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[Bouvet, J. P. R., Urbaneja, A., Pérez-Hedo, M., & Monzó, C. (2019). Contribution of predation to the biological control of a key herbivorous pest in citrus agroecosystems. *Journal of Animal Ecology*, 88(6), 915-926.]

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The final publication is available at

[\[https://doi.org/10.1111/1365-2656.12982\]](https://doi.org/10.1111/1365-2656.12982)

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Running title: Role of predation in perennial crops

Submitted to: Journal of Animal Ecology

Contribution of predation to the biological control of a key herbivorous pest in citrus agroecosystems

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Abstract

1. Biological control has traditionally simplified the view of trophic relationships between herbivorous pests and their natural enemies in agriculture. The success or failure of this pest management strategy is still mainly attributed to the ability of a few key natural enemies to suppress the pest density. For example, successful regulation of the California red scale (*Aonidiella aurantii*) a key citrus pest, is generally credited to specific parasitoids of the *Aphytis* genus. Currently, research is revealing how herbivore regulation in agroecosystems can be alternatively achieved with a greater number of trophic associations within the system.
2. The goals of the present study were: i) to unravel species-specific trophic links between *A. aurantii*, and its natural enemies in citrus agroecosystems, and ii) to assess their contribution to control of *A. aurantii*.
3. Predation and parasitism of this herbivorous pest were assessed through exclusion experiments. Species-specific trophic links between this herbivorous pest and its natural enemies were studied using gut-content analysis of field collected predators employing prey specific DNA molecular markers. Relative predation rates of the species involved in *A. aurantii* regulation were estimated.
4. Predation was found to be the main biotic component of *A. aurantii* mortality, causing reductions of more than 75% in recently settled cohorts. *Aonidiella aurantii* DNA was detected in the digestive system of 11 species of predators. Generalist and stenophagous predators, mainly associated with other citrus pests such as aphids, proved to be the most important biological control agents of this pest. Complex trophic relationships, such as apparent competition between two key citrus pests, were revealed.
5. The present study highlights the role of predation as biotic mortality factor of key pests in perennial agroecosystems; wherein, it is a rich complex of indigenous or naturalized generalist predators that are primarily responsible for this mortality. The results herein

presented may therefore offer another perspective on the biological control of one of the key worldwide citrus pests; at least in those regions where specific parasitoids are not able to successfully regulate the scale populations.

Keywords: Conservation biological control, exclusion, DNA prey detection, PCR, predator assemblages, integrated pest management, apparent competition, citrus, California Red Scale

Introduction

In recent years pesticide use in agriculture has become increasingly limited due to new territorial regulations (such as the European Regulation (EC) n° 1107/2009), market restrictions, environmental and human health concerns. Globally, these pressures have driven the implementation of less intensive pest management approaches, such as biological control (Tscharnkte et al., 2012). Conservation biological control is currently considered to be the most promising strategy for sustainable pest management in perennial crops (Rusch, Bommarco & Ekbom, 2017). Throughout recent decades the main foci of pest management research have become the development of pesticides which are more selective, the study of habitat complexity and arthropod community associations in agroecosystems, and the study of trophic relationships of natural enemies and herbivorous arthropod pests (Bale, van Lenteren, & Bigler, 2007; Gentz et al., 2010; Woltz, Isaacs & Landis, 2012; Dominik et al., 2018; Shields et al., 2018).

Predators have always been highly valued as natural enemies in agriculture. However, their complex biology and ecology has frequently hindered assessment of their true role as biological control agents (Symondson et al., 2002; Furlong & Zalucki, 2010). Theoretical developments were, at first, mostly concentrated on specialist predators, since these systems are easier to parameterize (Hassell & May, 1986; Van Driesche et al., 2010). Nevertheless, in recent decades, there is growing interest in the control exerted by associations of mainly indigenous and naturalized generalist predators who are recognized to have the ability to keep prey densities at stable equilibriums in non-density-dependent ways (Symondson et al., 2002, Harwood & Obrycki, 2005).

Direct observations in the evaluation of predatory activity are often very laborious and time consuming (Luck, Shepard & Kenmore, 1988); traits such as the high mobility of predators and their cryptic activity makes this task highly difficult. Interference with the observed phenomenon is also a risk associated with the observation approach (Symondson et al., 2002; Hodek, Emden & Honěk, 2012). *In-situ* post-evaluations usually underestimate the importance

of predation as a biotic mortality factor since frequently no prey remains are found after predation occurs (Sorribas & García-Marí, 2010).

Indirect methods offer an opportunity to assess the impact of predators on the population of a specific pest both quantitatively and qualitatively without directly interfering with the predation events. Quantifications are mostly based on comparisons between pest densities in the presence or absence of the predators (Michaud & Harwood, 2012; Harwood & Obrycki, 2005). Exclusion of natural enemies has been achieved with pesticides that selectively affect them and not the pest (pesticide exclusion design) and with experimental units that physically protect the pest (exclusion cage design) (Luck, Shepard & Kenmore, 1988; Monzó, Qureshi & Stansly, 2014). Predator gut-content studies permit the identification of components of the predator guild that are actually contributing to the predation of the target pest (Symondson et al., 2002). Among the existing *post-mortem* techniques, prey DNA detection by polymerase chain reaction (PCR) has proven to be a powerful tool. This technique is employed using species specific primers that amplify multicopy DNA gene regions such as mitochondrial cytochrome oxidase I (COI) or Internal transcribed spacer 1 (ITS1). With PCR one can obtain important information on trophic interactions that may otherwise be challenging to acquire (Agustí et al., 2003; King et al., 2008; Monzó et al., 2011; Wolf et al., 2018).

Citrus are perennial crops with high potentials to maintain agroecosystem functions and services. Not surprisingly, modern biological control research in agriculture began in this crop (Debach & Rosen, 1991). The high stability of citrus agroecosystems allows the harboring of rich complexes of natural enemies with the potential to keep most of the associated phytophagous species under economic injury levels, almost certainly through complex ecological interactions (Ciancio & Mukerji, 2010). Despite this, there are few research projects aimed at disentangling the trophic relationships between these phytophages and their potential natural enemies (Gómez-Marco et al., 2016; Pérez-Sayas et al., 2015). The success or failure of biological control

has commonly been attributed to the role of a reduced number of specialist natural enemies (Murdoch et al., 2005; Pekas, 2010; Sorribas & García-Marí, 2010).

There is a minor group of phytophagous species associated with citrus that escape the regulation by their natural enemies; control of them then relies mostly on the use of pesticides (Gómez-Marco et al., 2016; Tena et al., 2011). In the western Mediterranean citrus region, the California Red Scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) is one such pest and is consequently considered to be a key pest in the crop (Tena et al., 2011). Biological control of this pest is mostly attributed to specialist scale parasitoids of *Aphytis* genus (Hymenoptera: Aphelinidae) and to some specialist coccinellid species such as *Chilocorus bipustulatus* (Linnaeus, 1758), *Rhyzobius lophanthae* (Fabricius, 1787), and *Coccidophilus citricola* (Brèthes, 1905) (Hattingh & Samways, 1992; Murdoch et al., 2005; Sorribas & Garcia Mari, 2010). Conservation and augmentative strategies for these natural enemies have been developed and implemented in numerous citrus growing areas of the world (Vanaclocha, Verdú & Urbaneja, 2011; Dao et al., 2017). Some indigenous generalist predators such neuropterans have also been cited as potential natural enemies of this pest (Alvis, 2003). However, little is known of their impact.

The aim of this study was to unravel the role of predation in key pests (*A. aurantii*) associated with perennial citrus crops by quantifying the importance of species-specific trophic links employing, among others, molecular techniques. Specifically, i) the contribution of predation as a biotic mortality factor of *A. aurantii* was weighted using exclusion techniques and ii) the relative importance of potential predators was assessed through detection of prey DNA in their gut contents.

Materials and methods

Contribution of predation as A. aurantii mortality factor

Experiments were conducted in two citrus orchards in the region of Valencia, Spain; one in Moncada (39°35'17.43"N / 0°23'53.28"O; 0.18 ha) and one in Algimia (39°42'55.11"N /

0°18'57.46"O; 0.25 ha), during the 2014, 2015, and 2016 seasons. Blocks contained Clementine mandarins (*Citrus clementine* Hort. Ex Tan.), all approximately 15 years old. Trees were drip-irrigated and had not been treated with insecticides for at least two years before the experiment nor during the course of the study. The presence of *A. aurantii* had been recorded in both orchards for several years before the study.

The *Aonidiella aurantii* individuals used in the experiments were taken from a laboratory colony reared on lemons at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Valencia, Spain). This colony was initiated in 1999 from scale insects collected in the citrus fields of Alzira (Valencia, Spain). New individuals from the fields surrounding IVIA were added every 2–3 years to ensure genetic diversity of the experimental population.

The contribution of predation as a mortality factor of *A. aurantii* in citrus was assessed through exclusion techniques. Experiments were replicated twelve times: in both of the two orchards, one replicate during each of the three annual *A. aurantii* field generations, for two seasons. *Aonidiella aurantii* generations were predicted using the degree-day model proposed by Asplanato & García-Marí (2001) and Carot et al., (2003) with the data obtained from <http://gipcitricos.ivia.es>. In each experiment, *A. aurantii* cohorts were artificially initiated on leaves (1st, 2nd and 3rd generations) and fruit (2nd and 3rd generations only, as fruit is not yet present during the 1st generation). Females from the IVIA colony were synchronized by infesting between 100 and 125 lemons 50 days prior to the field experiments; during this period the females were maintained at 27° C and 75% HR. Under these conditions, females were expected to reach maturity just before the onset of each experiment. In each orchard, 10 to 13 trees with no *A. aurantii* present were randomly selected and four fruit and/or leaves, one per cardinal orientation were chosen to initiate the artificial cohorts. Lemons hosting gravid females from the IVIA colony were attached to the field selected fruit and/or leaves with adhesive tape and left for three days to facilitate the infestation of first instar nymphs (crawlers). In each orchard 45 colonies per plant part (fruit or leaves), containing between 10 and 30 recently settled *A.*

aurantii first instars, were selected and randomly assigned to the following treatments: i) 'predation' (colonies exposed to natural enemies), ii) 'exclusion' (colonies protected from natural enemies with a 45 x 20 cm muslin bag, and iii) 'semi-exclusion' (colonies covered with a muslin bag, open at one end to allow access of natural enemies; thus the same conditions as the exclusion treatment were mimicked).

Scale growth within cohorts was checked weekly; treatments were left in the field until females reached adult stage (42 to 60 days). At this point, leaves and/or fruit hosting the cohorts were removed from trees and taken to the laboratory. The number of scales present in each colony, as well as their developmental stage, evidence of predation, parasitoid host-feeding and parasitized scales were verified with stereoscope microscopy and compared to the initial number of attached first instars.

Seasonal-activity of predators

To identify the arthropod predatory species associated with *A. aurantii* biotic mortality and to estimate their relevance, the species present in the study citrus groves were monitored weekly. Monitoring was done throughout each *A. aurantii* generation by stem-tap sampling from the beginning to the end of the exclusion experiments. Stem-taps consisted of striking eight randomly selected branches (two per cardinal orientation) with a PVC pipe three times on 10 to 13 trees adjacent to those chosen for the exclusion trials. Arthropod predatory specimens collected in a 45 x 30 cm plastic tray placed under the stricken branches were individually moved to dram containers by using an entomological aspirator (Pooter). The containers were then filled with ethanol (80%) for specimen preservation. Collected material was immediately taken to the laboratory and identified to the species level with stereoscope microscopy. Specimens of interest for the gut-content studies were carefully rinsed; first with water and then with ethanol (80%). Cleaned specimens were individually stored in Eppendorf tubes containing 1 ml of ethanol (80%) and kept in the freezer at -20°C for posterior gut-content examination using molecular analyses.

Molecular gut-content analysis

Total DNA was extracted from 3 *A. aurantii* individuals of IVIA's colony following a modified Salting Out protocol (Monzó et al., 2011). DNA was then amplified by PCR (Polymerase Chain Reactions) using the universal primers for the mitochondrial gene cytochrome c oxidase subunit 1 (COI) LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al., 1994). PCR amplifications were performed in a thermal cycler (Eppendorf Mastercycler) for 35 cycles at: 95°C, 1 min; 45°C, 1 min; 72°C, 1.3 min. The 20 µl PCR reaction volumes contained: 1 µl of DNA from the *A. aurantii*'s extraction, 2 µl buffer (10x Standard Reaction, Biotools), 0.7 µl MgCl₂ (Biotools), 0.4 µl dNTPs (10 mM each 1 ml, Thermo Scientific), 0.4 µl of each primer, 1 µl of Taq DNA polymerase (1000 units - 1 U/µl, Biotools) and 14.10 µl free water. Firstly, the denaturation cycle was carried out at 94°C for 2 min, and lastly the extension cycle at 72°C for 10 min. The double-stranded PCR products were purified (NucleoSpin® Gel and PCR Clean-up; Macherey-Nagel) and sent to Institute of Molecular and Cellular Biology of Plants (IBMCP, Valencia, Spain) for sequencing. *Aonidiella aurantii* species specific primers were later designed and named as CRSCOI primer pair (CRSCOIF1 and CRSCOIR1), with these sequences using Primer3web 4.0.0 software (<http://primer3.ut.ee>). The oligo selected were later contrasted with that of other organisms to evaluate their specificity employing BLAST on the GenBank website (<https://blast.ncbi.nlm.nih.gov/>).

Primers were employed in a final volume of 20 µL which contained 10 ng total extracted DNA, 1x Standard Reaction buffer with MgCl₂ (Biotools B&M labs S.A.), 200 nM dNTPs (Thermo Fisher Scientific, Inc.), 0.4 µl of each primer, and 1 u Taq DNA polymerase (Biotools B&M labs S.A.). The amplification profile was as follows: an initial denaturation step at 94 °C for 4 min; 35 cycles of 92 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s; and a final extension step at 72 °C for 4 min. Amplifications were performed in a Mastercycler® ep gradient-S thermal cycler (Eppendorf).

Primer specificity was tested by assaying CRSCOIF1 – CRSCOIR1 primers with total DNA extracted from several individuals of two strains of *A. aurantii* (laboratory colony and field

strain), several closely related species, and a wide range of arthropods (prey and predators) potentially present in the same citrus agroecosystem. Two PCRs per sample were run. Sensitivity was determined by assaying primers with ten-fold dilutions of *A. aurantii* total DNA starting with 10 ng and progressing to a 1:10,000 dilution.

Stored material from the exclusion studies (see section '*Sampling of predators*') was used for the predator gut-content evaluations. The most abundant predator species and *A. aurantii* specialist predator species cited in the literature were selected (Hattingh & Samways, 1992; Vanaclocha, Verdú & Urbaneja, 2011). Prior to the DNA extraction, to reduce the proportion of predator/prey DNA in each tested specimen and to minimize risks of topical prey DNA contamination, the selected specimens were dissected under a stereoscope microscope. Heads, thoraxes and legs were removed; thus only abdomens were used for the DNA extraction (Monzó et al., 2011). DNA was later extracted following modified Salting Out Protocol and each sample was tested in duplicate by PCR using CRSCOI primers. For any case with contradictory results (6.6% of the samples) a third PCR was also conducted.

Statistical analysis

To calculate the reductions in numbers, *Aonidiella aurantii* recovered at the end of each experiment were compared to the initial numbers of first instars which had settled in each cohort of the three treatments: protected ('exclusion'), semi-exposed ('semi-exclusion') and exposed ('predation'). Parasitized scales and host-feeding damage from scale parasitoids were also recorded to differentiate between the two main sources of *A. aurantii* biotic mortality: parasitism and predation.

Differences in *A. aurantii* mortality between treatments ('predation', 'semi-exclusion' and 'exclusion') were studied using generalized linear mixed model (GLMM) analysis (Wolfinger & O'Connell, 1993) with a binomial error distribution of the variable. A multiplicative overdispersion parameter was added to the model. Mortality in the 'exclusion' treatment, in which cohorts were protected from natural enemies, was considered to be the natural mortality.

Differences in *A. aurantii* mortality (dependent variable) between seasons (year 1 and 2), locations (Moncada and Algimia) and plant parts (fruit and leaves) (fixed effects) in the 'exclusion' treatment (natural mortality) were also compared using GLMM analysis with a binomial error distribution of the variable. Percentage mortality in exposed cohorts corrected for mortality in protected cohorts was estimated using the Henderson and Tilton formula (Henderson & Tilton, 1955). Corrected mortality was therefore the one attributed to predators (Monzó, Qureshi & Stansly, 2014). Differences between seasons, *A. aurantii* generations (1st, 2nd and 3rd), locations, and plant parts (fixed effects) on *A. aurantii* corrected mortality (dependent variable) were studied using GLMM analysis. The interaction between the variables 'season' and 'generation' was also included in the model. Normal distribution of the variable was assumed based on Akaike and Bayesian information criteria.

Influence of *A. aurantii* generation, season, and location (fixed effects) on the seasonal-activity of the citrus predatory complex and on each predatory group (Araneae, Coleoptera, Dermaptera, Hemiptera, and Neuroptera) (dependent variables), monitored through stem-tap sampling, were evaluated using GLMM analyses with a binomial error distribution, and with two-way interactions between all fixed effects. The cases in which interactions were not significant were later removed from their respective models.

The predation rate of each predatory species on *A. aurantii* was estimated by multiplying the proportion of specimens testing positive in the PCR analyses in each generation, by the number of individuals collected through stem-tap sampling. The total predation rate of each predatory group and of the whole predatory assemblage was calculated by adding up the estimated predation rates of all the species included in each group. Changes in the importance of predation from all predators and of each predator group separately on *A. aurantii* was studied through GLMM analyses with 'predation rate' as the dependent variable and 'generation' as the explanatory variable. Negative binomial error distribution of the variable was selected in all the cases based on Akaike and Bayesian information criteria.

Two GLMM were used to determine if changes in the mortality of *A. aurantii* attributed to predation could be explained by the predator abundance and the predation rate estimated from the molecular analyses. In the first model *A. aurantii* corrected mortality was dependent on predator abundance (fixed factor), whereas in the second model corrected mortality was dependent on the estimated predation rate (fixed factor). In both models 'generation' was also included as a fixed factor. Normal distribution of the variables was assumed based on Akaike and Bayesian information criteria. Akaike information criterion (AIC) was used to check whether the model including 'predation rate' as response variable offered a better predictive accuracy of *A. aurantii* mortality than the model including 'abundance' as response variable (Aho et al. 2015).

Kenward and Roger Satterthwaite approximation for degrees of freedom was included in all the models of this study (Kenward & Roger, 1997). Post-hoc t-test (Tukey) comparisons were made in each case having a significant effect ($P < 0.05$) for all analyses.

Results

Contribution of predation as A. aurantii mortality factor

According to the degree-day model the three *A. aurantii* generations were predicted to reach of their maximums at the following dates: first generation: 10/06/2015 and 12/06/2016; second generation: 29/07/2014 and 27/07/2015; and third generation: 16/09/2014 and 18/09/2015 (**SS1**). Exclusion trials were therefore set at those dates as indicated in **table 1**.

Overall 652 *A. aurantii* cohorts were used for this study; 216 in the 'exclusion' treatment, 208 in 'semi-exclusion' and 228 in the 'predation' treatment. Mortality found in the cohorts under exclusion (natural mortality) was around 60% (**fig. 1**). No differences were found between generations ($F = 0.61$; $df = 2, 210$; $P = 0.54$) and seasons ($F = 0.07$; $df = 1, 210$; $P = 0.79$). Natural mortality was nevertheless different between locations ($F = 4.27$; $df = 1, 210$; $P = 0.04$) and higher on the leaves than on the fruit ($F = 13.94$; $df = 1, 210$; $P < 0.01$). Mortality within cohorts was

influenced by treatment ($F= 91.07$; $df= 2, 649$; $P< 0.01$). The highest mortality was found in the 'predation' treatment and the lowest in the 'exclusion' treatment; the 'semi-exclusion' treatment presented *A. aurantii* mortality levels between those found in the other two (**fig. 1**). Mortality attributed to parasitism accounted for less than 3% and 6% of all mortality in the 'semi-exclusion' and the 'predation' treatments respectively. No evidence of host feeding was observed.

Corrected mortality owing to predation of *A. aurantii*, on the other hand, ranged between 51% and 97% throughout the study. No differences in corrected mortality were found between plant parts ($F= 0.51$; $df= 1, 220$; $P= 0.48$), sites ($F= 0.57$; $df= 1, 220$; $P= 0.45$) and only marginal differences were observed between seasons ($F= 3.48$, $df= 1, 220$, $P= 0.06$). Corrected mortality was affected by *A. aurantii* generation ($F= 4.21$; $df= 2, 220$; $P= 0.02$); being higher during the first generation (**fig. 2**).

Seasonal-activity of predators

More than 10,000 specimens of potential *A. aurantii* predators from five taxonomic orders were collected by stem-tap sampling throughout the experiments (**SS2**). Araneae was the most diverse and frequently encountered predatory group; it accounted for 40% of all specimens collected; it presented more than 20 species. Coleoptera was the second most diverse and frequently found group with 18 species identified; all of which belong to Coccinellidae; it represented 34% of all specimens. Only 5 hemipteran species were found, but they accounted for 18.6% of all the predators captured. Neuroptera and Dermaptera were the least captured and diverse groups accounting for only 7% and 0.4% of captures; 4 and 2 species, respectively.

No differences in the mean number of predators per stem-tap and tree were found for any of the taxonomic groups between seasons (**table 2**). In general, predators were more frequently found in Moncada than in Algimia; with differences between taxonomic groups. Araneae and Hemiptera were more frequently found in Moncada than in Algimia; whereas the

contrary was observed for Coleoptera, Neuroptera and Dermaptera. Predators were most frequently found during the first *A. aurantii* generation with further differences between taxonomical groups. Coleoptera and Hemiptera were most frequently found during the first *A. aurantii* generation, whereas Araneae was mostly captured during the last generation (**fig. 3**). No differences between generations were found for Dermaptera and Neuroptera captures.

Molecular gut-content analysis

For the molecular analyses, the five most frequently found species of Coccinellidae and Araneae (except *Dipoena melanogaster* that is an ant predator) (Simon, 1997) were selected. In addition, the already known specific coccinellid predators of *A. aurantii*, *Rhyzobius lophanthae*, *Coccidophilus citricola* and *Chilocorus bipustulatus* were also included in the study. The three most frequently captured Hemiptera and Neuroptera species and the only two Dermaptera species captured were also chosen for study.

Sequenced *Aonidiella aurantii* COI fragments were deposited in the GenBank database (accession number: MH384792). The CRSCOI primer pair (CRSCOIF1 and CRSCOIR1) designed on the sequenced COI fragment amplified a 259 bp fragment from *A. aurantii* DNA (**table 3**). Detection threshold on the sensitivity test was of 10^{-5} dilution of *A. aurantii* total DNA.

From the 61 arthropod species tested (31 species were considered to be potential predators), no amplicon was found except in two *A. aurantii* closely related species of the Diaspididae family: *Aspidiotus nerii* and *Chrysomphalus aonidum* (**SS3**).

Aonidiella aurantii DNA was detected in the gut content of 11 of the 21 predatory species from the 1,676 specimens analyzed (**table 4**). *Pilophorus cf gallicus* had the highest proportion of positive detections followed by *R. lophanthae*, *S. aleyrodiformis* and *S. interruptus*. Fewer positive detections were obtained from *C. mildei* and *S. subvillosus* specimens. Positive detections were sporadic in *C. carnea*, *P. cespitum*, *B. chalybeus*, *R. cardinalis* and *I. hamatus* (less than 2% of all the specimens tested).

Changes in *A. aurantii* mortality attributed to predation throughout the season, at the two locations, and the two seasons were more accurately predicted by the model including the 'estimated predation rate' response variable (AIC: 82.69; $F = 6.14$; $df = 1, 8$; $P = 0.04$) than that including the 'abundance' response variable (AIC: 86.23; $F = 5.56$; $df = 1, 8.745$; $P = 0.05$).

According to predator abundance and subsequent PCR positive detections of gut contents, predation rates were estimated to be most relevant to *A. aurantii* biological control during the first generation and least in the second generation (**table 5**). Nevertheless, differences were found between predatory groups. During the first generation, Hemiptera (Miridae) and Coleoptera (Coccinellidae), had the highest predation rates on *A. aurantii*. Hemiptera, the most relevant order, decreased its relevance throughout the season. On the other hand, Coleoptera importance as an *A. aurantii* mortality factor was lower after the first generation, with no differences between the second and the third generations. Neuroptera increased its relevance as the season went on. Spiders presented low and variable relevance between generations.

The estimated relative importance at the species level also changed between *A. aurantii* generations (**fig. 3**). During the first *A. aurantii* generation, only two species, *P. cf gallicus* and *S. subvillosus*, seemed to be highly relevant in the control of *A. aurantii* as detected by positive PCR gut content analysis. In the second and third *A. aurantii* generations, relative abundance of *P. cf gallicus* reduced drastically, but nevertheless remained one of the most relevant predators. *Scymnus interruptus* importantly increased its relevance throughout the season. A similar trend was found with *S. aleyrodiformis* and to a lesser extent with *C. carnea*. The *A. aurantii* specific predator *R. lophanthae* seemed to only significantly contribute to the control of its preferential prey at the end of the season (in the third *A. aurantii* generation) and its relevance in terms of positive detections and seasonal-activity was lower than the generalist predatory species.

Discussion

The present study demonstrates the potential of predation as a determining regulating factor of a key pest in a perennial crop and reveals how this mortality factor is mostly attributed to the combined action of non-specialist predators. Under minimal biological control disruption management conditions in commercial citrus groves, the main biotic component of *A. aurantii* mortality is attributed to its predator complex; causing on average, reductions of more than 75% in recently settled cohorts. Though previous studies have named predation as a component of *A. aurantii* mortality, they were unable to accurately estimate its importance, hence it was typically underrepresented (Sorribas & Garcia Mari, 2010; Vanaclocha, Verdú & Urbaneja, 2011).

Our results present a very different view of the biological control of this herbivore than Murdoch et al. (2005) described for the citrus agroecosystems of California. In that system, *A. aurantii* populations rapidly reached temporal stability, maintaining their populations well below their economic injury levels. These authors attributed this demographic equilibrium to the single interaction between *A. aurantii* and the parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), and conclude that this interaction was purely mediated through life-history and physiological properties of only these two species. Demographic stability was consequently reached locally on each tree without the influence of metapopulation dynamics of both species. In the Western Mediterranean basin, the relationship between *A. aurantii* and *A. melinus* has been widely studied, but the stability in the parasitoid-host system described by Murdoch et al. (2005) has never been observed (Asplanato & García-Marí, 2001; Vanaclocha, Verdú & Urbaneja, 2011; Tena et al., 2011). Various factors, such as specific climatic conditions or the absence of sugar food resources for *A. melinus* adult females at the end of the season, have been named among the potential causes explaining the different parasitoid-host dynamics (Boyero et al., 2014; Tena et al., 2011). The high diversity of natural enemies and herbivores associated with Mediterranean citrus agroecosystems is probably another main difference between our system and that presented by Murdoch et al. (2005). As observed in the present study, *A. aurantii* biological control in Mediterranean citrus agroecosystems may alternatively

be achieved through more complex trophic interactions between this herbivore and a guild of specific and generalist natural enemies. *Aonidiella aurantii* mortality attributed to predation may be overrepresented owing to the difficulty in discriminating between mortality caused by predation and that resulting from probing and host-feeding of the *A. aurantii* specific parasitoids (Vanaclocha, Verdú & Urbaneja, 2011; Cebolla et al., 2018). Nonetheless, in this study, parasitism accounted for less than 5% of total CRS biotic mortality. We therefore believe that this factor would not importantly overrate our estimation of CRS mortality attributed to predators.

Mortality observed in the 'exclusion' cohorts, could be used as an estimation of *A. aurantii* mortality due to environmental conditions or biotic factors such as substrate quality. Although mortality in exclusion treatment seemed to be relatively high (60% of the initially settled nymphs died), values coincide with what has been observed in other studies which surveyed *A. aurantii* mortality factors in citrus crops; thus a potential negative influence of our experimental setup could be discarded (Hare, Yu & Luck, 1990; Vanaclocha, Verdú & Urbaneja, 2011). As expected, mortality in the exclusion treatment was not affected by season, or *A. aurantii* generation, and differences between locations may be explained by specific environmental conditions of each orchard. Natural mortality was also higher on leaves than on fruit. Earlier studies demonstrated higher survival of *A. aurantii* on fruit and concluded that this parameter is mediated by the availability of nutrients (Pekas, 2010; Dao et al., 2017). The mortality of *A. aurantii* attributed to predation was not affected by plant part or location and only partially affected by season.

The predation rate observed in the first *A. aurantii* generation could be explained by: i) climatic conditions are optimal for most predator species in the study area at the end of the spring and beginning of the summer; ii) offspring of the first generation or overwintering generation of *A. aurantii* are usually the least abundant because they are the progeny of

specimens that survived the winter, thus there are less individuals to prey upon (Atkinson, 1977; Campos-Rivela, Martínez-Ferrer & Fibla-Queral, 2012).

The most frequently found natural enemy species during the first generation of *A. aurantii* are those mainly associated with citrus aphids (Ciancio & Mukerji, 2010). The first *A. aurantii* generation coincides with the end of aphid infestations which is during the first and major citrus flushing period of the year (Gómez-Marco et al., 2016). In fact, stenophagous Coccinellidae, Dermaptera and Miridae species associated with aphids had population peaks in this generation, then progressively decreasing in number; whereas Neuroptera and Araneae generalist species that usually do not show a marked prey specific density-dependence (Riechert & Lockley, 1984; Symondson et al., 2002) progressively increased their numbers throughout the growing season as had been previously observed (Monzó, Qureshi & Stansly, 2014).

In contrast to generalist and stenophagous predators, the specialist coccinellid predators of the family Diaspididae, *R. lophanthae*, *C. bipustulatus* and *C. citricola* presented very low seasonal-activity and were mostly concentrated at the end of the season. Prey specific density-dependence of these natural enemies would explain the progressive increase of their activity throughout the growing season in concordance with the build-up of *A. aurantii* populations (Vanaclocha, Verdú & Urbaneja, 2011).

Stem-tap sampling revealed a larger number of potential *A. aurantii* predators present in citrus agroecosystems. Nevertheless, some groups such as phytoseiid mites, dipterans, thrips as well as predator species with nocturnal activity that could also contribute to *A. aurantii* mortality (Vanaclocha, Verdú & Urbaneja, 2011; Campos-Rivela, Martínez-Ferrer & Fibla-Queral, 2012; Sorribas & García-Marí, 2010) cannot be accurately sampled with this methodology, and we can therefore not determine the importance of these species.

The molecular markers designed for the present study (CRSCOI primers) were able to detect *A. aurantii* DNA within the gut content of numerous predator species associated with the

citrus agroecosystem. Although CRSCOI primers were not species specific, the only two positive detections obtained with non-target organisms were found in two closely related Diaspididae species that are not found in citrus groves of the study region (Simon, 1997) and therefore, no risk of obtaining false positive detections could be expected. Seasonal changes in *A. aurantii* mortality attributed to predation were more accurately explained by the estimated relevance of *A. aurantii* predators from the gut-content studies than by their abundance. This consequently illustrates the great utility of applying molecular tools in field studies to better understand the trophic relationships that modulate the demography of economically important pests.

Predator gut-content studies with CRSCOI primers revealed, contrarily to what has traditionally been cited (Murdoch et al., 2005), a relatively rich complex of predators exploiting *A. aurantii*. In addition, most of these species are generalist or stenophagous predators never before cited as important predators of this pest. The use of the information obtained through the PCR analyses to estimate the relative predation rates of different predator groups and species creates a new view on the *A. aurantii* predatory complex contribution to its regulation. The mirid *P. cf gallicus* seems to be the most predator on *A. aurantii* in terms of positive detections and seasonal-activity, especially during the first generation but also throughout the growing season. Species of this genus have been traditionally associated with aphid control and never cited as an *A. aurantii* predator (Schuh & Schwartz, 1988). We speculate that this mirid species exploits *A. aurantii* as an alternative prey source to survive when its preferential prey is not readily available (Schuh & Schwartz, 1988; Hosseini, Schmidt & Keller, 2008). A similar case could be argued for the two *Scymnus* species. These coccinellid species, although previously cited as potential *A. aurantii* biological control agents, are also mostly associated with aphid consumption (Tawfik et al., 1973; Atlihan & Güldal, 2009; Lundgren, 2009; Hodek, Emden & Honěk, 2012). Although these generalist predators may not be the most efficient predators of *A. aurantii*, our analyses show their importance as potential *A. aurantii* biological control agents in citrus agroecosystems. The high predation rate of CRS observed during the first

generations is therefore attributed to predators which have been traditionally associated with aphid control in citrus clementines (Gómez-Marco et al. 2016). This observation unveils the phenomenon of apparent competition between these two key citrus pests, mediated through the action of the shared generalists and stenophagous predator complex (Holt and Bonsall, 2017).

Specialist *A. aurantii* predator species of the Coccinellidae family, in contrast, were seemingly less important as predators on *A. aurantii*. The abundance of *C. bipustulatus* and *C. citricola* was too low to offer a significant contribution to *A. aurantii* control, whereas *Rhyzobius lophanthae* was an important predator at the end of the growing season. Specialist natural enemies typically show a strong prey specific density-dependency and their population densities only increase once the target pest has built up. On the other hand, the late presence of specialists would complement the action of generalist predator species present at the beginning of the season by reducing the number of overwintering *A. aurantii* and thus to lessening the risk of severe demographic increases during the first generation of the following year. Reduction of overwintering specimens by any means in pests that reproduce during the citrus growing season has been demonstrated to significantly affect the demographic potential of the first generation of the year (Qureshi & Stansly 2009).

Chrysopidae and Coniopterygidae families (Neuroptera) have been previously cited as predators of *A. aurantii*. In our study the most captured and relevant species were *C. carnea* and *S. aleyrodiformis*. Both species seem to be more relevant at the end of the season; the latter was one of the most relevant *A. aurantii* predators in this study during the third *A. aurantii* generation. In this and other citrus growing regions similar seasonal-activity trends for this group of predators have been illustrated; however, no information about its predatory activity on *A. aurantii* had previously existed (Alvis, 2003; Monzó, Qureshi & Stansly, 2014).

The probability of obtaining positive detections in predators which had fed upon the target pest is conditioned by the type of digestion of each predatory group and species. For the same molecular markers post-digestion detection can strongly vary between predator species (Monzó et al., 2011). The post-digestion time periods in laboratory studies help to understand this variability. Nevertheless, this kind of evaluation is not viable for studies in which a large predator range is tested. In this sense, the estimated relevance of the species associated with *A. aurantii* control could be partially conditioned by differences in digestion times. According to the extensive literature dedicated to digestion times of different predatory groups; the relevance of spiders may be overrated due to their prolonged digestions times, whereas Miridae and Coccinellidae relevancies would be underrated (Riechert & Lockley, 1984; Agustí, De Vicente & Gabarra, 1999; Hoogendoorn & Heimpel, 2001).

In conclusion, the present study reassess the importance of conservation biological control in perennial crops by highlighting the primary role of predation as the biotic mortality factor of key pests and by exposing how a rich complex of indigenous or naturalized generalist and stenophagous predators is mostly responsible for this mortality. Results herein presented may therefore help to change the biological control management paradigm of one of the key citrus pests worldwide, at least in those areas where specific parasitoids of *Aphytis* genus are not able to successfully regulate their populations, and would serve as an example for other systems similar to this.

Author contribution statement

JPRB, CM, MP-H and AU conceived and designed the research. JPRB and CM participated in data collection and analyses. JPRB and CM wrote the manuscript. All authors read and approved the manuscript.

Acknowledgements

514 Funding for this research: European Union through FP7-PEOPLE-2013-IRSES project (APHIWEB;
515 ID: 611810) and Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana.
516 Technical assistance: Ana Cristina Izquierdo García and Carlos Rubio Bosca.
517 Taxonomic assistance: Armand Matocq (Muséum National d'Histoire Naturelle, France)
518 Citrus groves for the experiments: IVIA and Emilio Pérez Escrig.
519 JPRB was recipient of a research fellowship from INTA-Argentina.
520 CM was recipient of a Juan de la Cierva program grant from Ministerio de Economía y
521 Competitividad, Spain.
522 MP-H was the recipient of a research fellowship from the INIA Spain (Subprogram DOC-INIA-
523 CCAA).
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Tables

Sites	Season	Generation	Establishment date	Ending date	Exposure time (days)
Moncada	1	1	3-June-2015	15-July-2015	42
		2	5-Aug.-2014	16-Sept.-2014	42
		3	3-Oct.-2014	2-Dec.-2014	60
	2	1	4-June-2016	27-July-2016	53
		2	29-July-2015	17-Sept.-2015	50
		3	2-Oct.-2015	26-Nov.-2015	55
Algimia	1	1	1-June-2015	13-July-2015	42
		2	8-Aug.-2014	19-Sept.-2014	42
		3	1-Oct.-2014	2-Dec.-2014	62
	2	1	7-June.-2016	28-July.-2016	51
		2	31-July.-2015	16-Sept.-2015	47
		3	1-Oct.-2015	26-Nov.-2015	56

Table 1. Calendar for the exclusion trials conducted during the three *A. aurantii* generations at two locations and two different seasons. Establishment dates of the experimental units, ending dates of each trial, and exposure time of cohorts in the field are displayed.

		Araneae	Coleoptera	Hemiptera	Neuroptera	Dermaptera	Total predators
Season	1	5.42 ± 0.25a	5.36 ± 0.34a	2.02 ± 0.18a	0.74 ± 0.1a	0.04 ± 0.02a	15.11 ± 0.64a
	2	5.12 ± 0.16a	4.44 ± 0.2b	1.43 ± 0.09b	0.90 ± 0.05a	0.04 ± 0.01a	13.01 ± 0.33b
	F – value	1.1	6.59	10.39	2.72	0.07	9.22
	df	1, 774	1, 771	1, 771	1, 771	1, 771	1, 771
	P	0.30	0.01	0.01	0.10	0.79	< 0.01
Plot	Moncada	6.29 ± 0.23a	3.92 ± 0.21b	3.21 ± 0.21a	0.64 ± 0.06b	0.01 ± 0.01b	14.9 ± 0.51a
	Algimia	4.4 ± 0.17b	6.05 ± 0.29a	0.9 ± 0.08b	1.05 ± 0.08a	0.1 ± 0.02a	13.2 ± 0.44b
	F – value	49.2	37.94	147.19	20.48	11.79	6.74
	df	1, 774	1, 771	1, 771	1, 771	1, 771	1, 771
	P	< 0.01	< 0.01	< 0.01	< 0.01	0.01	0.01
Generation	1	4.6 ± 0.19b	6.09 ± 0.3a	3.77 ± 0.23a	0.71 ± 0.06a	0.06 ± 0.02a	16.63 ± 0.55a
	2	5.2 ± 0.25b	4.65 ± 0.35b	1.06 ± 0.13b	0.87 ± 0.1a	0.03 ± 0.02a	12.84 ± 0.65b
	3	6.15 ± 0.28a	4.09 ± 0.26b	1.22 ± 0.11b	0.9 ± 0.09a	0.03 ± 0.02a	12.91 ± 0.55b
	F – value	11.75	13.14	75.24	1.86	0.76	15.09
	df	2, 774	2, 771	2, 771	2, 771	2, 771	2, 771
	P	< 0.01	< 0.01	< 0.01	0.16	0.47	< 0.01

Table 2. Mean number (±SE) of predators collected per stem-tap and tree during the exclusion trials. Captures are grouped by taxonomical orders, season, study site and *A. aurantii* generation. Different letters within taxonomical groups indicate significant differences between treatments: season, site and *A. aurantii* generation (Tukey's test, P < 0.05).

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Primer	Sequence (5'-3')	Ta (°C)
CRSCOIF1	AATGAAGGAAATAAAAAATCAGAATC	48.4
CRSCOIR1	GGAATTTGATCAGGAATAATAGGAA	50.9

688 Ta, annealing temperature.

689 **Table 3.** Primers designed from COI sequence of *Aonidiella aurantii*

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	Specimens analyzed				% Positives			
	1	2	3	Total	1	2	3	Total
<i>Pilophorus cf gallicus</i>	40	38	27	105	45	57.89	92.59	61.90
<i>Rhyzobius lophanthae</i>	10	2	14	26	40	0	21.43	26.92
<i>Semidalis aleyrodiformis</i>	19	37	40	96	10.53	10.81	17.5	13.54
<i>Scymnus interruptus</i>	40	40	40	120	0	10	15	8.33
<i>Cheiracanthium mildei</i>	40	40	40	120	0	10	5	5
<i>Scymnus subvillosus</i>	40	27	24	91	10	0	0	4.40
<i>Chrysoperla carnea</i>	40	40	33	113	2.5	0	3.03	1.77
<i>Philodromus cespitum</i>	40	40	40	120	0	0	5	1.67
<i>Ballus chalybeus</i>	36	28	34	98	0	3.57	0	1.02
<i>Rodolia cardinalis</i>	40	40	38	118	2.5	0	0	0.85
<i>Icius hamatus</i>	40	40	40	120	0	2.5	0	0.83
<i>Stethorus punctillum</i>	27	35	40	102	0	0	0	0
<i>Coccidophilus citricola</i>	0	1	3	4	0	0	0	0
<i>Chilocorus bipustulatus</i>	0	1	1	2	0	0	0	0
<i>Cryptolaemus montrouzieri</i>	11	34	27	72	0	0	0	0
<i>Conwentzia psociformis</i>	7	7	21	35	0	0	0	0
<i>Orius laevigatus</i>	32	16	40	88	0	0	0	0
<i>Cardiastethus fasciiventris</i>	27	27	30	84	0	0	0	0
<i>Forficula auricularia</i>	12	7	6	25	0	0	0	0
<i>Euborellia annulipes</i>	7	4	8	19	0	0	0	0
<i>Philodromus albidus</i>	40	40	38	118	0	0	0	0
TOTAL				1,676				

Table 4. Total number of predatory arthropods analyzed per *A. aurantii* generation and proportion of positive *A. aurantii* DNA detections by gut-content PCR analysis.

Generation	Araneae	Coleoptera	Hemiptera	Neuroptera	Total predators
1	0.04 ± 0.01b	0.31 ± 0.02a	1.87 ± 0.14a	0.02 ± 0.01b	2.2 ± 0.12a
2	0.08 ± 0.02a	0.2 ± 0.02b	0.46 ± 0.06b	0.03 ± 0.01b	0.79 ± 0.07b
3	0.05 ± 0.02ab	0.2 ± 0.02b	0.25 ± 0.04c	0.11 ± 0.02a	0.64 ± 0.06ab
F – value	2.13	9.84	96.77	10.2	89.76
df	2, 778	2, 778	2, 778	2, 778	2, 778
P	0.12	< 0.01	< 0.01	< 0.01	< 0.01

Table 5. Mean number ($\pm$ SE) of estimated relevance (proportion of positive detections by number of captures) of *A. aurantii* predators grouped by taxonomical orders for each generation. Different letters within taxonomical groups indicate significant differences between generations (Tukey's test, $P < 0.05$).

Figure captions

Figure 1. Mortality (%) of *A. aurantii* cohorts from the beginning of the experiments until the end, in the following treatments: exclusion, semi-exclusion and predation. Different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

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710

711 **Figure 2.** Corrected mortality presumed to be due to predation of *A. aurantii* calculated by the
712 Henderson and Tilton formula in the three generations of *A. aurantii*. Different letters indicate
713 significant differences between treatments (Tukey’s test, $P < 0.05$).
714

Figure 3. Relative abundance and relative predation of *A. aurantii* predators out of the most abundant predatory species found in citrus through stem-tap sampling during the three *A. aurantii* generations: A) first generation; B) second generation; C) third generation.