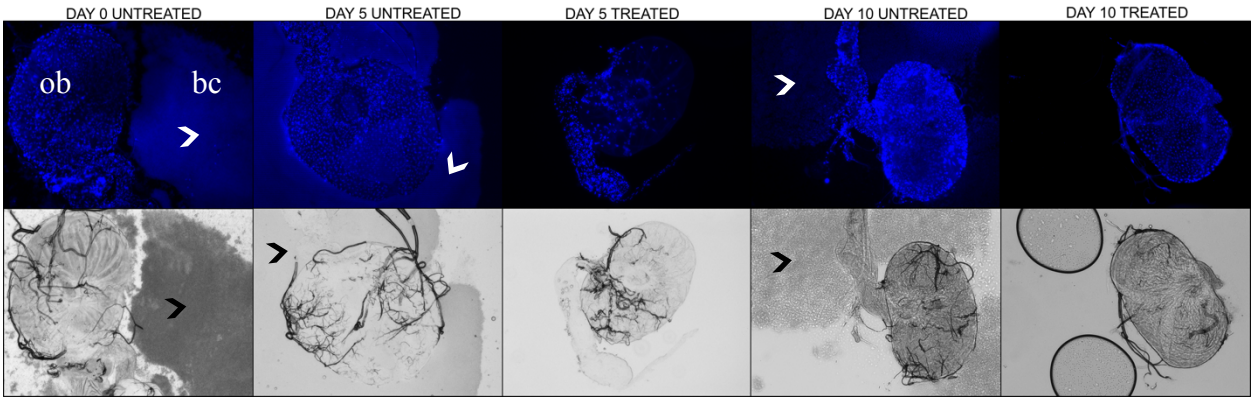


Figure 4.5. Fluorescent microscopy revealed an elimination of the bacterial cells in the majority of the oesophageal bulbs that were examined. During the ten-day antibiotic treatment, treated and untreated (DAY 0) females were sacrificed and the oesophageal bulbs were dissected out of their heads, in order to detect any possible presence of the bacterial symbiont partners. On the fifth day of the treatment, 14 out of 20 dissected bulbs were devoid of bacterial cells, whereas 6 bulbs still carried bacteria. On the final day of the treatment (DAY 10), 17 out of 20 dissected bulbs were devoid of bacteria, whereas 3 bulbs still carried bacteria, compared to the untreated-control samples; ob: oesophageal bulb, bc: bacterial "cloud".

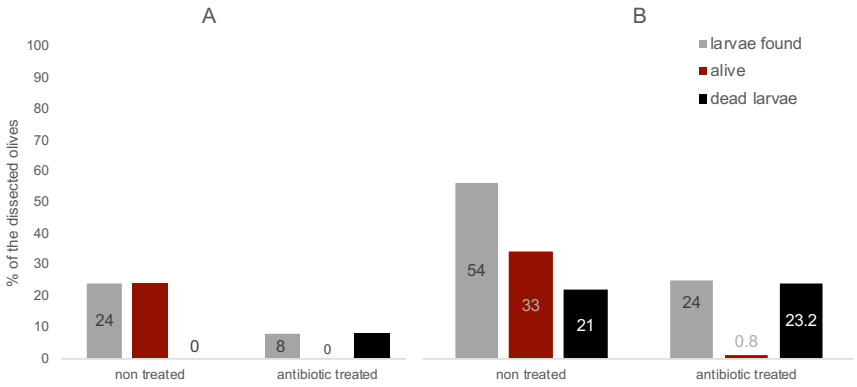


Figure 4.6. Fitness cost of piperacillin treated olive flies. 5-7 days after the oviposition of G0 antibiotic-treated female flies, the olives were dissected and the fitness cost of the developing G1 larvae was examined. Very few larvae emerged from antibiotic treated G0 mothers and almost all of them showed a developmental arrest at first instar larval stage, in contrast to the untreated control samples, where only alive second instar larvae were detected, during the same time frame; (A) small cage experiment (B) big cage experiment; y axis: % of the olives that were dissected.

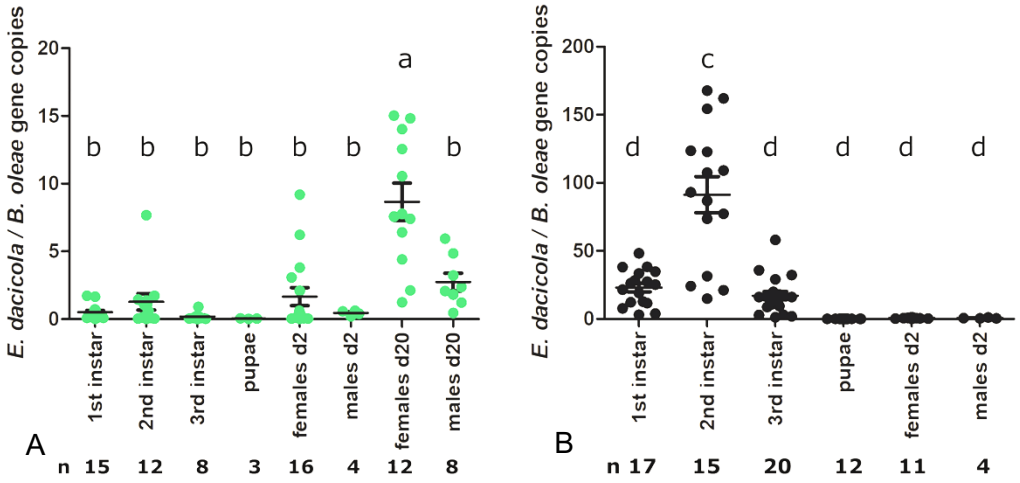


Figure 4.7. Real time qPCR analysis revealed fluctuations of *Ca*. E. dacicola in field populations developing in unripe (green) and ripe (black) olives. The abundance of the major bacterial partner *Ca*. E. dacicola was estimated relatively to the *actin* gene of *B. oleae*. Each sample consists of a pool of five individuals. Number of samples (n) is shown beneath the X-axis. In total, 390 individuals were collected and tested from unripe olives and 395 from ripe olives. (A) Samples of *B. oleae* individuals recovered from unripe (green) olives. Individuals from all three larval stages (first, second and third instar larval stage), pupae and adult males and females 2 and 20-day-old were tested. (B) Samples derived from ripe (black) olives. The same stages were tested except that only 2-day-old adults (and not 20-day old) were analysed (ANOVA, p =2.57e-11). A few differences among the developmental stages in both samples from the unripe and the ripe olives were significant (ANOVA, p =2.57e-11, and Tukey HSD test p<0.001). More specifically, the *Ca*. E. dacicola levels of the 20-day-old females developing in unripe olives (group “a”) were significantly higher when compared to the other developmental stages of individuals developing in unripe olives (group “b”). Likewise, the bacterial levels in the second instar larvae developing in ripe olives (group “c”) were significantly higher compared to other developmental stages of individuals developing in ripe olives (group “d”). Error bars, S.E.M.

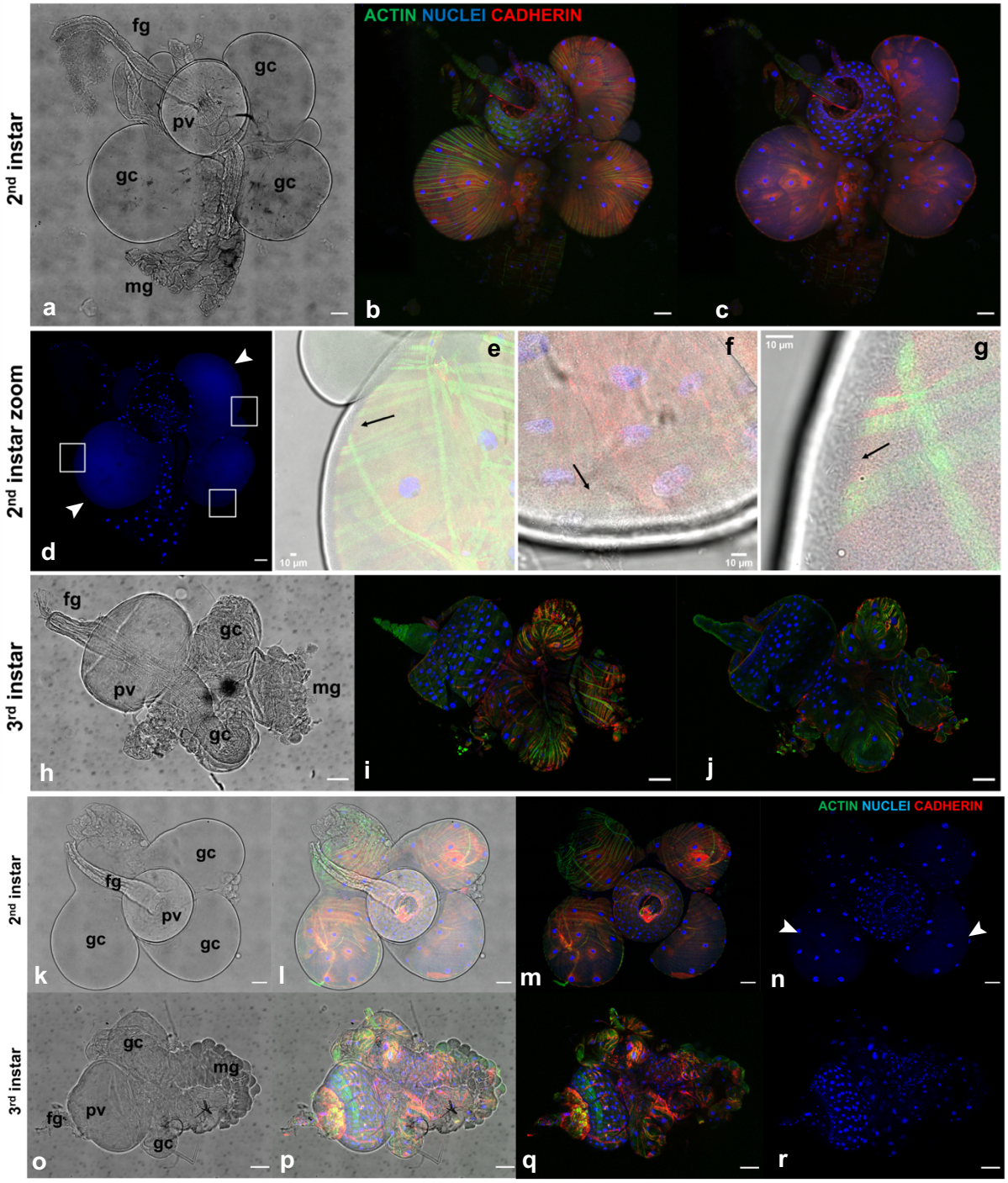


Figure 4.8. Localization of the bacterial symbionts in larvae developing in ripe olives. Dissected gastric caeca from second (a-g, k-n) and third instar (h-j, o-r) larvae were analysed with confocal scanning microscopy. Blue color marks nuclei (DAPI), green actin filaments (phalloidin) and red E-cadherin; a, h, k, l: Bright-field images; b, c, i, j, m, n, q, r: Optical sections from two or three (two bottom lines) different focal planes of each sample; d, n, f: Confocal sections with only the blue channel (DAPI) reveals a cloud of bacterial cells inside the gastric caeca (white arrowheads, d, n); black arrows mark the bacterial cells. Scale bars in a-d, h-j: 100 µM, e-g: white boxes indicate the zoom panels with higher magnification, scale bars: 10 µM. fg, foregut; gc, gastric caecum; mg, midgut; pv, proventriculus.

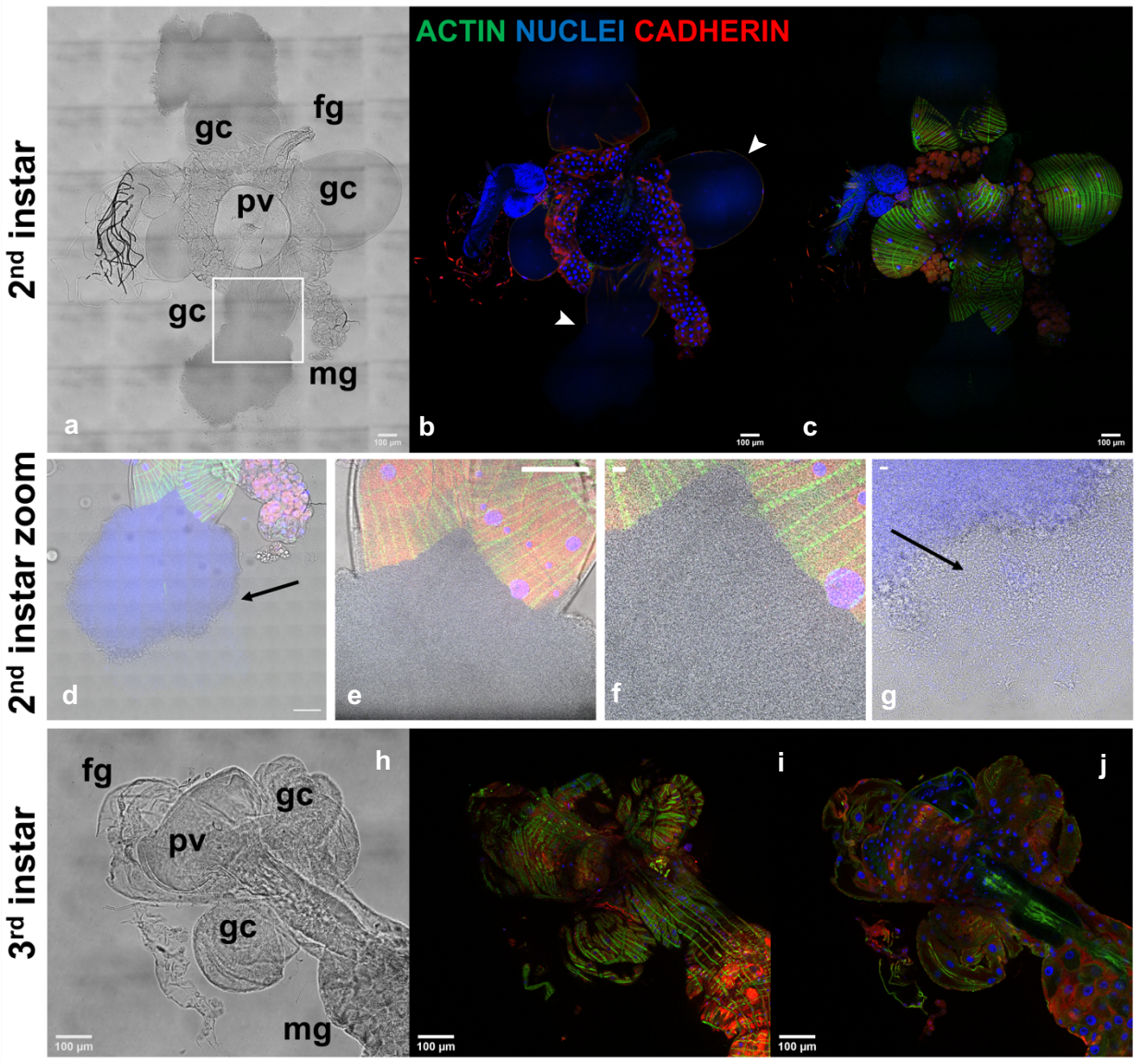


Figure 4.9. Localization of the bacterial symbionts in larvae developing in unripe olives. Dissected gastric caeca from second (a-g) and third instar (h-j) larvae were analysed with confocal scanning microscopy. Blue color marks nuclei (DAPI), green actin filaments (phalloidin) and red E-cadherin; a, h: Bright-field images; b, c, i, j: Optical sections from two different focal planes of each sample; b: Confocal section only with the blue channel reveals a cloud of bacterial cells inside the gastric caeca (white arrowhead) only in second instar larvae; black arrows mark the bacterial cells (d and g); Scale bars in a-d, h-j: 100 µM, e-g: 10 µM. fg, foregut; gc, gastric caecum; mg, midgut; pv, proventriculus.

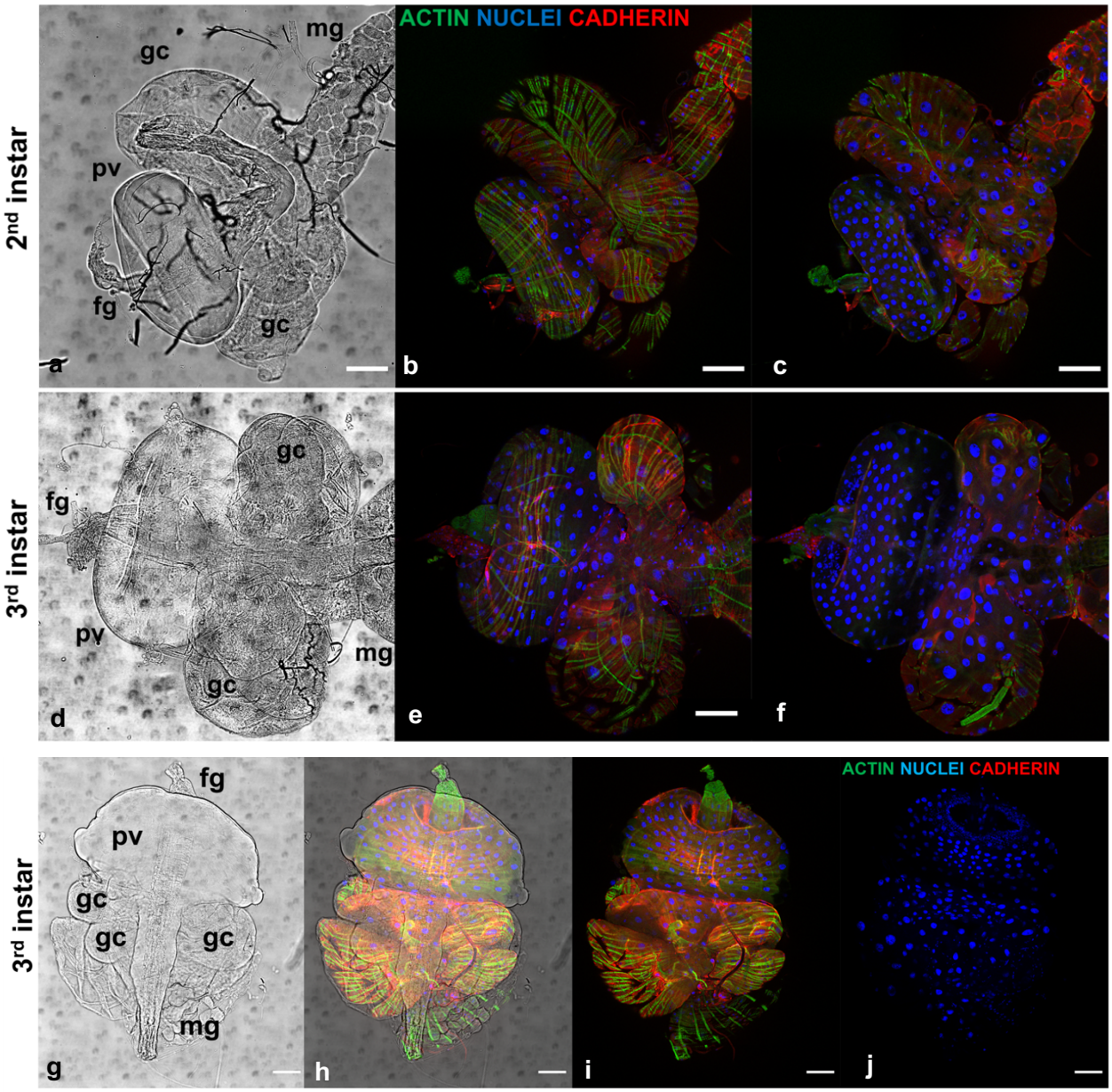


Figure 4.10. Localization of the bacterial symbionts in laboratory reared-hybrid larvae. Dissected gastric caeca from second (a-c) and third instar (d-f) larvae from the HYB strain were analysed with confocal scanning microscopy. Blue color marks nuclei (DAPI), green actin filaments (phalloidin) and red E-cadherin. a, d, g: Bright-field images; b, c, e, f, h, I, j: Optical sections from two or three (bottom row) different focal planes of each sample; Scale bars a-j: 100 μM. fg, foregut; gc, gastric caecum; mg, midgut; pv, proventriculus.

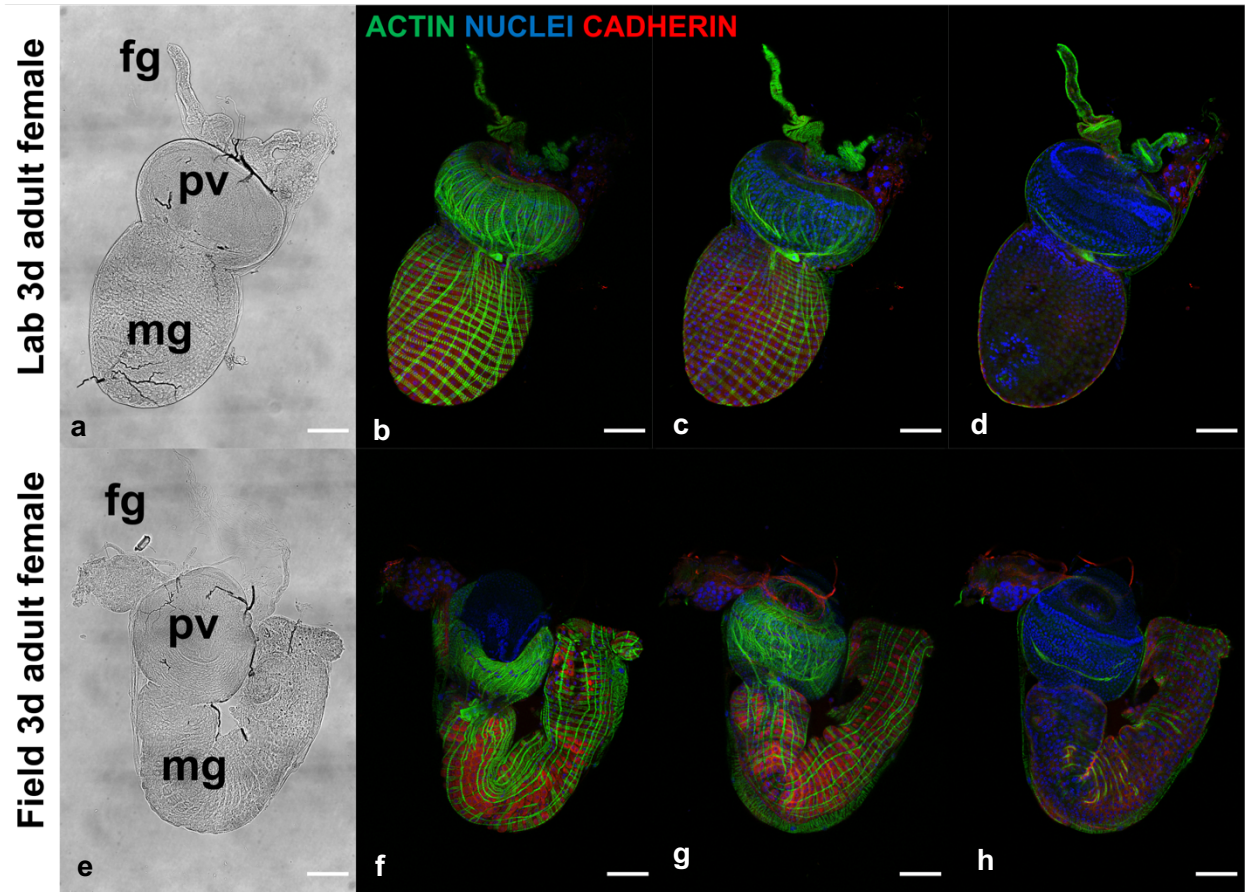


Figure 4.11. Localization of the bacterial symbionts in the gut of young adult olive flies. Dissected gut from laboratory-reared 3-day-old hybrid adults (a-d) and adults which had developed in black olives (e-h) were analysed with confocal scanning microscopy. Blue color marks nuclei (DAPI), green actin filaments (phalloidin) and red E-cadherin. The gastric caeca epithelial cells were reported eradicated, probably histolyzed; a, e: Bright-field images; B-d, f-h: Optical sections from three different focal planes of each sample; Scale bars: 100 μM; fg, foregut; gc, gastric caecum; mg, midgut; pv, proventriculus.

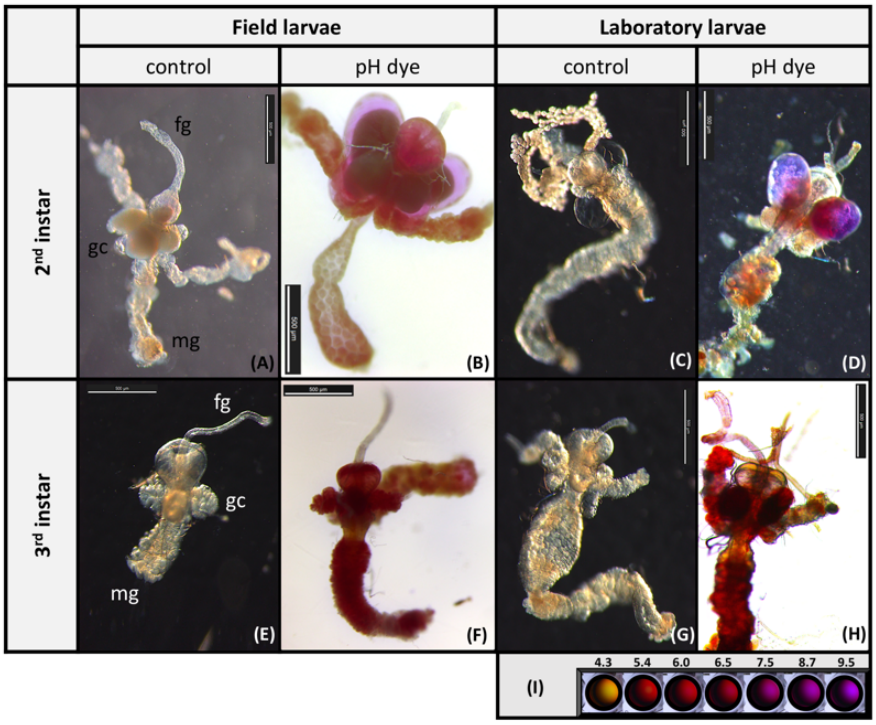


Figure 4.12. Investigation of the pH of gastric caeca in olive fly larvae. Alimentary canals from second and third instar olive-developing and hybrid larvae were stained with Bromocresol Purple pH indicator dye. Dissected organs without staining represent control images. For interpretation of the colours, standard buffered solutions containing the dye were prepared (lower right). fg, foregut; gc, gastric caeca; mg, midgut; gc, gastric caeca; Scale bars, 500 µm. (A-D) Part of the gut from 2nd instar olive-developing (A, B) and laboratory-reared hybrid (C, D) larvae with (A, C) and without (B, D) pH indication dye incubation. (E-H) Same for gut parts dissected out of 3rd instar larvae (I) Buffered standard solutions ranged from pH values 4.3 to pH 9.5, containing 0.1% Bromocresol Purple.

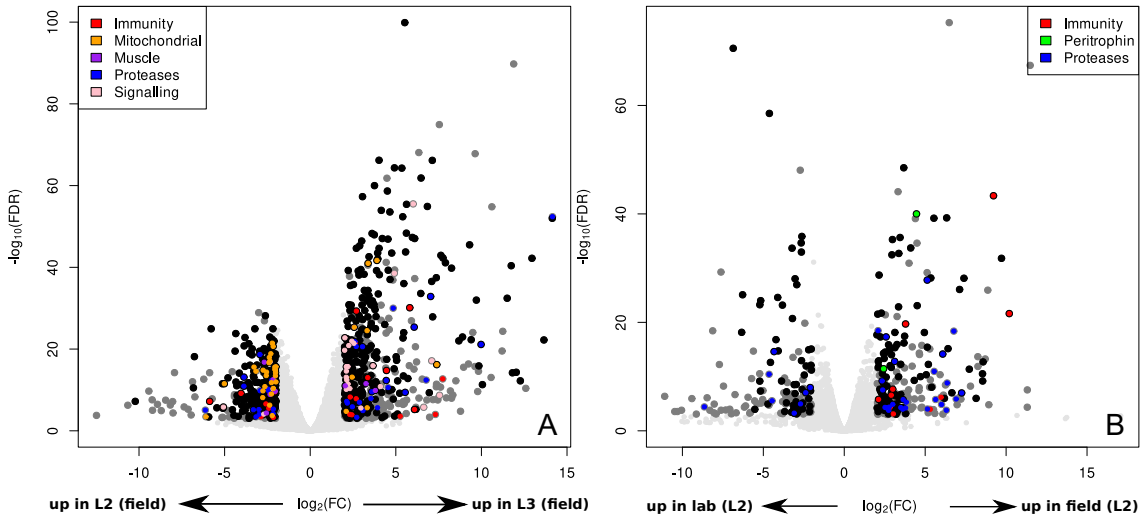


Figure 4.13. Summary of the differentially expressed genes. Each dot represents one gene, with the x-axis showing the magnitude of the up-regulation as the logarithm of the fold change (log_2FC), while the y-axis shows the negative logarithm of the false discovery rate (FDR), which represents the statistical significance of the up-regulation. Differentially expressed genes are shown in dark grey and black, with the latter corresponding to nearly full-length genes that are also not lowly expressed. A. Summary of differentially expressed genes between gastric caeca of the two different developmental larval stages L2 and L3, from olive-developing larvae. Genes with a negative log_2FC are upregulated in the gastric caeca of second instar (L2) larvae (left side), whereas genes with a positive log_2FC (right side) are upregulated in the third instar (L3) larvae. Colored dots highlight genes of interest (see Results and Discussion for more details). B. Summary of the differentially expressed genes between gastric caeca of L2 larvae from field-collected (developing in olives) and HYB populations (laboratory-reared). Genes with a negative log_2FC are upregulated in the hybrid population (left side), whereas genes with a positive log_2FC (right side) are upregulated in the field population. Colored dots highlight genes of interest (see Results and Discussion for more details).

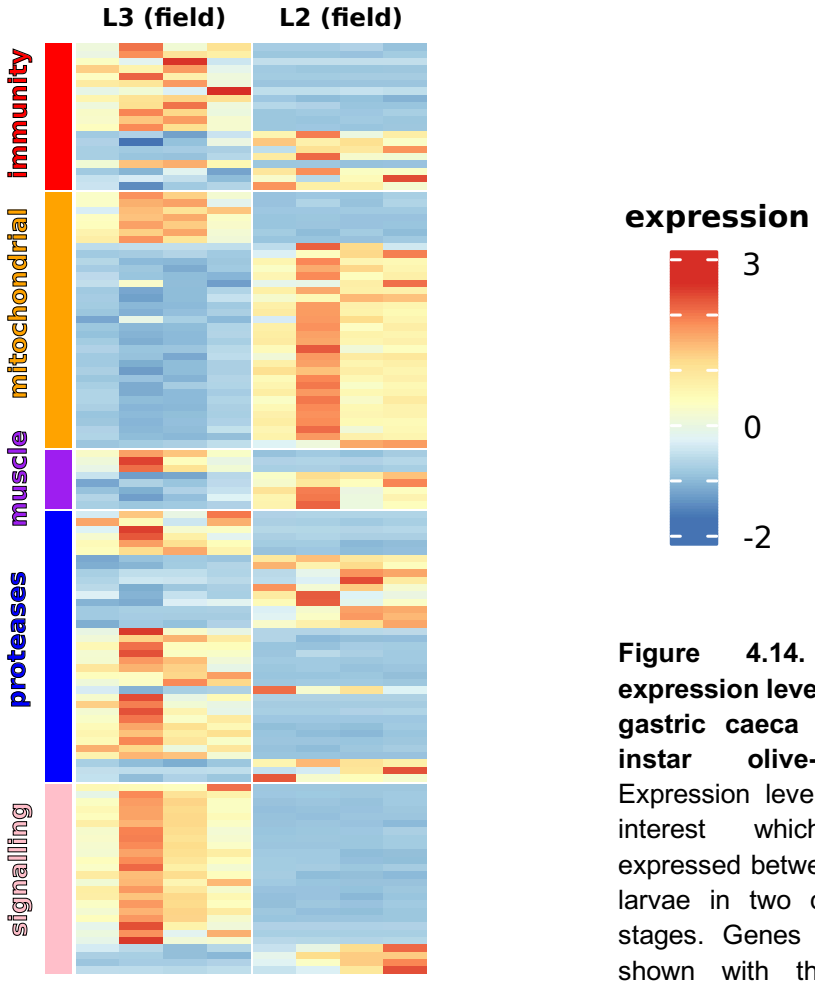


Figure 4.14. Differential gene expression levels of selected genes in gastric caeca of second and third instar olive-developing larvae. Expression levels of specific genes of interest which are differentially expressed between the gastric caeca of larvae in two different developmental stages. Genes from five families are shown with their expression levels depicted as normalized z-scores.

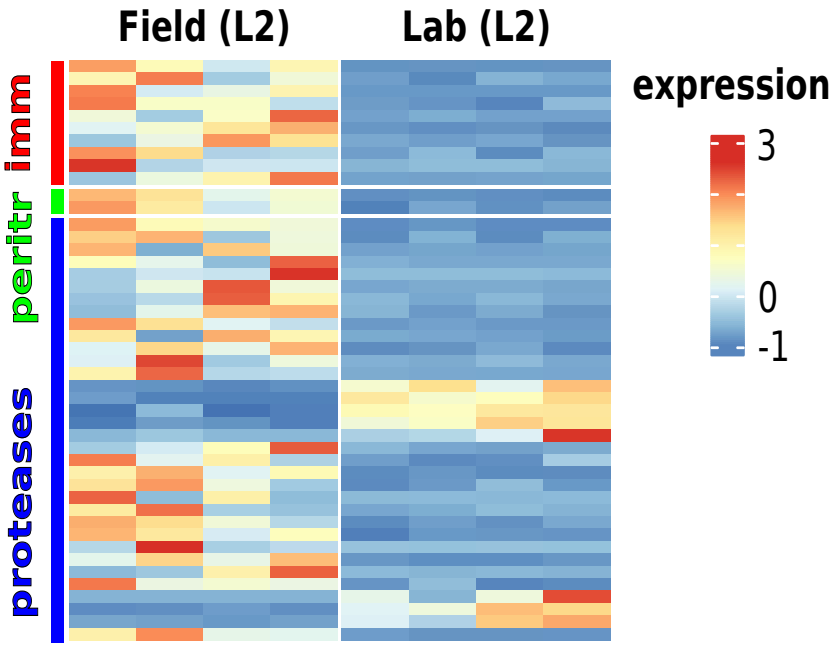


Figure 4.15. Differential gene expression levels of selected genes in gastric caeca of second instar olive-developing and laboratory reared larvae. Expression levels of specific genes of interest that are differentially expressed between the gastric caeca of larvae in two different populations. Genes from three families are shown with their expression levels depicted as normalized z-scores.

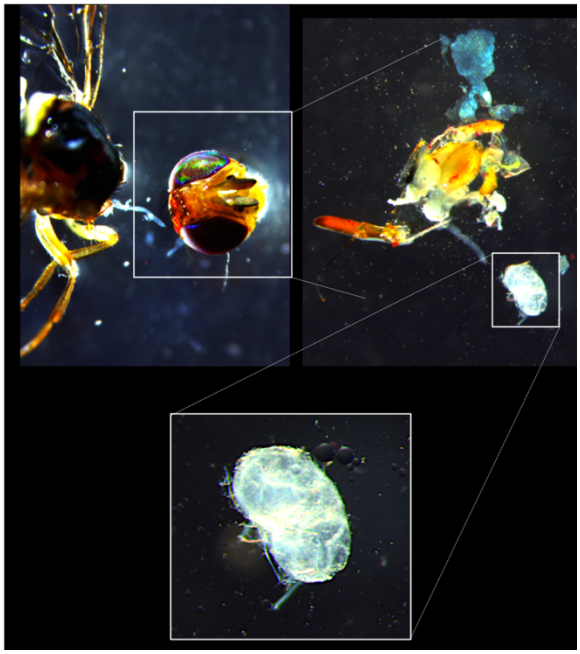
4.8. Supplementary material

Supplementary Table ST4.1. Piperacillin antibiotic diet (M. Ben-Yosef et al. 2014; Michael Ben-Yosef, Jurkevitch, and Yuval 2008)
○ 50ml DDW ○ 10g sucrose ○ 150mg yeast hydrolysate ○ 5.5mg piperacillin

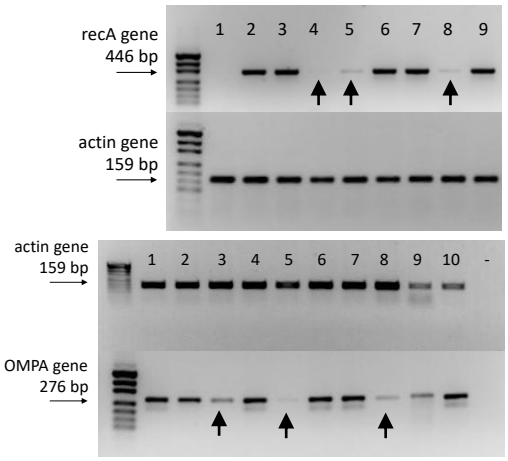
Supplementary Table ST4.2. PCR primers (5' > 3')	
Bo_actinF	CGGTATCCACGAAACCACAT
Bo_actinR	ATTGTTGATGGAGCCAAAGC
Ed_recAF	TCTACCGGTTCGTTATCCCTG
Ed_recAR	AACGATAATCACGTCAACAGCAC
Ed_OmpAF	AGGATGAAGCAGCTGCTCCAG
Ed_OmpAF	GGTCAACTACAGACTGTGCACG
Ed_q_recAF	TACCGGTTCGTTATCCCTGGATAT
Ed_q_recAR	AACGATAATCACGTCAACAGCACCG

Supplementary Table ST4.3. Viability rates upon antibiotic treatment.

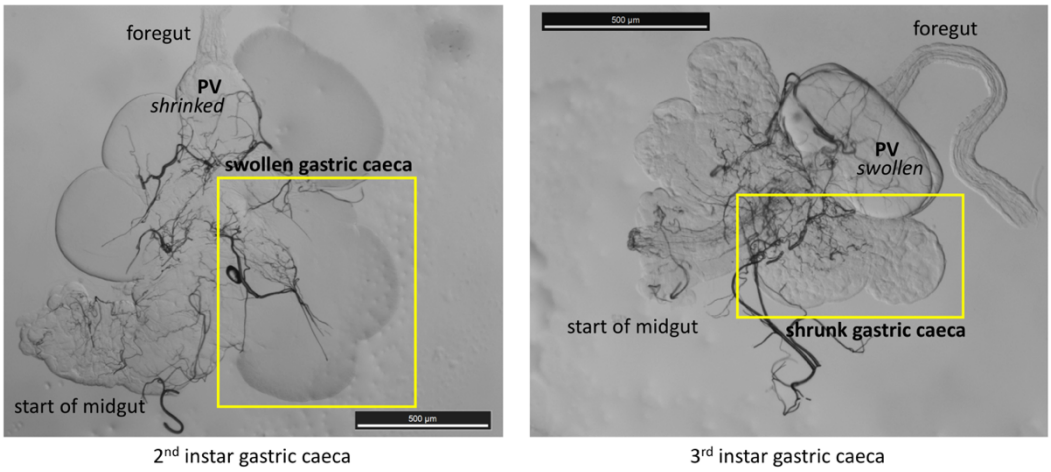
Small cage experiment			**Big cage experiment**		
	NO TREATMENT	**TREATMENT**		**NO TREATMENT**	**TREATMENT**
♀ flies	60	60	**♀ flies**	150	130
olives dissected	114	145	**olives dissected**	105	123
larvae found	27	12	**larvae found**	57	30
% larvae/olives	24%	8%	**% larvae/olives**	54%	24%
alive	27	0	**alive**	35	1
% alive	100%	0%	**% alive**	61%	3%
dead	0	12	**dead**	22	29
% dead	0%	100%	**% dead**	39%	97%



Supplementary Figure SF4.1. The oesophageal bulb of the olive fly. The esophageal bulb, which is located in the cephalic organ of the insect, dissected out of a female adult in PBS 1X, under the stereoscope.

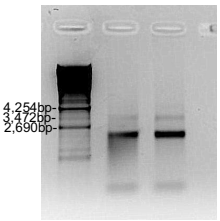


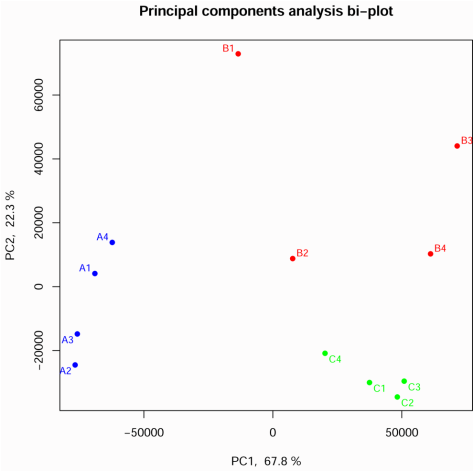
Supplementary Figure SF4.2. PCR reaction for the detection of *Ca.* E. dacicola upon the piperacillin antibiotic treatment. PCR reactions amplifying *Ca.* E. dacicola *recA* and *OmpA* and *B. oleae actin* genes as positive loading control. PCR samples differ between the 2 panels. **Upper panel:** *recA* gene: 1. Lab fly (-control), 2. Day 0 untreated, 3. Day 5 untreated, 4. Day 5 treated, 5. Day 5 treated, 6. Day 10 untreated, 7. Day 10 untreated, 8. Day 10 treated, 9. Untreated field fly (+ control). **Bottom panel:** *OmpA* gene: 1. Day 0 untreated, 2. Day 5 untreated, 3. Day 5 treated, 4. Day 10 untreated, 5. Day 10 treated, 6. Day 10 untreated, 7. Day 10 untreated, 8. Day 10 treated, 9. (+ control) field fly, 10. (+ control) field ob mix, (-) negative control H_2O. The reactions are loaded on a 2% agarose gel.



Supplementary Figure SF4.3. Dissected gastric caeca of second and third instar larvae, developing in olives (ripe). Left panel: Gastric caeca dissected out of a second instar larva. The gastric caeca are filled with bacterial cells, resulting in a milky white colour and a bloated appearance. Right panel: Gastric caeca dissected out of a third instar larva depict a convoluted-shrunk appearance; pv, proventriculus; Scale bar in all panels: 500 µm.

Supplementary Figure SF4.4. 1.2% agarose gel loaded with RNA samples for sequencing, in order to check RNA integrity. 28S (approx. 2kb) and 18S (approx. 3.5kb) bands seem to be in approximately 2:1 ratio, which is a good indication that the RNA is intact. First lane: λ-DNA/Sty I Digest Ladder (EnzyQuest), second lane: 2nd instar field-derived gastric caeca, third lane: 3rd instar field-derived gastric caeca.





Supplementary Figure SF4.5. Principal component analysis (PCA) of gene expression levels for the four replicates from each biological sample. The samples represent gastric caeca from field second instar (L2) and third instar (L3) larvae as well as gastric caeca from HYB (laboratory-reared) larvae (L2). WT L3, A1-A4; WT L2, B1-B4; HYB L2, C1-C4.

Supplementary table ST4.4. Differentially expressed (DE) genes upon comparison of gastric caeca from L2 (2nd instar) and L3 (3rd instar) olive-developing larvae.

Proteases	
Digestive enzymes	**Metalloproteases**
lysosomal aspartic protease	ADAMTS 9
anionic trypsin-1-like	stall
trypsin alpha-3-like	asticin like zinc metalloproteinase
aminopeptidase N	asticin metalloprotease
Nna1 carboxypeptidase	ADAMTS Zinc-dependent
carboxypeptidase B	ER metallopeptidase 1
trypsin theta	neprilysin like
trypsin theta-like	zinc metalloproteinase nas-4
trypsin alpha-like	**Other proteases/ peptidases**
trypsin I-P1	Peroxisomal leader peptide-processing protease
carboxypeptidase B	Gamma-glutamyltranspeptidase
lysosomal aspartic protease-like	ATP-dependent Clp protease
Protease involved in development	protein with peptidase domain
serine proteinase stubble	transmembrane protease serine 9
Protease inhibitors	glutathione hydrolase 1 proenzyme
Kunitz-type serine protease inhibitor	serine protease K12H4.7
Kunitz type trypsin inhibitor	transmembrane protease serine 9
Mitochondrial proteins	
clpX-like	isocitrate dehydrogenase [NAD] subunit alpha
adrenodoxin-like protein 2	60S ribosomal protein L33
alpha-aminoadipic semialdehyde synthase	ATP synthase lipid-binding protein
cytochrome P450 12a4	aminomethyltransferase
Cytochrome P450 12b1,	specific acyl-CoA dehydrogenase
glycerol-3-phosphate acyltransferase 1	malate dehydrogenase
succinate--CoA ligase subunit alpha	aspartate aminotransferase
glutamine synthetase 1	dihydrolipoyllysine-residue acetyltransferase
basic amino acids transporter	ATP synthase subunit delta
aldehyde dehydrogenase X	pyruvate dehydrogenase E1 subunit beta
sulfide:quinone oxidoreductase	enoyl-CoA hydratase
pyruvate carrier 2	ATP synthase subunit O
inner membrane translocaseTim8	ATP synthase-coupling factor 6
D-2-hydroxyglutarate dehydrogenase	fumarate hydratase
ATP synthase subunit alpha	cytochrome c oxidase subunit 7A
D-2-hydroxyglutarate dehydrogenase	cytochrome P450 12b2
ATP synthase subunit g	cytochrome c oxidase subunit 6A
D-beta-hydroxybutyrate dehydrogenase	
Signaling proteins	
Ror	PA-phosphatase related DDB_G0275547
tyrosine-phosphorylation-regulated kinase 2	orphan steroid hormone receptor 2
orphan steroid hormone receptor 2	ecdysis-triggering hormone receptors
hepatocyte growth factor-regulated tyrosine kinase substrate	hormone receptor 4
phosphatidylinositol 4-kinase type 2-alpha	LLR GPCR5
serine/threonine-protein kinase S6KL	glycoprotein hormone receptor
S6 kinase like	nuclear hormone receptor FTZ-F1
serine/threonine-protein kinase mig-15 isoform X7	juvenile hormone epoxide hydrolase 1
serine/threonine-protein kinase mig-15	Juvenile hormone esterase
isoform X9 ## serine/threonine-protein	myogenesis-regulating glycosidase
kinase mig-15 isoform X10	inositol-3-phosphate synthase
protein kinase 3	dual specificity protein phosphatase 18
serine/threonine-protein kinase minibrain	protein phosphatase 2C 52
serine/threonine-protein kinase grp	serine/threonine-protein phosphatase 2B
inositol-trisphosphate 3-kinase	phosphatidate phosphatase
Muscle function	
myosin heavy chain 95F	myosin heavy chain, muscle
MICAL-like protein 2	myosin light chain alkali
muscle segmentation homeobox	troponin C
myosin-VIIa	

Supplementary table ST4.5. Differentially expressed (DE) genes upon comparison of gastric caeca from L2 olive-developing (2nd instar) and L2 hybrid (laboratory-reared) larvae (2nd instar).

Digestive enzymes
chymotrypsin-2
chymotrypsin-2
trypsin theta-like
trypsin alpha-3-like
trypsin
trypsin
trypsin alpha-like
trypsin
dipeptidase 1
serine carboxypeptidase
zinc carboxypeptidase-like
lysosomal aspartic protease-like
carboxypeptidase B
anionic trypsin-1
isoaspartyl peptidase/L-asparaginase GA20639
aminopeptidase N
zinc carboxypeptidase
Carboxypeptidase B
Peritrophins
peritrophin-44-like
peritrophin-48

Metalloproteases
zinc metalloproteinase nas-13
zinc metalloproteinase nas-4
ER metallopeptidase
Protease inhibitors
Kunitz type trypsin inhibitor
Kazal type serine protease inhibitor
chymotrypsin inhibitor
serine protease inhibitor 42Dd
Other proteases
Protein with trypsin and CLIP domains
Peptidase C1A subfamily like
transmembrane protease serine 2-like
serine protease K12H4.7
transmembrane protease serine 9
serine proteinase stubble
serine proteinase stubble
angiotensin-converting enzyme
serine protease gd

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Chapter 5

General Discussion and Future Research Aspects

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The objective of this PhD thesis was to provide new insights and characterize potent mechanisms implicated in insecticide resistance and in the interactions of the olive fly, *Bactrocera oleae,* the major pest of olive orchards worldwide, with symbiotic bacteria of the species *Candidatus* Erwinia dacicola. The thesis (apart from the general introduction and the discussion chapters), is divided in three sections.

In the **first section,** entitled **"The development and application of the biotechnology-based CRISPR/Cas9 tool in olive fly, *Bactrocera oleae*: a proof-of-concept approach"**, we report the first successful demonstration of the CRISPR/Cas9 gene editing tool in *B. oleae,* by targeting the *scarlet* eye-color gene. Embryo micro-injections of the Cas9/sgRNA complex, which targeted the *scarlet* coding sequence, yielded multiple heritable genetic variants, and the mutants showed a striking shift in eye color phenotype from dark-red-brown to yellow. We establish that this technology is applicable to this non-model organism, and can be further exploited for the better comprehension of various molecular mechanisms (i.e. insecticide resistance and symbiosis) and the subsequent development of alternative genetic tools and strategies, in order to efficiently control this destructive tephritid species.

The **second section** entitled **"Characterization of genes associated with insecticide resistance in *B. oleae*"** was further divided in two parts. In the first part we attempted the functional validation of the Δ3Q mutation in acetylcholinesterase gene (AChE), which has been associated with organophosphorus resistance. The deletion of three amino acids in the last exon of AChE (Δ3Q) has been associated with organophosphorus (OP) resistance in *B. oleae,* after field screens and *in vitro* experiments, since 2008 (Kakani and Mathiopoulos 2008). However, this hypothesis has not been supported with *in vivo* evidence yet. The Aim of our study was to investigate this hypothesis *in vivo*, and more specifically by introducing the Δ3Q mutation, with the CRISPR/Cas9 gene editing system, which was developed in the previous chapter, in a susceptible genetic background of a laboratory-reared *B. oleae* strain and study the mutant phenotype upon OP insecticide application.

For this purpose, a strategy was implemented in which purified and commercially available Cas9 protein along with multiple sgRNAs and a synthetic donor DNA template (ssODN) were administered into early olive fly embryos by microinjections, in order to knock-in the Δ3Q, by homologous recombination (HDR mechanism). Prior to the embryo microinjections, the components were evaluated firstly *in vitro*. The results showed that although *in vitro* five out of the total seven sgRNAs direct the Cas9 to the desired sites, in order to cleave the DNA and integrate the donor template (including the Δ3Q instead of the WT 5Q, in the end of AChE), the corresponding result was not accomplished *in vivo*. Specifically, a total of approximately 2,174 olive fly embryos were injected and sequenced in groups. Sequencing results did not show any DNA cleavage. Subsequently, we didn't exceed to confirm or dismiss the association of this 9-bp deletion in the olive fly AChE gene, with resistance to OPs, with *in vivo* data. Further investigation

is required, since any solid functional *in vivo* evidence that proves the implication or not of this mutation in OP resistance, will contribute in an improved pest management strategy.

These experimental data, combined with the low efficiency of the mutagenesis method which was initially observed in the previous chapter, indicate that in order to successfully introduce mutations and study them functionally with the CRISPR/Cas9 genome modification tool, the knock-in method needs to be improved. The micro-injection efficiency, the hatching rate and the screening methodology for the detection of incorporated mutations, through sequencing or other visualization tools, are only a few of the fields that require further improvements. Upgraded directions will include the establishment of *B. oleae* transgenic lines, which will express endogenously and under tissue specific promoters the Cas9 nuclease, similarly to *Drosophila* and aim to improve the efficiency of the method, as well as facilitate the injection and the screening procedures. An alternative approach may include the delivery of the Cas9 RNP complex straight to the insect's germline, namely ReMOT (Receptor-Mediated Ovary Transduction of Cargo), through the injection of adult females, avoiding the injection of early embryos. Such protocols have been already applied in mosquitos (Chaverra-Rodriguez et al. 2018; Macias et al. 2020) and a Lepidopteran species (Hunter, Gonzalez, and Tomich 2018) and soon will expand in other arthropod species.

In the second part of the **second chapter**, we searched for new genes of *B. oleae,* implicated in pyrethroid resistance mechanisms, using transcriptome sequencing on olive fly malpighian tubules (MTs), one of the proposed detoxification tissues in insects. The objective of this study was to compare gene expression in MTs, between an insecticide resistant and a susceptible olive fly strain and identify candidates not immediately apparent in whole organism RNA sequencing data.

In an attempt for a better understanding of the insecticide detoxification in olive flies, MTs were dissected out of two populations; a resistant to α-cypermethrin field population and a susceptible laboratory-reared strain. As expected, many genes that are well known to be implicated in insecticide resistance to pyrethroid compounds in other insect species were identified. Moreover, several other genes were also identified, but their possible role in the detoxification of the insecticides remains to be elucidated via further investigation. The highlighted in the RNAseq data over-expression of two P450 genes, known to be implicated in DDT resistance in *D. melanogaster* (CYP4P6 and CYP6G28) (Seong, Coates, and Pittendrigh 2019; Fusetto et al. 2017), were further validated with quantitative PCR analysis. The functional characterization of one of them (CYP4P6), revealed a promising phenotype of increased mortality rates (21% mortality compared to 4% of the control) upon gene silencing and subsequent exposure to α-cypermethrin. Taken together, this unique MT-specific RNA sequencing dataset revealed several promising genes, possibly associated with the olive fly detoxification of the pyrethroid compounds. The results of this work will contribute to a better understanding of the mechanisms conferring pyrethroid resistance in the olive fly, upon their functional characterization with *in vitro* assays, which will confirm their potent implication (i.e. expression studies in *E. coli*, baculovirus and *D. melanogaster* heterologous systems and insecticidal substrate metabolism assays).

In the **third section** entitled **"Unraveling the unique adaptation of the olive flies to olive fruits, via the symbiotic relationship with bacteria of the species *Candidatus* Erwinia dacicola",** the aim was to gain further information underlying the symbiotic relationship between the olive fly and its bacterial symbiont partners, especially *Ca*. E. dacicola, through qPCR, transcriptomic profiling and confocal microscopy analysis, depending on the fly developmental stage and the presence or absence of the symbiont partner.

The determination of the relative abundance of *Ca.* E. dacicola during the life cycle of the olive fly, from the larval to the adult stage, comparing flies developing in unripe and ripe olives, assayed with qPCR analysis, revealed that the bacterial titre is fluctuating between the different developmental stages. Higher abundance of the major symbiont partner was detected during the second instar larval stage, followed by a decrease during the third instar stage to pupal transition and finally an increase during the adult stage, in both populations which were examined, confirming older and more recent data (Anne M. Estes et al. 2012; Ben-Yosef et al. 2010; Hammer and Moran 2019; Jing, Qi, and Wang 2020; Campos et al. 2022).

Furthermore, a unique tissue transformation was reported with the use of confocal scanning microscopy, between two different larval stages of insects developing both in unripe and ripe olives, as well as under artificial diet and laboratory rearing conditions. Larvae at second instar stage were characterized with distended gastric caeca, filled with a thick bacterial mass, the bacterial symbiont partner *Ca.* E. dacicola, while during the third instar stage the gastric caeca lobes were much smaller and convoluted. This finding suggests that more likely an activation of the gastric caeca muscles rather than the housing symbiotic bacteria *per se* consists the catalytic factor for this transformation pattern. Similarly, the symbiont partner *Ca.* E. dacicola does not affect the pH of the gastric caeca, upon gut pH measurement assays, which revealed a common pH pattern between gastric caeca of larvae developing in olives and under artificial rearing systems.

Moreover, through microscopy observations, we report the bacterial cells extracellularly, in the lumen of the gastric caeca, rejecting a previous indication about their intracellular localization (A. M. Estes et al. 2009). Furthermore, imaging analysis of adult tissues (same part of the gut) revealed a completely eradicated state of the gastric caeca lobes, probably due to histolysis, confirming previous indications (Hammer and Moran 2019). Additionally, this observation is in consistency with previous ones, confirming the different tissue that houses the symbiont partners of the olive fly during adulthood, the oesophageal bulb, which is located in the cephalic organ (Capuzzo et al. 2005).

Furthermore, the transcriptomics analysis on the gastric caeca of second and third instar larvae that were developing in olives (field samples), in comparison to gastric caeca obtained from a laboratory strain, devoid of *Ca.* E. dacicola, revealed many potential genes, probably with an important role in the symbiotic relationship, between the olive fly and the major symbiont bacterial partner. In brief, two pairwise comparisons were set in order to identify genes potentially involved in the developmental transition between the larval stages of the host and to identify olive fly genes potentially involved in the establishment and regulation of this symbiosis, since wild-type insects

contain huge numbers of the bacterial partner, contrary to the laboratory-reared ones, and subsequently significant changes in the transcript expression levels were reported. The analysis of the differentially expression levels highlighted critical groups of genes, including genes encoding proteases, mitochondrial proteins, proteins involved in signalling pathways and in muscle function, peritrophins, as well as genes related to immunity and response to microbial infection (detailed reviewed in the Results and Discussion parts of the fourth chapter).

However, functional characterization experiments are required for a better understanding of the functions and their putatively roles of these genes in symbiosis; part of the genes that were reported in this study can possibly be functionally validated with the use of the newly established protocols of reverse genetics approaches, the gene knock-down (RNAi) and the gene knock-out (CRISPR/Cas9) molecular tools, and confirm a possible implication in this unique interaction.

Future approaches

Since the fundamental inquiry referring to the mechanism allowing the olive flies to utilize toxic unripe olives remains, new pest control strategies which will act via targeting certain genes and pathways, either of the host or of its symbiont partners, and focus on the disruption or inhibition of this symbiotic relationship, will be in the heart of the up-coming research.

The sequencing data provided in the frame of this PhD thesis can be further mined in order to gain a deeper understanding of the olive fly biology. Up-coming experimental approaches could provide insights for the crucial larval developmental stages and shed light on the molecular basis of the strict monophagy which is observed in this pest species. On the other hand, insecticide resistance can be further studied by functionally characterizing promising gene candidates, which are not yet defined to be involved in the detoxification of insecticides. New protocols developed for reverse genetics approaches like the RNAi, or the CRISPR/Cas9 which were established here, will be considered as indispensable post genomic tools for the scientific community. Candidate genes and mechanisms (detoxification enzymes, immune response genes etc.) can be further characterized by an integrated experimental approach and devoid of the withdraws of the heterologous expression in different systems.

Furthermore, taking under consideration the provided detailed view of the abundance of the major bacterial partner *Ca*. E. dacicola in the olive fly, during the host developmental stages, and according to the ripening level of the olives, practical field methodologies, such as copper or propolis applications, may be tested at critical time points. Since the bacteria are substantially reduced when the larva exits the olive mesocarp, such applications, which aim to target the symbiont partner, may have a higher impact if applied then; reduction of the bacterial load, will subsequently result in a potent reduction of the olive fly population.

Moreover, future research directions aiming to target microorganisms that the insect definitely necessitates, resulting in insect mortality or reduced levels of its development or fecundity, as this was partially discussed in Chapter 4, can be accompanied through three strategies (Arora and Douglas 2017): (a) the construction of heterologous associations with microbial partner(s) that do not naturally associate with the focal insect species, (b) the genetic modification of the microbial

partner(s); and (c) the perturbation of the microbial partners. For each strategy, two applications are proposed: the suppression of the insect pest population and the modification of the insect traits. Concerning the first strategy (a), the first step in olive flies has been already demonstrated; a previous study has reported the successful transinfection of *Wolbachia* in an olive fly strain, inducing cytoplasmic incompatibility (CI) and subsequent embryonic mortality (Apostolaki et al. 2011).

Furthermore, since the major obligate symbiont partner of olive fly, *Ca.* E. dacicola, is an un-culturable bacterial species (A. M. Estes et al. 2009, Petri 1909), the genetic manipulation of it cannot be accomplished. However, future approaches may include the genetic manipulation of other obligate microbial partners of the host, since the whole spectrum of the symbiont partners of the olive flies has just been released (Campos et al. 2022). Genetic technologies can be applied to modify microorganisms in a way that they express traits that are virulent to the host, commonly referred as Trojan horse strategy (Arora and Douglas 2017). Two classes of genetic elements have been used to confer specific toxicity against insect pests: dsRNA against essential genes of the host and protein toxins produced by bacterial species (*Photorhabdus* and *Xenorhabdus*) associated with entomopathogenic nematodes. Previous studies have shown that when the Enterobacteriaceae BFo2 was transformed with dsRNA against the insect α-tubulin gene and then administered to *Frankliniella accidentalis*, insect mortality was significantly elevated (Whitten et al. 2016). Additionally, the Tc toxin of the entomopathogenic bacterium *Photorhabdus*, which was engineered into a culturable bacterial symbiont, *Enterobacter cloacae*, of the termite *Coptotermes formosanus*, caused high levels of mortality to the host (Zhao et al. 2008). Since *Enterobacter sp.* represents an obligate symbiont of *B. oleae* too (A. M. Estes et al. 2009), similar approaches may potently be applied. A different strategy may include the use of a horizontally-transmissible gut microorganism, as a delivery vehicle of microbicidal agents against the microorganisms required by the insect, combining the previously described approaches (Nobre 2019). Previous studies have reported similar techniques (Husseneder, Donaldson, and Foil 2016; Tikhe et al. 2016). Nevertheless, the several “less obvious” horizontally transferred symbiont partners of the olive fly might be considered in an attempt to design approaches to manage this insect pest.

Moreover, the elimination of the vertically-transmitted obligate symbiont partner *Ca.* E. dacicola (previously discussed in Chapter 4) may involve the use of specific symbiocides i.e. effectors (conventional antibiotics or inorganic antimicrobial compounds) or small molecule antagonists, peptide/protein toxins and RNAi-mediated suppressors of insect genes, which possess critical roles in the symbiotic relationship (Arora and Douglas 2017).

Taken together, the data provided though this study, including the recent development of a genome modification technology for application in olive flies (CRISPR/Cas9) and the resistance-specific and symbiosis-specific released gene datasets, provide the opportunity to study the molecular basis of insecticide resistance and symbiosis-based mechanisms, of this pest species, through versatile approaches, and moreover, utilize the acquired knowledge towards the development of innovative pest control strategies, that will go beyond the traditional approaches and that will manage to efficiently control this destructive pest species.

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