UNIVERSIDAD DE TALCA

Instituto de Ciencias Biológicas

Programa de Doctorado en Ciencias, mención Ingeniería Genética Vegetal

Bases genómicas y transcriptómicas de la plasticidad en el uso de hospederos del parasitoide *Aphidius ervi* Haliday (Hymenoptera: Braconidae)

Molecular mechanisms underpinning host fidelity in the parasitoid wasp *Aphidius ervi* Haliday (Hymenoptera: Braconidae)

Tesis entregada al Instituto de Ciencias Biológicas de la Universidad de Talca en cumplimiento parcial de los requisitos para optar al grado académico de:

Doctor en Ciencias

Mención Ingeniería Genética Vegetal

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Agosto de 2018

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Fecha inicio de Tesis: Diciembre 2015

Fecha término de Tesis: Enero de 2018

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Talca, 2019

ÍNDICE GENERAL

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

RESUMEN GENERAL

La agricultura moderna está basada en el uso de cultivos homogéneos y susceptibles al ataque de plagas, enfermedades y malezas, las que son controladas principalmente a través de aplicaciones de grandes cantidades de pesticidas de origen sintético. No obstante existen estrategias de control alternativas, tales como el uso de enemigos naturales introducidos, naturalizados, naturales o liberados para el control de plagas. Uno de los grupos de enemigos naturales de insectos más utilizados son las microavispas parasitoides, organismos que poseen un ciclo de vida que involucra el parasitismo de un insecto hospedero que finalmente ocasiona su muerte. En general, los parasitoides presentan una alta especificidad a un hospedero particular y son muy eficientes como controladores de la especie blanco. Sin embargo, se ha descrito que existen especies de parasitoides que presentan un amplio rango en el uso de hospederos y una gran variación fenotípica asociada a la elección y oviposición sobre diferentes insectos hospederos. Este es el caso del parasitoide *Aphidius ervi*, capaz de parasitar dentro de más de 20 especies de áfidos, por lo que se usa como controlador biológico de áfidos de importancia agrícola a nivel mundial. En Chile, este parasitoide fue introducido en la década de los 70s como agente controlador del áfido plaga de cereales *Sitobion avenae*. Sin embargo, en Chile también se han encontrado poblaciones de *A. ervi* sobre el pulgón de las leguminosas *Acyrthosiphon pisum*. De forma muy interesante, las poblaciones de *A. ervi* en Chile no evidencian signos de diferenciación genética entre poblaciones a pesar de que exhiben especialización conductual y preferencias por su hospedero natal (fidelidad al hospedero) respecto de hospederos alternativos no-natales. En efecto, parasitoides *A. ervi* no muestran diferencias en cuanto a su desempeño cuando se compara su tasa de reproducción entre *S. avenae* y *A. pisum*. En consecuencia, se ha propuesto que la preferencia hacia un hospedero natal en particular (fidelidad de hospedero) observada en *A. ervi* dependería tanto de factores ambientales (por ej., señales químicas del insecto hospedero y del cultivo atacado) como de factores heredables (por ej., marcas epigenéticas). En la siguiente tesis se propone que la fidelidad al hospedero en *A. ervi* sería consecuencia de una plasticidad transcripcional para genes relacionados con la percepción de estímulos químicos. La expresión diferencial de estos genes explicaría la plasticidad para rasgos fenotípicos como la elección y uso de hospederos. Además, se propone que algunos de estos mecanismos de detección de señales química estarían siendo regulados por mecanismos epigenéticos que actuarían como intermediarios entre el genotipo del individuo y el ambiente en donde se ha desarrollado (aprendizaje asociativo).

En primer lugar, en esta tesis se determinó el efecto del desarrollo del parasitoide *A. ervi* dentro de un cierto áfido hospedero (*S. avenae* versus *A. pisum*) sobre los perfiles transcripcionales de hembras adultas a escala de transcriptoma completo, lográndose identificar un primer grupo de genes diferencialmente regulados entre parasitoides provenientes de ambos hospederos que podrían estar involucrados en la formación de la fidelidad al hospedero. Los resultados efectivamente sugieren que *A. ervi* presenta plasticidad fenotípica a nivel transcripcional, la que a su vez depende del origen poblacional de los parasitoides y que podría ser mediada por la relación planta-hospedero, descartándose que las avispas parasitoides utilicen las mismas estrategias a nivel molecular para parasitar hospederos natales y no-natales.

En segundo término, se determinaron los efectos de realizar un transplante recíproco entre hospederos natales y no-natales sobre los niveles de expresión génica para genes candidatos codificantes para proteínas quimiosensoras en dos líneas de *A. ervi* provenientes de dos hospederos diferentes (*A. pisum* y *S. avenae*). Dado que los parasitoides son multiplicados en insectarios con fines comerciales para ser vendidos y luego liberados al campo (control biológico), adicionalmente se estudió el efecto de la crianza sobre el éxito del parasitismo a través de la comparación de niveles de expresión génica para el mismo set de genes quimiosensoriales estudiados previamente, entre poblaciones naturales de campo y poblaciones endogámicas de laboratorio. La falta de fidelidad al hospedero descrita previamente para poblaciones con altos niveles de endogamia (comparado con sus contrapartes de campo) pudo relacionarse con variaciones en mecanismos moleculares específicamente involucrados en la olfacción.

Finalmente, se secuenció y ensambló *de novo* el genoma de *A. ervi*, utilizando una estrategia híbrida de ensamble que consistió en combinar librerías Illumina con una librería PacBio. Esta estrategia permitió obtener un ensamble genómico de alta calidad con bajo nivel de fragmentación (5,778 *scaffolds*). Con este genoma se logró predecir 20,226 genes codificantes a partir de la evidencia transcripcional obtenida previamente para *A. ervi* en esta tesis (capítulo II). Sorpresivamente no se encontró evidencia respecto de la metilación del DNA como mecanismo epigenético involucrado en la regulación génica de *A. ervi*, como sí ha sido descrito para otros himenópteros, en donde la metilación del DNA estaría involucrada en la regulación transcripcional en respuesta a cambios/factores ambientales. Estos resultados sugieren que, en *A. ervi*, la metilación del DNA no tendría un rol *per se* sobre la plasticidad fenotípica observada, a diferencia

de lo que ocurre en otros himenópteros. Este genoma y los genes predichos utilizando evidencia transcripcional están disponibles públicamente y constituyen un aporte a la comunidad científica. En efecto, actualmente se están llevando a cabo una serie de estudios colaborativos que están dando luces de los mecanismos moleculares que posee *A. ervi* y que le permiten enfrentar ambientes cambiantes, lo que a su vez llevará a un mejor entendimiento de los factores que inciden en la eficacia de los parasitoides como agentes biocontroladores.

ABSTRACT

Modern agriculture is mostly based on homogeneous crops, which are pest, disease and weed susceptible; this situation requires a permanent, mandatory input of synthetic pesticides into these oversimplified agroecosystems. Alternatively, biological control strategies are becoming an important part of sustainable pest control. These strategies are based on using introduced, naturalized, natural or released antagonistic agents to regulate population densities of other organisms. Parasitoids wasps are among the most widely used agents in biological control programs, as they lay eggs inside the body of host's juvenile stages or adults, subsequently killing them during their development. Overall, parasitoids have been shown to be host specialist and can be employed in pest control without negative side effects due to potential impacts on non-target organisms. However, many parasitoid species exhibit broad host ranges, as well as phenotypic variation in selection and parasitization of different insect hosts. This is the case of *Aphidius ervi*, a parasitoid wasp capable of parasitizing over 20 different aphid species, being widely used in biological control programs of several relevant aphid pests worldwide. In Chile, this parasitoid was introduced from Europe in the late 70's as a biological control agent of a cereal pest, the grain aphid *Sitobion avenae*. However, the introduced Chilean *A. ervi* wasps also parasitize the pea aphid *Acyrthosiphon pisum*, populations. Interestingly, even when parasitizing different aphid species, Chilean *A. ervi* populations do not exhibit signatures of genetic differentiation between them. Surpringsinly, although they do display variation in terms of host preference towards their natal host (host fidelity) compared to alternate hosts (non-natal hosts), no differences in fitness were observed in terms of reproductive rate between *S. avenae* and *A. pisum.* Hence, it has been proposed that the strong preference towards their natal host (host fidelity) observed in Chilean populations of *A. ervi* would depend on both environmental factors (i.e., chemical signals derived from the host insect and the plant species) and on heritable factors (i.e., epigenetic marks). In the following Thesis, it has been proposed that host fidelity in *A. ervi* is a consequence of transcriptional plasticity for genes involved in perception of chemical stimuli. Differential expression on these genes would in turn explain the phenotypic plasticity observed in terms of host selection and host preference. Additionally, it has been proposed that the perception of some of these stimuli is modulated by epigenetic mechanisms, which would be acting as mediators between the individual's genotype and the environment where it developed, producing specific phenotypes.

In this thesis we first examined the effect of rearing and development in two different aphid host species (*Sitobion avenae* versus *Acyrthosiphon pisum*) on the transcriptome of adult *A. ervi* females. This approach allowed us to identify several differentially expressed genes (which could be involved in host fidelity) when comparing parasitoids reared on different aphid host species. Our results suggest that *A. ervi* displays a significant phenotypic plasticity at transcriptional levels, which would depend on the aphid-plant complex that the parasitoid populations developed on. Hence, it is unlikely that these parasitoid wasps use the same strategies and/or molecular mechanisms to parasitize and exploit either a natal or non-natal aphid host.

Second, the effects of reciprocal transplants between natal and no-natal hosts were determined in the expression levels of genes coding for chemosensory proteins in two different *A. ervi* lines reared on two different aphid hosts (*A. pisum* and *S. avenae*). As parasitoids are reared in small caged populations in commercial insectaries under laboratory conditions before being released to farms and/or natural environments, the effect of long-term rearing on the parasitism success was also studied by comparing the gene expression levels for the same chemosensory candidate genes between natural, field *A. ervi* populations and highly inbred populations reared in laboratory. In this way a relationship could be established between variations in molecular mechanisms specifically involved in olfactory perception with the reduced host fidelity described previously for highly inbred populations of *A. ervi* (compared to their counterparts from field).

Finally, the *A. ervi* genome was sequenced and *de novo* assembled, using a hybrid assembly strategy which combined both Illumina libraries (short reads) and a Pacific Biosciences library (long reads) into a hybrid assembly. Using this strategy, a high-quality genome draft was obtained with reduced fragmentation levels (5,778 *scaffolds*). From this genome assembly, 20,226 coding genes were predicted by using as reference the transcriptional evidence for *A. ervi* obtained within this thesis (Chapter II). Surprisingly, no evidence was found supporting the existence of DNA methylation as an epigenetic mechanism involved in gene regulation of *A. ervi*, unlike other Hymenoptera species, where it has been described as an important mechanism involved in transcriptional regulation in response to environmental factors/changes. Thus, these results suggest that, in *A. ervi*, DNA methylation would not be responsible *per se* for the observed phenotypic plasticity. This genome assembly and predicted coding genes using transcriptional data are publicly available and constitute a valuable resource to the scientific community. Indeed, collaborative

studies are currently being performed, aimed at addressing the molecular mechanisms present in *A. ervi* involved in responding to changing environments. This will lead to a better understanding of several key aspects of parasitoids' biology affecting their efficacy as biological control agents of agricultural aphid pests.

Chapter I

General introduction

1.1 Introduction

Oversimplified agroecosystems, based mostly on pest, disease and weed susceptible crops, which demand permanent usage of synthetic pesticides, are the present situation of modern agriculture. However, pests, disease and weeds are reducing food availability and security considerably, despite the increasingly costly inputs of pesticides into agroecosystems (Birch et al., 2011). Current insect pest management strategies also consider the usage of introduced, naturalized, natural or released antagonistic agents in biological control programs to regulate population densities of other organisms such as aphids (Orr, 2009). Biological control is considered as one of the most successful, non-chemical approaches to pest management (Dhawan & Peshin, 2009). Parasitoids wasps are among the most widely used agents in biological control programs; several parasitoids have been shown to be host specialist and can be employed in targeted pest control without side effects due to potential impacts on non-target organisms (Henry et al., 2008). Hence, parasitoids have been introduced worldwide for partial/complete suppression of relevant arthropod pests (such as aphids) (Orr, 2009). Primary parasitoids of aphids are found in two taxa, the sub-family Aphidiinae (Hymenoptera: Braconidae) and the genus *Aphelinus* (Hymenoptera: Aphelinidae) (Le Ralec et al., 2010). These two groups, specialized on aphids, are endoparasitoids, which means they lay eggs inside the body of host juvenile stages or adults aphids, killing them during their larval development. The hatching larva then develops through three larval stages to become a pupa, protected inside the hardened host body called "mummy", from which an adult wasp emerges (Figure 1.1). Aphidiinae wasps, like their hosts, can be found in almost all climatic regions in the world and in a large variety of habitats, making them an ideal and effective biocontrol agent to reduce population densities of the aphid target species (Le Ralec et al., 2010), while contributing to saving billions of dollars annually in crop losses due to invasive species and by reduction of pesticide usage (Simpson et al., 2011). However, many parasitoid species exhibit variation in their ability to prefer, select and parasitize a natal host (i.e., the aphid host-plant complex from which parasitoids emerged; Davis 2008) compared to a non-natal host. Thus, oviposition is preferred on the same host from which females emerged; this is also known as host fidelity, and it has been

considered as an important trait in parasitoids searching for a suitable host (Henry et al., 2008), but which could negatively affect parasitoids' efficacy as biocontrol agents (Godfray, 1994; Le Ralec et al., 2010).

This is the case of *Aphidius ervi*, a worldwide distributed koinobiont endoparasitoid widely used species in biological control programs of several Macrosiphinae aphid species such as the pea aphid *Acyrthosiphon pisum* (Henry et al., 2010; Stilmant et al., 2008) and the grain aphid *Sitobion avenae* (Cameron et al., 1984). This parasitoid is of Palaearctic origin and was introduced into Chile from Europe in the late 70's as part of an aphid biological control program in cereals. The introduced *A. ervi* wasps successfully parasitized both *A. pisum* on legumes (e.g., alfalfa) and *S. avenae* on cereals (e.g., wheat) (Peñalver-Cruz et al., 2017; Zepeda-Paulo et al., 2013) although these two aphids differ in several important aspects (e.g., host range usage, body size and colour, semiochemicals present in the cuticle, cornicular secretions, defensive behaviors, etc.) (Daza-Bustamante et al., 2003). The natural occurrence of *A. ervi* attacking different aphid species and displaying significant variation in host preference and acceptance could in turn lead to host differentiation or even the formation of host-races and speciation, as a result of genetic differentiations among parasitoid populations (ecological speciation; Abrahamson & Blair, 2008; Schluter, 2001).

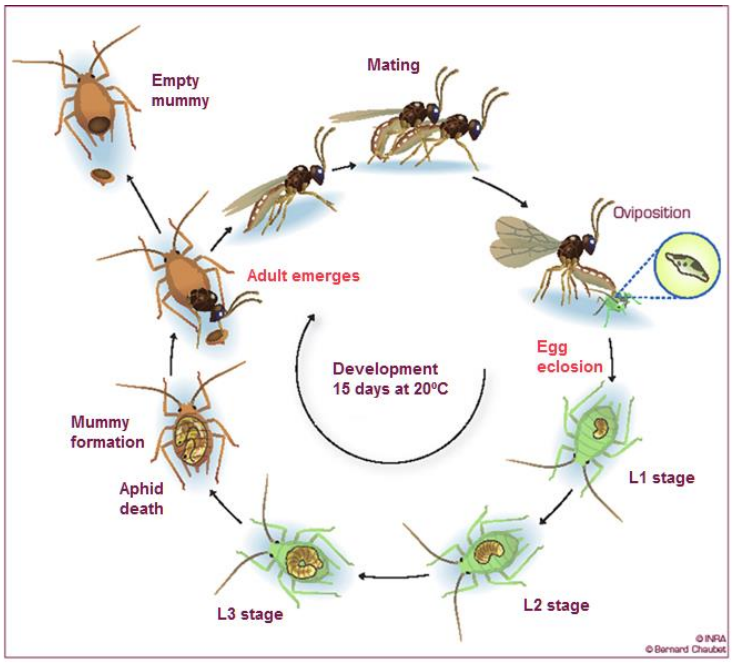

Figure 1.1. Life cycle of a hymenopteran endoparasitoid wasp on an aphid host (adapted from Turpeau et al., 2011)

In parasitoids, phenotypic variation producing host-specific traits could arise due to genetic differentiation between host lineages or differential regulation depending on the host environment during parasitoid ontogeny. The former idea is the basis of the sequential host-association differentiation hypothesis (HAD hypothesis, Abrahamson & Blair, 2008; Dickey & Medina, 2012; Stirema et al., 2006). Interestingly, this would be the case in *A. ervi*, where the usage of different hosts could promote local adaptation and host-associated genetic differentiation (Henry et al., 2008). In the Palaearctic region, *A. ervi* has been described as a complex genetic mosaic of more or less distinct host races, although they interbreed freely (Emelianov et al., 2011). Furthermore, these races may show morphological, behavioural and molecular divergences (including differences in parasitoids' fitness) (Emelianov et al., 2011) which would be explained by the reported genetic differences between races (Henry et al., 2008; Emelianov et al., 2011). However, Bilodeau et al. (2013) reported that the distribution of neutral genetic diversity among introduced *A. ervi* populations attacking different host races of *A. pisum* in Canada does not support the HAD hypothesis, i.e., there was no genetic differentiation between different *A. ervi* host races at the nuclear level. Similarly, population genetic studies of *A. ervi* in Chile also failed to support HAD among distinct aphid hosts (for example, in parasitoids collected from pea aphid, grain aphid, bird cherry-oat aphid) (Zepeda Paulo et al., 2013). Instead, it has been shown that field populations of *A. ervi* from different crops are characterized by sharing close ancestry while no evident genetic structures between host races are found. This could be due to a strong male-driven gene flow between host races (Zepeda-Paulo et al., 2015) or the consequences of a genetic bottleneck due to the recent (in the late 70's) introduction of a limited number of individuals in Chile (Zepeda-Paulo et al., 2016). Hence, the high phenotypic plasticity for host preference and host selection traits displayed in *A. ervi* in Chile cannot be attributed to genetic differences at population levels (Daza-Bustamante et al., 2002; Zepeda-Paulo et al., 2013). An alternative explanation for the observed host race fidelity could be that phenotypic plasticity (defined as the property of a given genotype to express different phenotypes in response to distinct environmental conditions; Pigilucci, 2001) is being regulated by signals from the host a female parasitizes or originated from epigenetic effects.

In Hymenoptera, epigenetic mechanisms such as DNA methylation have been implicated in behavioral plasticity through dynamic regulation of gene and transcriptional activity, unlike its role as a fairly stable epigenetic mark in mammals (Yan et al., 2014). DNA methylation has been

associated with modulation of gene expression levels (Herb et al., 2012) or regulation of alternative splicing (Lyko et al., 2010). As DNA methylation levels within coding regions of protein-coding genes are higher compared with genome-wide methylation levels (Bewick et al., 2016), this epigenetic mechanism has been implicated in behavioral plasticity in several Hymenoptera (e.g., ants, bees, wasps and sawflies), all which also possess the enzymatic machinery involved in DNA methylation (cytosine methyltransferases DNMT1 and DNMT3) (Glastad et al., 2015; Yan et al., 2014). Hence, DNA methylation could explain the high phenotypic plasticity displayed in *A. ervi* in the absence of a strong host-related genetic differentiation. Under this scenario the genome of *A. ervi* would be able to control the expression of distinct phenotypes depending on the habitat (e.g., aphid host), which in turn could behave as host-adapted even in the absence of population-wide genetic differences (Zepeda-Paulo et al., 2013). Thus, phenotypic plasticity could be playing a key role in the observed host fidelity (Henry et al., 2008; Zepeda-Paulo et al., 2013). However, it is unclear how much phenotypic plasticity the parasitoid can use to exploit different hosts or whether they can use identical mechanisms and strategies when searching and parasitizing different hosts.

In parasitoids, host search and selection are not a random processes, even in the case of naïve females without previous oviposition experience (Hoedjes et al., 2011). As reproductive success in parasitoids is closely linked to host finding ability, host search behaviours and subsequent host selection made by the ovipositing mother is critical in order to maximize their reproductive performance and it has been proposed that natural selection is predicted to act strongly on the efficiency of finding hosts (Gols et al., 2012). In particular, plant volatiles produced in response to herbivory (herbivore-induced plant-volatiles, HIPVs) have been demonstrated to play an important role in host finding behaviour by parasitoids (Gols et al., 2012). Hence, parasitoid wasps rely on these chemical cues for both host finding and acceptance (Godfray, 1994). However, parasitoids display significant differences in terms of host preference between their natal host (i.e., host fidelity) rather than alternative, non-natal hosts (Zepeda-Paulo et al., 2013). It has been proposed that this preference for a specific natal host observed in adult parasitoids would be a consequence to its exposure, during larval stages, to both host and host-plant related chemical volatiles and cues (Volatile Organic Compounds, VOCs) which are emitted by the plant-host complex upon aphid infestation (Gols et al., 2012) and which trigger behavioural responses upon recognition (Rehman & Powell, 2010). Hence, detection and processing of chemical signals play

a crucial role during host searching and selection process in adult parasitoids (Wajnberg & Colazza, 2013; Wang et al., 2003) and may be modulated by maternal experience and/or previous oviposition experience. Given that olfactory behavioral responses depends on both odorant recognition and signal propagation/processing, variations in the ability to perceive and respond to chemosensory cues from the host or host-plant complex would also provide a target for adaptive evolution or phenotypic plasticity (Arya et al., 2015). Indeed, phenotypic plasticity in the expression levels of chemosensory genes has been documented in response to different developmental, physiological and social conditions (Zhou et al., 2009), even between individuals of the same species but exhibiting differences in their ecological preferences (Glaser et al., 2015). Hence, it has been proposed that the main mechanism participating in insect adaptation to varying environments would be transcriptional regulation rather than sequence changes in coding sequences/proteins (Smith et al., 2013).

Although many studies have been conducted on the biology of *A. ervi* (He & Wang, 2006; He et al., 2005; Sasso et al., 2009), their efficacy in biocontrol programs (Boivin et al., 2012; Starý, 1993), its ability to be mass-reared and stored before release (Frère et al., 2011; Ismail et al., 2014), and the significant variation on host preference for the natal host (i.e., host fidelity) occurring naturally (Sepúlveda et al., 2017; Zepeda-Paulo et al., 2013), no studies have been conducted regarding the molecular basis and evolution of both host preference and host specific adaptations in this parasitoid species. A possible approach to elucidate the molecular mechanisms involved in certain behavioral phenotypes would be to study the contribution of individual genes to such behavior ("candidate genes"), which has been used successfully in cases such as the rover-sitter polymorphism in *D. melanogaster* that influences foraging strategies and is associated with variants of the *foraging* gene (a cGMP-dependent protein kinase) (Kent et al., 2009). However, understanding the parasitoid behavior using only single-gene approaches has limitations (Neville & Goodwin, 2012) as it is becoming increasingly accepted that many hundreds of sequences, coding and non-coding, may act in concert to influence behavioral phenotypes, as observed in the case of oviposition behavior in *Nasonia vitripennis*, which may be studied using a transcriptomic approach (Pannebakker et al., 2013). The use of a genome/transcriptome approach to identify genes are associated with complex phenotypes such as behavior or to determine whether changes in gene regulation could be linked to changes on these phenotypes (e.g., gene expression regulation mechanisms) is possible in parasitoids, but would require as a starting point either a fully

sequenced/annotated genome and/or reference annotated transcriptomes, such as in the case of *N. vitripennis* (Pannebakker et al., 2013; Werren et al., 2010). In the case of *A. ervi*, to the best of our knowledge, no genomic nor transcriptomic resources are available, making it extremely difficult to study the genomic/transcriptomic basis of phenotypic plasticity on host preference displayed by this parasitoid species, and which could be the molecular mechanisms underpinning such host preference.

Furthermore, it is unclear whether *A. ervi* displays phenotypic plasticity in terms of variations of gene expression when reared on different hosts, and whether epigenetic mechanisms such as DNA methylation and/or chromatin modification/histone protein modifications (Smith et al., 2013) would be implicated in phenotypic plasticity through transcriptional regulation of genome-wide gene expression (Yan et al., 2014), which have not been described nor studied in *A. ervi*. Alternatively, it is possible that *A. ervi* could use the same strategy when parasitizing two different aphid hosts; in that case, transcriptomes should be very similar in terms of transcriptome-wide gene expression, while the expression levels of chemosensory genes should be similar between hosts even when the same *A. ervi* lineage is reciprocally transplanted between two different aphid hosts.

1.2 Thesis aim, structure and outline

The natural occurrence of *A. ervi* attacking different aphid species and displaying significant variation in host preference and acceptance opens interesting questions regarding the molecular basis and evolution of both host preference and host specific adaptations in this parasitoid species. Although these differences should lead to the formation of host-races and speciation as a result of genetic differentiation among *A. ervi* populations, the available evidence suggest that the phenotypic differences observed in Chilean *A. ervi* populations seem to be not related to host race specific genetic differentiation, but rather to environmental variation (e.g., host aphid, aphid-plant interaction) and phenotypic plasticity, which could be playing a key role in the observed host fidelity. **Hence, we hypothesize that I) phenotypic plasticity in host selection and preference traits displayed by different lineages of *A. ervi* parasitizing different aphid host species is**

characterized by a wide plasticity at the transcriptome level and, II) DNA methylation would be the epigenetic mechanism underlying that phenotypic plasticity.

Therefore, this thesis aims to address the following questions: **i)** Does *A. ervi* display phenotypic plasticity at the transcriptome level when parasitizing different aphid host species? **ii)** Can differences in expression levels of chemosensory genes explain the phenotypic plasticity expressed by *A. ervi* lineages when they are parasitizing a novel (non-natal) plant-host complex? **iii)** What chemosensory genes underpin the phenotypic plasticity of both host specialization and host interaction related traits, and could other molecular mechanisms (including DNA methylation) explain that plasticity?

To answer those questions we conducted the following experiments which are organized in three separate chapters.

Chapter II: Expression differences in *Aphidius ervi* (Hymenoptera: Braconidae) females reared on different aphid host species

A transcriptomic RNAseq approach was used to *de novo* assemble and annotate the first representative transcriptome for *A. ervi*. The experimental setup allowed the identification of a set of differentially expressed genes (including some genes related to chemosensory traits) between *A. ervi* parasitizing two different host species (*Acyrthosiphon pisum* and *Sitobion avenae*). This was achieved after comparing gene expression profiles in two different body parts (head and body) and the same host species (*A. pisum*) reared on alfalfa and pea. Overall, this Chapter presents evidence of how much phenotypic plasticity at the transcriptome level *A. ervi* shows when parasitizing different hosts.

Chapter III: Chemosensory genes may be involved in the formation of host fidelity in the parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae)

Phenotypic plasticity in chemosensory gene expression was studied in two lines of *A. ervi* naturally parasitizing different aphid species (*A. pisum* and *S. avenae*). The experimental set up included parasitoids reared on their own host (natal host) or transplanted to a non-natal host. This allowed us to study if the exposure to a novel environment (non-natal host-plant complex) would have an impact on the expression levels of candidate chemosensory genes, which could explain parasitoids'

natal host fidelity. Additionally, chemosensory gene expression levels were compared between *A. ervi* parasitoids collected from the field and parasitoids obtained from a laboratory-reared line. This experiment was conducted in order to decipher the mechanisms that account for the loss of host fidelity observed in inbred populations reared in caged *A. ervi* under laboratory conditions.

Chapter IV: Draft genome of the endoparasitoid wasp *Aphidius ervi* and its utility to provide insights into the dynamics of biological control

Here the aim was to obtain the first *de novo A. ervi* genome sequence. Using a hybrid assembly strategy that combined Illumina libraries (short reads) and a Pacific Biosciences library (long reads), a high-quality genome draft was obtained. Interestingly, no evidence was found supporting the occurrence of DNA methylation in *A. ervi*, which is a major epigenetic mechanism widely present in Hymenoptera related to transcriptional regulation and phenotypic plasticity. These results suggest that DNA methylation per se would not be responsible for the behavioral phenotypic plasticity observed in *A. ervi*. The genome assembly and predicted gene sets using transcriptional data (derived from the recently published *A. ervi* transcriptome; Chapter II) constitutes a valuable foundational dataset that is currently contributing to genetic and genomic research on several key aspects of parasitoids' biology, opening avenues for improving the utility of *A. ervi* as a biocontrol agent of agricultural aphid pests.

1.3. References

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Chapter II[§]

Expression differences in *Aphidius ervi* (Hymenoptera: Braconidae) females reared on different aphid host species

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§ This Chapter was published in *PeerJ* and may be cited as: Ballesteros GI, Gadau J, Legeai F, Gonzalez-Gonzalez A, Lavandero B, Simon J, Figueroa CC. (2017) Expression differences in *Aphidius ervi* (Hymenoptera: Braconidae) females reared on different aphid host species. PeerJ 5:e3640 https://doi.org/10.7717/peerj.3640

2.1 Abstract

The molecular mechanisms that allow generalist parasitoids to exploit many, often very distinct hosts, are practically unknown. The wasp *Aphidius ervi*, a generalist koinobiont parasitoid of aphids, was introduced from Europe into Chile in the late 1970ies to control agriculturally important aphid species. A recent study showed significant differences in host preference and host acceptance (infectivity) depending on the host *A. ervi* were reared on. In contrast, no genetic differentiation between *A. ervi* populations parasitizing different aphid species and aphids of the same species reared on different host plants was found in Chile. Additionally, the same study did not find any fitness effects (in terms of reproductive rate) in *A. ervi* if offspring were reared on a different host as their mothers.

Here, we determined the effect of aphid host species (*Sitobion avenae* versus *Acyrthosiphon pisum* reared on two different host plants alfalfa and pea) on the transcriptome of adult *A. ervi* females. We found a large number of differentially expressed genes (between host species: head: 2765; body: 1216; within the same aphid host species reared on different host plants: alfalfa versus pea: head 593; body 222). As expected, the transcriptomes from parasitoids reared on the same host species (pea aphid) but originating from different host plants (pea versus alfalfa) were more similar to each other than the transcriptomes of parasitoids reared on a different aphid host and host plant (head: 648 and 1524 transcripts; body: 566 and 428 transcripts). We found several differentially expressed odorant binding proteins and olfactory receptor proteins in particular, when we compared parasitoids from different host species. Additionally, we found differentially expressed genes involved in neuronal growth and development as well as signaling pathways. These results point towards a significant rewiring of the transcriptome of *A. ervi* depending on aphid-plant complex where parasitoids develop, even if different biotypes of a certain aphid host species (*A. pisum*) are reared on the same host plant. This difference seems to persist even after the different wasp populations were reared on the same aphid host in the laboratory for more than 50 generations. This indicates that either the imprinting process is very persistent or there is enough genetic/allelic variation between *A. ervi* populations. The role of distinct molecular mechanisms is discussed in terms of the formation of host fidelity.

2.2 Introduction

Parasitoids are widely used in biological control programs, which are based on introduced, naturalized, natural or released parasitoid wasps (Starý et al., 1993). Most parasitoids used for controlling aphids are endoparasitoids, which lay eggs inside their host as part of their life cycle and eventually killing it. Endoparasitoids may attack many related host species (e.g., same family) (Loxdale & Harvey, 2016). However, they have at the same time the problem to overcome or avoid different defense mechanisms of their varied host species in order to survive through adulthood (Jones et al., 2015). One way to do this is the formation of host races, i.e. populations that are adapted to a specific host (Stireman et al., 2006). Alternatively, the parasite changes its "phenotype" depending on the host it encounters and parasitizes, i.e. it shows adaptive phenotypic plasticity (Crispo, 2008). A third alternative is that several host species can be parasitized by the parasitoid using the same strategy/phenotype. The former two mechanisms will lead to differences in host preference and host acceptance, affecting behavioral traits associated to host selection (Zepeda-Paulo et al., 2013) but should not necessarily affect or change parasitoid fitness (Rivero, 2000; Wang et al., 2016).

One of the most widely used species in biological control programs is *Aphidius ervi*, a worldwide distributed koinobiont endoparasitoid of several Macrosiphinae aphid species such as the pea aphid *Acyrthosiphon pisum* (Henry et al., 2010; Stilmant et al., 2008) and the grain aphid *Sitobion avenae* (Cameron et al., 1984). The parasitoid *A. ervi* was introduced into Chile from Europe in the late 70's as part of an aphid biological control program in cereals. The introduced *A. ervi* wasps successfully parasitized both *A. pisum* on legumes (e.g., alfalfa) and *S. avenae* on cereals (e.g., wheat) (Starý, 1993; Zepeda-Paulo et al., 2013) although these two aphids differ in several important aspects (e.g. host range usage, body size and colour, semiochemicals present in the cuticle, cornicular secretions, defensive reactions, etc.) (Daza-Bustamante et al., 2003). The recent introduction of a small number of individuals and natural occurrence of *A. ervi* attacking different aphid species opens interesting questions regarding the molecular basis and evolution of host preference and host specific adaptations (host races). Adaptations to different hosts have been described and confirmed as a plausible speciation mechanism (ecological speciation; Abrahamson & Blair, 2008; Schluter, 2000). Interestingly, whereas differences in host preference and acceptance have been described previously from parasitoids reared or collected from different aphid host species, no fitness effects were detected in terms of reproductive rate, if those parasitoids

were forced to lay their eggs in suitable hosts they were not reared on (Zepeda-Paulo et al., 2013). These results suggest that the preference for the natal host (i.e., host fidelity) is not under direct selection and those parasitoids may show adaptive phenotypic plasticity (Daza-Bustamante et al., 2002; Zepeda-Paulo et al., 2013). Indeed, no host race specific differentiation has been detected in Chilean *A. ervi* populations (Zepeda-Paulo et al., 2013) and a high gene flow predominantly mediated by male dispersion was found between populations (Zepeda-Paulo et al., 2015). Therefore, phenotypic plasticity should be playing a key role in the observed host fidelity (Henry et al., 2008; Zepeda-Paulo et al., 2013). However, it is unclear how much phenotypic plasticity the parasitoid needs to exploit different hosts or whether they can use identical mechanisms and strategies when parasitizing different hosts. One way to test between these options is to compare the transcriptomes of *A. ervi* females reared on different hosts. If *A. ervi* uses the same strategy for parasitizing both hosts their transcriptome should be very similar.

Despite its widespread use in applied and fundamental research, no genomic or transcriptomic information are available for *A. ervi* (Colinet et al., 2014). This study uses RNAseq to *de novo* assemble and annotate the first representative transcriptome for *A. ervi*. Additionally, our experimental setup allowed us to identify differentially expressed genes between *A. ervi* parasitizing two different host species (*Acyrthosiphon pisum* and *Sitobion avenae*) for two different body parts (head and body) and the same host species (*Acyrthosiphon pisum*) reared on two different host plants (alfalfa and pea). This will allow us to determine how much phenotypic plasticity at the transcriptome level *A. ervi* shows and whether it can use the same strategy when it parasitizes different hosts.

2.3 Materials and Methods

Insect collection and rearing

Parasitized aphids (two host races of *Acyrthosiphon pisum* and *Sitobion avenae*) were collected from fields of legumes and cereals in two different geographic zones in Chile: Region de Los Rios (S 39° 51´, W 73° 7´) and Region del Maule (S 35° 24´, W 71° 40´). The alfalfa race of *A. pisum* (APA) was sampled on alfalfa (*Medicago sativa* L.) and the pea race (APP) was sampled on pea (*Pisum sativum L.*) (Peccoud et al., 2008), while the grain aphid *Sitobion avenae* (SA) was sampled on wheat (*Triticum aestivum* L.). Pea aphids (from both alfalfa and pea races) were maintained in the laboratory on broad bean (*Vicia faba* L.) while grain aphids were maintained on barley

(*Hordeum vulgare* L.). Both host plants have been used previously for aphid and parasitoid rearing in other studies (Sepúlveda et al., 2016; Zepeda-Paulo et al., 2013). Parasitized aphids were reared under laboratory conditions that allowed continuous reproduction (20°C, D16/N8 photoperiod) (Zepeda-Paulo et al., 2013). *Aphidius ervi* parasitoids were collected as larvae from parasitized aphids, recognizable as mummies and kept separated in vials until adult parasitoids emerged. Species and sex of each emerging parasitoid was determined using a standard taxonomic key (Starý, 1995). In order to establish inbred populations, a single, isolated naive *A. ervi* virgin female was mated with a naive virgin male for 24 hours in a petri dish with diluted honey and water for sustenance. Mated females were then offered new aphid hosts *ad libitum* in a separate cage. These inbred lineages were propagated for approximately 60 generations before samples were taken for the RNAseq experiments. All *A. ervi* parasitoids were reared on the same host aphid species from which they were originally collected (further on called natal host). Thus, three different and highly inbred *A. ervi* laboratory populations were established: (i) *A. ervi* population originally collected from *A. pisum* living on alfalfa (Ae-APA) (ii) *A. ervi* population originally collected from *A. pisum* living on pea (Ae-APP) and (iii) *A. ervi* population originally collected from *S. avenae* living on wheat (Ae-SA) (Figure 2.1). Each week, new aphid infested plants were introduced into the *A. ervi* rearing cages for parasitoid population maintenance, together with vials containing diluted honey and water for adult parasitoid feeding. All aphid populations were free of known secondary endosymbionts; their presence was evaluated using the amplification of specific 16S rDNA from whole-body aphid DNA based on the set of known primers described by Peccoud et al., 2014. This method allows screening of different symbionts, including the protective bacteria *Hamiltonella defensa* and *Regiella insecticola*, and also *Serratia symbiotica*, *Rickettsia, Rickettsiella* and *Spiroplasma* (Sepulveda et al., 2017a)

RNA collection and sequencing

Adult female parasitoids were collected alive from each one of the three caged parasitoid populations (N=20 per population) (Figure 2.1) and stored in 1.5 ml centrifuge tubes containing RNALater (QIAGEN) at -20°C until dissection and RNA extraction. Heads and bodies were dissected on ice using a sterile scalpel and pooled in six different samples. For each sample, total

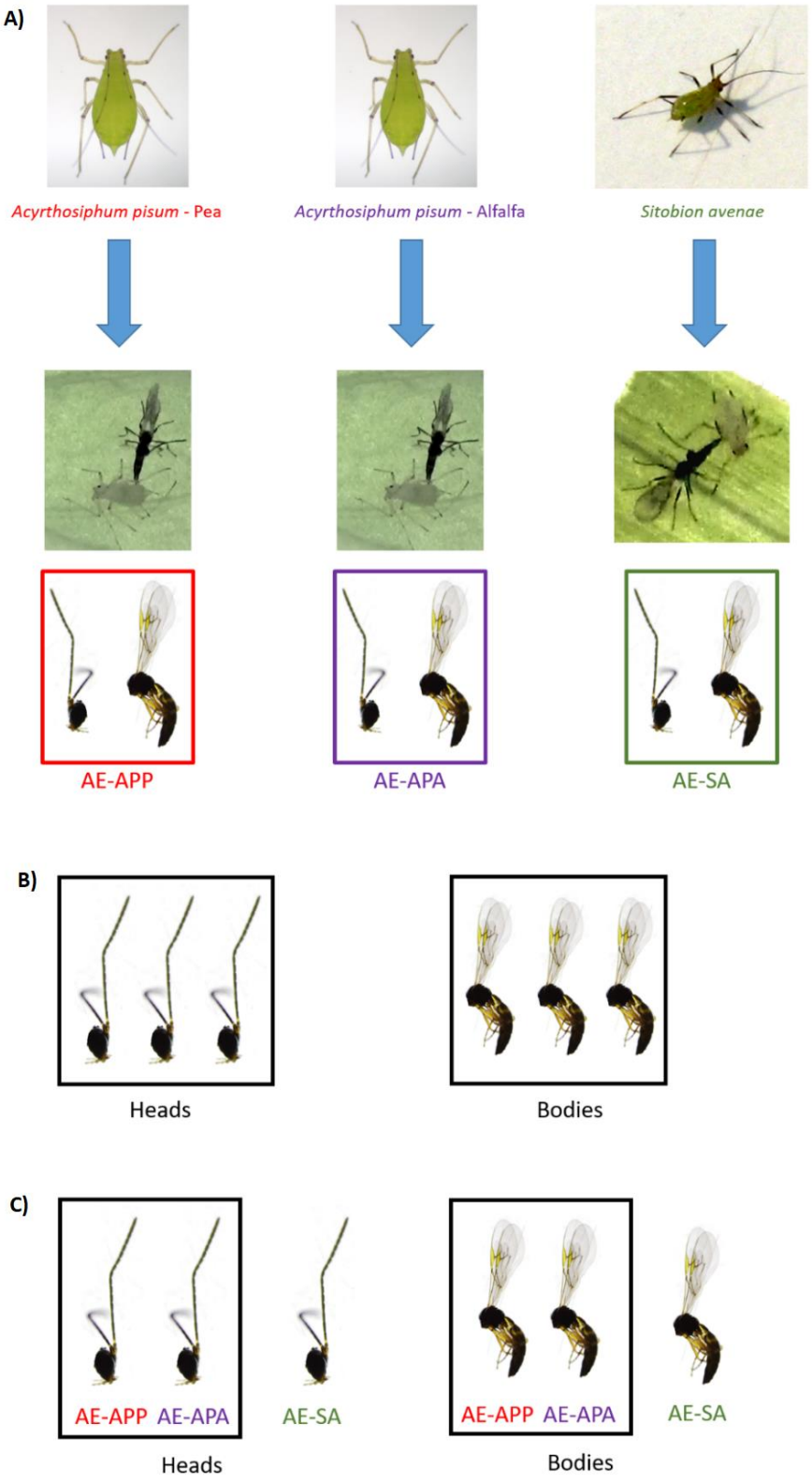

Figure 2.1. Sampling design for RNA sequencing and differential expression analysis in the aphid parasitoid wasp *Aphidius ervi*. A) Parasitoid populations. B) DE analysis between heads and bodies (3 libraries/tissue). C) DE analysis between parasitoid host races.

RNA was extracted using the RNEasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions and eluted in 50 µl of RNAse free water. Total RNA was quantified by spectrophotometry (Epoch Microplate Spectrophotometer, Biotek) and fluorometry (Qubit 3.0, Qubit RNA Broad Range Assay Kit), and integrity checked in a Bioanalyzer 2100 RNA Nano Kit (Agilent Santa Clara, CA, USA). Recovered total RNA was precipitated using 0.1 volumes of Sodium Acetate 3M and 2 volumes of 100% Ethanol and shipped to Macrogen Korea for library preparation and sequencing. Ribosomal RNA was depleted from total RNA using the Ribo-Zero rRNA Removal Kit for enrichment of both insect mRNA and non poly-adenilated mRNA that might be present in *A. ervi* sequenced samples. Remaining RNA was used for library construction using TruSeq Stranded Total RNA Sample Preparation Kit (Illumina), tagged, pooled and sequenced using an Illumina HiSeq 2000 (2x100bp, Paired End libraries; Macrogen, Korea). Raw transcriptome data was deposited in NCBI's Sequence Read Archive database under BioProject ID: PRJNA377544.

Illumina sequence processing and transcriptome assembly

Illumina RNA-seq libraries were quality checked with FastQC ver. 0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc) in order to assess the presence of adapters derived from sequencing, overrepresented kmers, read length and overall read quality scores. All libraries were processed with Trimmomatic ver. 0.35 (Bolger et al., 2014) to remove any remaining TruSeq adapter sequence and to eliminate low quality bases ($Q < 3$) from reads. After sequence processing, all remaining sequences shorter than 36 bp long were also removed from all datasets. Clean Illumina datasets were pooled *in silico* by concatenating library files. Before assembly, ribosomal RNA reads were removed by mapping the libraries using Bowtie ver. 1.1.1 (Langmead et al., 2009) against a custom rRNA database created from insect ribosomal sequences downloaded from NCBI and keeping non-mapped reads. The remaining high quality reads were *de novo* assembled with Trinity ver. 2.0.6 using default parameters. Metrics for *de novo* assembly were obtained with QUAST ver. 2.3 (number of contigs, total length, N50, largest contig, %GC, etc.) (Gurevich et al., 2013) while transcriptome completeness was assessed by benchmarking the assembled transcriptome using BUSCO (benchmarking universal single-copy orthologs) v.1.1b1 (Simão et al., 2015). To determine whether this transcriptome encodes for one or more set of core genes conserved across a range of Arthropod species, a "completeness score" was calculated

(Moreton et al., 2016). A total of 2,675 near-universal single-copy orthologs from Arthropod species were used as reference core genes (available at busco.ezlab.org; Simão et al., 2015). Additionally, reference protein sequences of *A. ervi* were downloaded from both the Non Redundant (NR) and Transcriptome Shotgun Assembly (TSA) sequence databases available at NCBI (N=422; Colinet et al., 2014). These sequences were used as a custom reference protein database for BLASTx alignments using the assembled transcriptome as query.

Annotation and functional gene classification

Homology searches of contigs from the assembled *de novo* transcriptome were performed locally with BLASTx using the NR database (NCBI) as reference (April 2016 version), setting an e-value of 1^{e-5} as threshold. Any contig that showed homology with at least one gene or protein was designated as a hit contig, while contigs with no hits were disregarded for any of the follow up analysis. Contigs with top-hit to non-insect species (e.g., prokaryote, yeast, vertebrates, etc.) were removed from the assembly and stored separately for future analysis because we were focused only on insect genes. Insect species reference lists available in NCBI Taxonomy browser were used to identify contigs with top-hits for insects (search criteria "insecta", 200 levels displayed). To further improve the accuracy of differentially expressed genes, all contigs aligning to the same protein were grouped using BLASTx homology results and were sorted by alignment BitScore. The sequence with the highest BitScore was considered as the best blast hit, selected and designed as an annotated contig (Ono et al., 2015). The insect-filtered, non-redundant contig fasta dataset was loaded into Blast2GO ver. 2.8 (Conesa & Götz, 2008) altogether with BLASTx results in XML format. We also performed InterPro annotation, Gene Ontology (GO) term assignment, enzyme code and pathway annotation using Kyoto Encyclopedia of Genes and Genomes (KEGG) term integrated into Blast2GO. Successfully annotated transcripts were categorized and assigned to GO terms from different GO categories (molecular function, cellular component and biological process). The final contig annotation table was obtained from the combination of "Top-Blast table" and "sequence table", both exported from Blast2GO.

Differential gene expression analysis

The insect filtered, non-redundant reference transcriptome was used as a basis for differential gene expression studies between tissues (separate for head and body) (Figure 2.1). Note: each individual sample was based on 20 individual females. Additionally, differential expression studies were

carried out in order to detect and describe unique expressed transcripts for parasitoid lines from different aphid hosts (Ae-APP = *A. pisum* – Pea; Ae-APA = *A. pisum* – Alfalfa; Ae-SA= *S. avenae*) (Figure 2.1). Gene abundance estimation was performed by separately aligning the libraries to the reduced reference transcriptome using the align_and_estimate script included in Trinity (ver. 2.0.6). This script automated the reference transcript, performed library read alignment to the reference using Bowtie2 (ver. 2.2.4; Langmead et al., 2009), and estimated read abundances from mapping results per library with the RSEM package included in Trinity (ver. 2.0.6.; Li& Dewey, 2011), and it was used to interpret and analyze Bowtie2 mapping results. RSEM was used to combine each count matrix and to build up a raw transcript expression matrix and a TMM-normalized expression matrix (script abundance_estimates_to_matrix.pl); this raw counts matrix was further used for Differential expression (DE) analysis at tissue level (heads vs. bodies, 3 libraries per sample), aphid host species and host rearing plant species. DE analysis was performed with edgeR Bioconductor package implemented in R using the provided run_DE_analysis script in Trinity ver. 2.0.6. The package edgeR was selected as it has a relatively high sensitivity and specificity in DE analysis of pooled samples compared to other methods of analysis. Genes that had at least 4-fold change values with a FDR-corrected *p-value* of 0.01 or lower were considered as significantly differentially expressed between libraries/tissues. The annotation of DE contigs was performed by combining TMM-normalized expression profiles for each contig with the annotated transcriptome tables generated in Blast2GO together with BLASTx results. Uniquely, differentially expressed contigs were detected for all libraries. In the case of the SA lineage (both for heads and bodies), a contig was considered as overexpressed only if its expression profile was 4-fold times higher when compared to both APA and APP populations. For both *A. pisum* races, contigs were considered as overexpressed only if their fold-change was at least 4-fold times higher (FDR-corrected *p-value* < 0.01) compared to the SA race in both APA and APP populations. The GO term enrichment was performed using Fisher's exact test in Blast2GO.

2.4 Results

Aphidius ervi *reference transcriptome assembly*

We generated a *de novo* transcriptome for *A. ervi* using transcriptomic datasets obtained from the sequencing of six Illumina libraries (NCBI SRA accession PRJNA377544). These datasets were

obtained from pooled female adult parasitoids reared from different aphid hosts (APP = *A. pisum* – Pea; APA = *A. pisum* – Alfalfa; SA = *S. avenae*) (Table 2.1).

Table 2.1. Summary of *Aphidius ervi* transcriptomic libraries and assembly statistics

Sequencing		
Library sequenced	**Raw reads**	**Filtered Reads**
Ae-APA	122,819,778	115,025,660
Ae-APP	124,329,988	113,456,324
Ae-SA	184,088,012	161,916,086
Total	431,237,778	390,398,070
Minus rRNA		237,214,294

Assembly			
	Reference *de novo*	**BLAST Hits**	**Insect only**
Total transcript number	135,676	33,853	17,763
Total length	91,710,298	47,294,638	25,440,092
longest transcript	19,479	19,479	19,479
N50	1,516	2,027	1,983
%GC	30.15%	31.02%	30.67%

All transcripts were concatenated to generate a reference transcriptome library that could act as further reference for other studies involving *A. ervi* (GenBank accession GFLW01000000). The RNAseq generated 431,237,778 raw reads (2x100bp, paired end libraries). After pre-processing and removal of ribosomal RNA reads, 237,214,294 reads remained. Using Trinity, filtered reads were assembled into 135,659 contigs (N50 of 1,516bp, mean length of 675.95bp). Among the assembled contigs, 38,567 were less than 300bp (28.4%), 75,458 contigs were between 301bp and 1000bp (55.6%), while 21,634 contigs had a size over 1000bp (16%). The assembly completeness was also evaluated using BUSCO (benchmarking universal single-copy orthologs) showing that 76.6% complete conserved genes were found in our assembly, 13.7% corresponded to fragmented conserved genes while only 9.8% of single-copy ortholog genes were missing. Our results are

similar to BUSCO metrics reported for other *de novo* insect transcriptomes assemblies such as the Western tarnished plant bug *Lygus hesperus* assembly performed with Illumina datasets (Tassone et al., 2016). Additionally, all previously known *A. ervi* sequences (N=422) retrieved from NCBI were present in our transcriptomic assembly.

Sequence annotation

The BLASTx alignments revealed that 33,853 contigs were annotated to a known protein within the NR database (24.9% of total contigs) (Table 2.1). Most transcript sequences with protein hits matched to other braconid endoparasitoids such as *Diachasma alloeum* (parasitoid of the apple maggot *Rhagoletis pomonella*), *Fopius arisanus* (parasitoid of Tephritid fruit flies) and *Microplitis demolitor* (parasitoid of noctuid larvae) (Figure 2.2), all species for which *de novo* transcriptomes have been published (Burke & Strand, 2012; Calla et al., 2015). This dataset was further filtered to remove redundant contigs by using best-BLAST hit criteria (see Materials and Methods section). Additionally, annotated sequences from non-insect organisms were removed; both steps filtered out 16,090 contigs. The remaining 17,763 contigs were the basis for all follow up analyses (annotation and differential expression). A total of 10,492 contigs (59.1%) could be annotated based on their sequence homology with GO terms. As contigs can be assigned to more than one GO category, 14,614 contigs were assigned to biological process, 7,945 contigs were classified under molecular function, and 5,976 were classified in cellular component (Figure 2.3). This GO term distribution is congruent with other insect transcriptomes already sequenced (Hwang et al., 2016).

Transcriptomic differences between tissues and function of transcripts with different expression levels

We used our filtered transcriptome as a reference dataset to perform Differential Expression (DE) analysis for *A. ervi* reared on the three different hosts. Our results indicate that there is a good correlation within head samples and within body samples, while clear differential expression patterns are observed for different body parts (Figure 2.4; supplementary Figure 2.1, appendix A). Differential expression analysis showed that 6,389 transcripts are being differentially expressed between head and body samples (N=3,445 up-regulated in heads, N=2,944 up-regulated in bodies). The GO Term enrichment analysis was performed between differentially expressed genes and indicated the most enriched GO terms for bodies and heads (Figure 2.4). “Signal transduction” and

"signal transducer activity" were among the most enriched GO terms for heads, hence indicating that signaling and response stimulus associated transcripts are prevalent in the head transcriptomes, which is expected as RNA was extracted from whole heads including the brain and chemical sense organs such as antennae (Glaser et al., 2015). In the case of bodies, "ribosome biogenesis" and "peptidase activity", were, amongst others, the most enriched GO terms found. As we extracted RNA from headless bodies without further dissection, the enriched "peptidase activity" could probably reference to venom proteins such as serine proteases or gamma-glutamyl transpeptidases, which have also been described previously for *A. ervi* (Colinet et al., 2014). These venom proteins are injected into the host at oviposition and would have a role in the modulation of the aphid physiology by inducing apoptosis of host ovaries and arresting host reproduction (Colinet et al., 2014; Falabella et al., 2007).

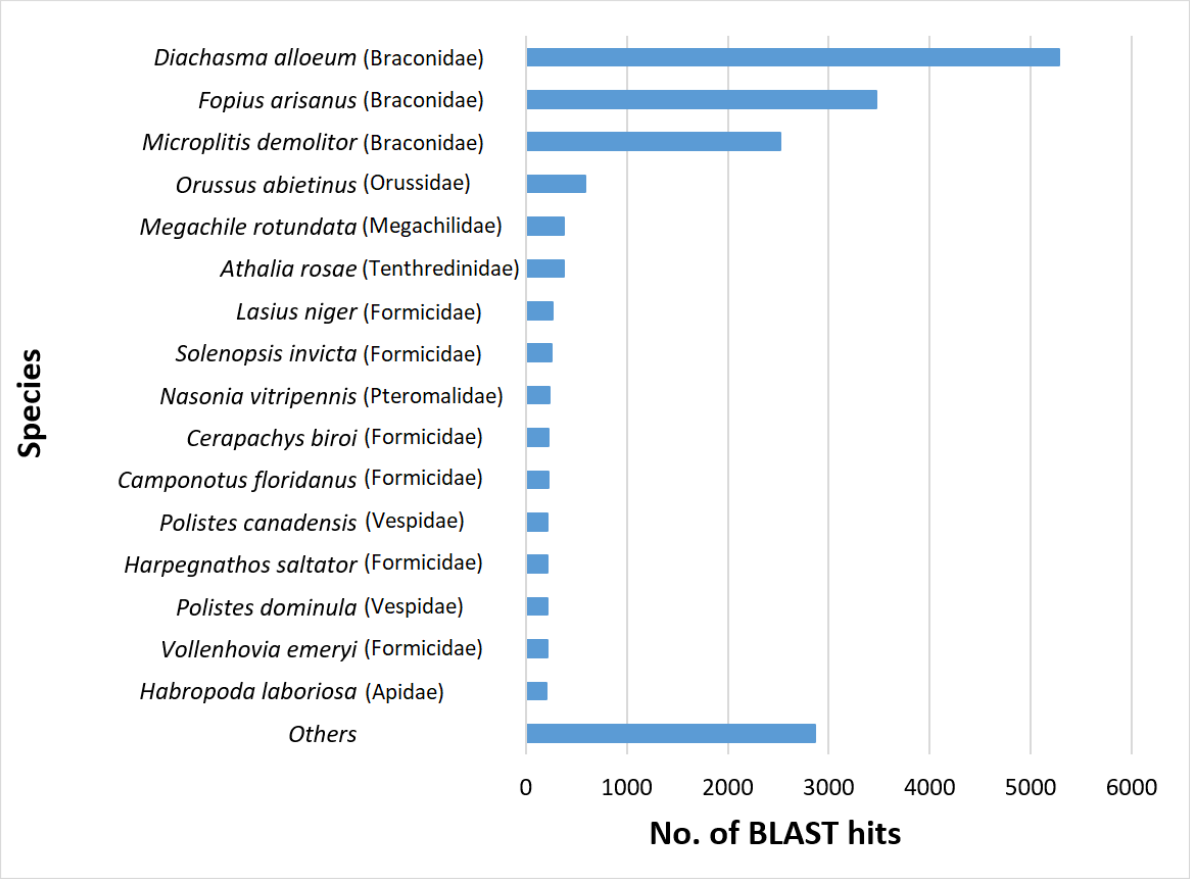

Figure 2.2. Species distribution of unigene sequences of the aphid parasitoid wasp *Aphidius ervi* transcripts to other insect species using homologous BLASTx hits and NR-NCBI database. Species' family is shown in brackets.

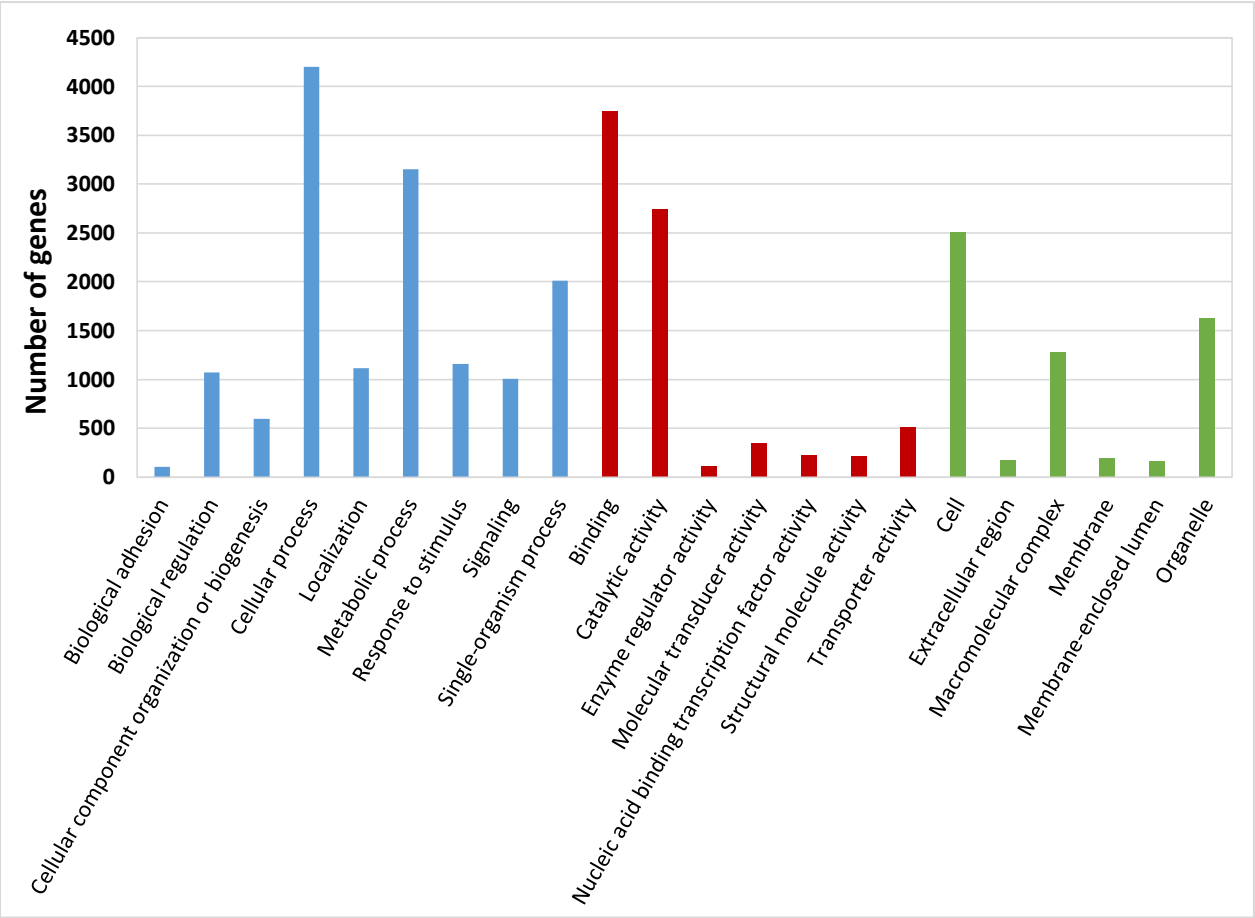

Figure 2.3. Gene Ontology (GO) annotations for the reference transcriptome of *A. ervi* separated by GO categories (Biological process: blue. Molecular function: red. Cellular component: green).

Transcriptomic differences between parasitoid lines and function of transcripts with different expression levels

At body level, we found 239 transcripts with differential expression patterns between *A. ervi* populations that were originally collected from *A. pisum* on alfalfa and pea, respectively (149 up-regulated genes for Ae-AP and 90 up-regulated genes in Ae-SA; *q-value* < 0.01, fold-change > 4), while at head level 390 transcripts showed differential expression (219 upregulated in Ae-AP and 171 upregulated in Ae-SA). All differentially expressed transcripts were annotated using the results from BLASTx alignments while GO terms were assigned using Blast2GO mapping results, which in turn was used to identify the functions of genes displaying different expression patterns between populations and tissues (Figures 2.5 and 2.6); this approach was used because GO term enrichment analysis failed to find any enriched term. Top 20 differentially expressed gene lists (ranked first

according to fold-change) are reported in Table 2.2 (Complete lists in supplementary file 2.1, appendix A).

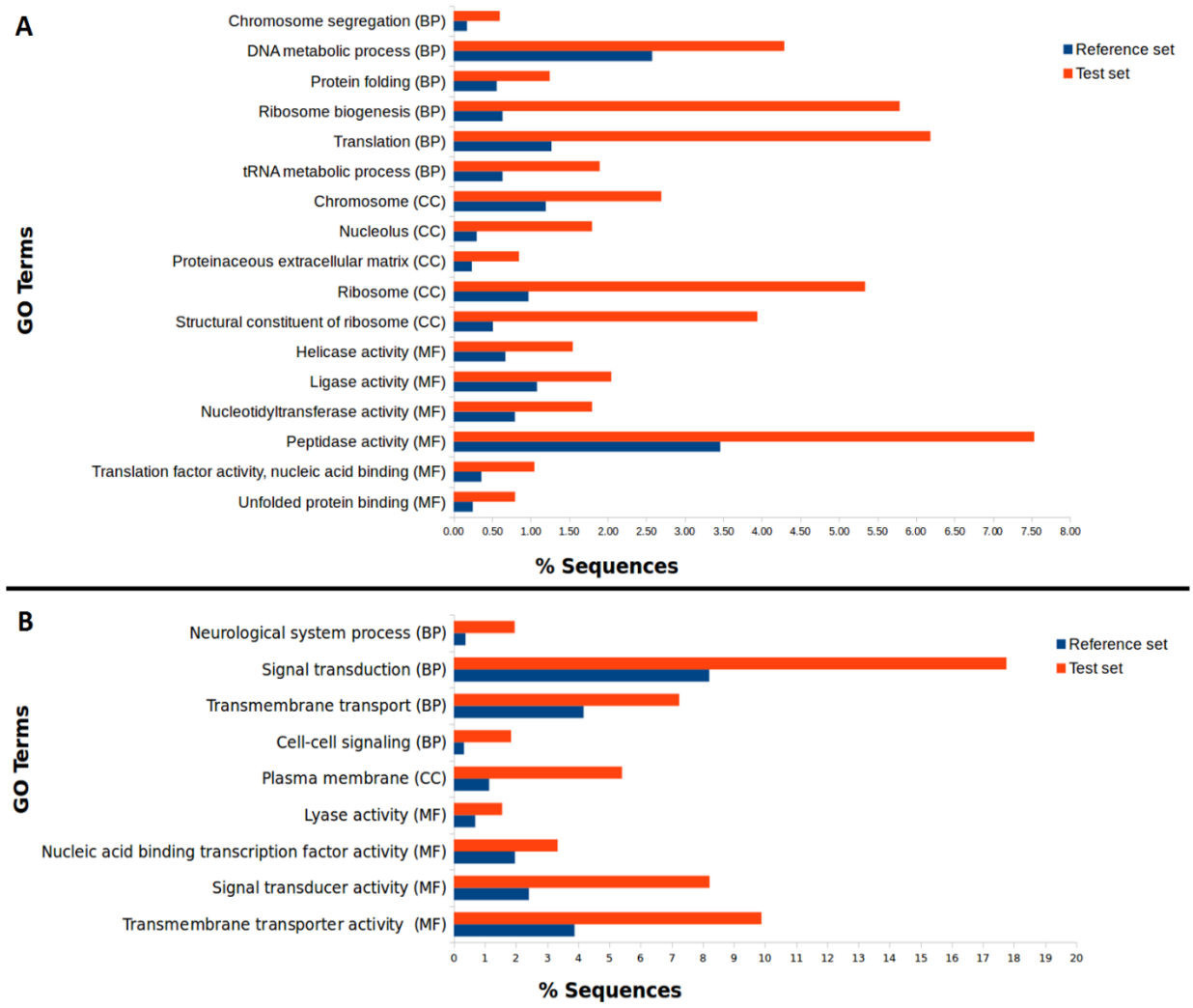

Figure. 2.4. Differential GO term distribution between *Aphidius ervi* bodies and heads (Blast2GO Fisher's exact test with FDR correction). Reference set: full *A. ervi* transcriptome. Test set: % of sequences associated to GO-enriched terms. A) enriched GO-terms in bodies. B) enriched GO-terms in heads. CC: Cellular Component; MF: Molecular Function; BP: Biological Process.

Identification of putative chemosensory and olfaction-related genes

Olfaction plays a crucial role in insect behavior such as mate recognition, foraging, host location and host discrimination, or finding shelter in complex environments (Suh et al., 2014). Behavioral differences in host preference and host acceptance based on aphid host species and plants have been reported previously for *A. ervi* (Zepeda et al., 2013). This ability to differentiate between hosts almost certainly involves chemical signal perception (Takemoto et al., 2011). As olfactory behavior differences depend on both odorant recognition and signal propagation/processing, genes coding for odorant perception such as Odorant Binding Proteins (*OBP*s), Chemosensory Proteins (*CSP*s) and Odorant Receptors (*OR*s) and genes coding for signal propagation such as voltage-gated sodium channels are prime candidate genes underlying the observed differences in host-preference in *A. ervi* (Zepeda et al., 2013). This variation in the ability to perceive and respond to chemosensory cues would provide a target for adaptive evolution (Arya et al., 2015) or phenotypic plasticity. The identification of genes potentially involved in olfactory behavior in *A. ervi* and the highlighted gene expression differences between wasp populations exploiting different aphid lineages provide the first clues to understand the molecular basis of host-fidelity (Li et al., 2013; Zepeda-Paulo et al., 2013; Sepúlveda et al., 2017b). Homology analyses using NR database identified 91 contigs belonging to gene families involved in insect chemoperception such as *OBP*s (10 transcripts), *CSP*s (2 transcripts), *SNMP*s (1 transcript), *OR*s (76 transcripts, including the conserved odorant co-receptor, *ORco*) and ionotropic receptors (*IR*s; 2 transcripts). Compared to other parasitoids in which these gene families had been annotated using genomic approaches or transcriptomic sequencing from antennal tissues, we found less *OR*s (76 *OR*s, 10 *OBP*s, 2 *CSP*s, 1 *SNMP*) compared to *Cotesia chilonis* (117 *OR*s, 8 *OBP*s, 2 *CSP*s, 3 *SNMP*s) (Qi et al., 2015), but more than *Sclerodermus sp.* (8 *OR*s, 10 *OBP*s, 10 *CSP*s, 2 *SNMP*s) (Zhou et al., 2015). However, these numbers have to be taken with caution because these gene families are extremely difficult to annotate automatically. We found several significantly differentially expressed genes involved in chemical perception between our three *A. ervi* populations, two *OBP*s and five *OR*s were expressed at a higher level in the Ae-AP host-race while only one *OBP* had a higher expression value on the Ae-SA host-race (Table 2.3). Additionally, we found higher expression of an *IR* (the glutamate receptor kainate 2) in the Ae-AP host-race relative to Ae-SA host-race (Table 2.3). Interestingly, increased expression levels of glutamate receptor kainate 2 have been linked to olfactory responses in the salmon louse *Caligus rogercresseyi* (Núñez-Acuña et al., 2014).

Cellular signaling and neural development

Within the *A. ervi* reference transcriptome, genes coding for proteins participating in neuronal development and synaptic function of the nervous system were also found, which include the Rho family of GTPases. As Rho signaling activity plays a key role in neural morphological plasticity through dendritic reorganization and structural remodeling of synapses (Tolias et al., 2011), it has been linked to long-term memory formation, including olfactory learning (Dobrin & Fahrbach, 2012). Additionally, variation in olfactory behavior showed by different lines of *Drosophila melanogaster* has been linked to variants in genes involved in nervous systems development and function, such as Rho signaling (Arya et al., 2015).

Remarkably, we found a higher expression for Rho GTPase activating protein 190 (*Rho-GAP 190*) (Table 2.2) and Rho guanine nucleotide exchange factor 7 (*Rho-GEF 7*) (supplementary file 2.1, appendix A), in the heads of Ae-AP compared to Ae-SA heads. We also found a higher expression for a gene coding for a sodium channel (sodium channel protein 60e) in Ae-AP heads compared to Ae-SA heads (supplementary file 2.1, appendix A). In *D. melanogaster*, this sodium channel participates in processing olfactory information and regulates olfactory acuity, so its reduced expression impairs olfaction (Kulkarni et al., 2002; Zhang et al., 2013). Furthermore, this sodium channel in *D. melanogaster* is particularly expressed in olfactory organs (third antennal segment and maxillary palps) and brains (Gosselin-Badaroudine et al., 2016; Kulkarni et al., 2002). Other protein involved in cellular signaling is Calmodulin (CaM), a highly-conserved protein that contains four EF-hand domains that allow binding Ca^{+2} ions (Park et al., 2008). Conformational changes in CaM allow its interaction with several target proteins including ORco (through ORco's CaM binding motif) (Bahk & Jones, 2016) and modulate insect OR function (Mukunda et al., 2014); repetitive subthreshold odor stimulation of olfactory neurons sensitizes ORs, and inhibition of *CaM* expression abolishes sensitization (Mukunda et al., 2016). Higher expression for a transcript coding for a CaM protein was found in Ae-AP heads compared to Ae-SA heads (neo-calmodulin-like isoform x4; 4.76 logFC; supplementary file 2.1, appendix A).

Table 2.2. Top 20 genes with differential expression patterns between *A. ervi* - AP and *A. ervi* - SA on two different tissues (heads and bodies)

ID	Library	Sequence description	Log2-fold change	P-Value	FDR-adjusted p-value
TR52610-c0_g1_i1	Ae-AP Body	sodium hydrogen exchanger 7 isoform x4	13.57	6.48E-20	3.73E-16
TR27559-c2_g1_i1	Ae-AP Body	rho gtpase-activating protein 190 isoform x1	13.27	1.18E-19	4.82E-16
TR36885-c0_g2_i1	Ae-AP Body	oxidoreductase glyr1 homolog	12.17	2.86E-16	3.71E-13
TR42270-c3_g5_i10	Ae-AP Body	calcium-activated potassium channel slowpoke	12.12	3.43E-16	6.80E-13
TR27536-c4_g1_i1	Ae-AP Body	disco-interacting protein 2 isoform x1	11.84	3.76E-10	7.65E-08
TR42293-c6_g1_i8	Ae-AP Body	lon protease mitochondrial isoform x1	11.44	3.38E-14	2.23E-11
TR52097-c0_g1_i3	Ae-AP Body	synaptojanin-1 isoform x1	11.14	2.84E-13	1.85E-10
TR30823-c0_g1_i18	Ae-AP Body	zinc finger protein rotund isoform x3	10.98	3.93E-12	9.37E-10
TR55124-c1_g1_i1	Ae-AP Body	ryanodine receptor isoform x6	10.95	2.18E-11	6.15E-09
TR55070-c8_g1_i6	Ae-AP Body	dynamin isoform x2	10.91	3.11E-12	7.91E-10
TR4006-c7_g2_i9	Ae-AP Body	down syndrome cell adhesion molecule-like protein dscam2 isoform x30	10.88	1.15E-12	4.56E-10
TR13038-c0_g3_i4	Ae-AP Body	excitatory amino acid transporter isoform x1	10.84	3.07E-12	8.35E-10

TR42270-c3_g5_i5	Ae-AP Body	calcium-activated potassium channel slowpoke isoform x7	10.75	8.45E-11	2.06E-08
TR37837-c6_g1_i2	Ae-AP Body	inorganic phosphate cotransporter isoform x1	10.74	6.56E-12	2.22E-09
TR28738-c0_g3_i2	Ae-AP Body	embryonic polarity protein dorsal-like isoform x3	10.66	4.77E-12	1.46E-09
TR20185-c0_g1_i1	Ae-AP Body	transposable element p transposase	10.64	1.09E-11	3.53E-09
TR29865-c1_g2_i1	Ae-AP Body	cordon-bleu 1	10.57	2.74E-10	5.85E-08
TR24635-c2_g2_i3	Ae-AP Body	zinc finger cchc domain-containing protein 4	10.48	6.09E-11	1.16E-08
TR16911-c9_g1_i6	Ae-AP Body	dynein heavy cytoplasmic isoform x2	10.26	2.05E-08	2.66E-06
TR19336-c1_g1_i6	Ae-AP Body	uncharacterized protein LOC100740589 isoform X3	10.18	3.73E-07	3.52E-05

ID	Library	Sequence description	Log2-fold change	P-Value	FDR-adjusted p-value
TR41810-c12_g5_i10	Ae-SA Body	vinculin isoform x9	11.09	3.07E-12	1.12E-09
TR42270-c3_g5_i12	Ae-SA Body	calcium-activated potassium channel slowpoke isoform x6	10.99	5.80E-12	1.95E-09
TR21226-c0_g1_i1	Ae-SA Body	nfu1 iron-sulfur cluster scaffold mitochondrial-like	10.94	8.07E-12	2.58E-09
TR19336-c1_g1_i10	Ae-SA Body	fh1 fh2 domain-containing protein 3 isoform x4	10.83	1.69E-11	4.93E-09
TR31615-c2_g1_i1	Ae-SA Body	PREDICTED: uncharacterized protein LOC105456969	10.75	2.70E-11	7.44E-09
TR31615-c7_g1_i1	Ae-SA Body	ubiquitin carboxyl-terminal hydrolase 17-like partial	10.46	1.66E-10	3.67E-08
TR32717-c6_g1_i1	Ae-SA Body	paired amphipathic helix protein sin3a	9.93	3.40E-09	5.60E-07
TR4388-c16_g1_i1	Ae-SA Body	whirlin isoform x1	9.66	1.71E-08	2.32E-06
TR32865-c1_g1_i2	Ae-SA Body	a disintegrin and metalloproteinase with thrombospondin motifs 16 isoform x1	9.64	2.06E-08	2.71E-06
TR13691-c0_g1_i3	Ae-SA Body	membrane metallo-endopeptidase-like partial	9.61	7.62E-13	3.88E-10
TR45167-c1_g2_i2	Ae-SA Body	kielin chordin-like protein isoform x2	9.36	9.44E-08	1.07E-05
TR9096-c9_g1_i6	Ae-SA Body	piezo-type mechanosensitive ion channel component 1 isoform x2	9.28	6.66E-12	2.21E-09
TR49009-c9_g1_i7	Ae-SA Body	voltage-dependent calcium channel subunit alpha-2 delta-3 isoform x2	9.26	1.66E-07	1.76E-05
TR47041-c0_g1_i4	Ae-SA Body	PREDICTED: uncharacterized protein LOC106789540 isoform X2	9.21	2.10E-07	2.14E-05
TR27559-c2_g1_i4	Ae-SA Body	rho gtpase-activating protein 190 isoform x1	9.02	6.48E-07	5.69E-05

TR23225-c0_g2_i6	Ae-SA Body	serine threonine-protein kinase ick-like isoform x2	8.85	1.49E-06	1.17E-04
TR37577-c0_g1_i2	Ae-SA Body	probable 28s rrna (cytosine-c)-methyltransferase	8.79	2.00E-06	1.52E-04
TR48980-c1_g2_i1	Ae-SA Body	creb-binding protein isoform x5	8.77	2.55E-10	4.86E-08
TR45475-c0_g2_i2	Ae-SA Body	ras-related protein m-ras-like	8.61	5.06E-06	3.38E-04
TR34000-c0_g1_i1	Ae-SA Body	neprilysin-2 isoform x1	8.56	9.29E-10	1.50E-07

ID	Library	Sequence description	Log2-fold change	P-Value	FDR-adjusted p-value
TR3953-c4_g1_i15	Ae-AP Head	sorbin and sh3 domain-containing protein 1 isoform x3	14.77	5.90E-23	3.41E-19
TR27559-c2_g1_i1	Ae-AP Head	rho gtpase-activating protein 190 isoform x1	14.07	6.39E-22	1.19E-18
TR42270-c3_g5_i10	Ae-AP Head	calcium-activated potassium channel slowpoke	14.04	8.41E-21	1.52E-17
TR16911-c9_g1_i6	Ae-AP Head	dynein heavy cytoplasmic isoform x2	13.97	1.95E-20	2.81E-17
TR27544-c9_g3_i1	Ae-AP Head	bromodomain adjacent to zinc finger domain protein 2b-like isoform x7	13.31	3.90E-18	2.42E-15
TR41810-c12_g5_i6	Ae-AP Head	vinculin isoform x5	12.85	1.59E-18	1.10E-15
TR4006-c7_g2_i9	Ae-AP Head	down syndrome cell adhesion molecule-like protein dscam2 isoform x30	12.76	2.79E-18	1.77E-15
TR36885-c0_g2_i1	Ae-AP Head	oxidoreductase glyr1 homolog	12.74	1.20E-17	6.36E-15
TR52610-c0_g1_i1	Ae-AP Head	sodium hydrogen exchanger 7 isoform x4	12.7	2.18E-17	8.18E-15
TR9088-c5_g1_i4	Ae-AP Head	heterogeneous nuclear ribonucleoprotein k	12.16	1.66E-16	6.35E-14
TR52097-c0_g1_i3	Ae-AP Head	synaptojanin-1 isoform x1	12.07	1.21E-15	3.58E-13
TR53811-c0_g2_i6	Ae-AP Head	e3 ubiquitin-protein ligase hectd1 isoform x4	11.76	3.20E-15	8.91E-13
TR23225-c0_g2_i1	Ae-AP Head	serine threonine-protein kinase ick-like isoform x1	11.75	2.83E-15	7.29E-13
TR8499-c8_g1_i3	Ae-AP Head	netrin receptor unc5c	11.64	7.35E-12	7.20E-10

TR13228-c0_g1_i1	Ae-AP Head	proteasome subunit alpha type-3	11.63	4.91E-14	1.03E-11
TR45093-c0_g1_i2	Ae-AP Head	adamts-like protein 4 isoform x2	11.47	3.37E-14	5.61E-12
TR11878-c8_g1_i2	Ae-AP Head	e3 ubiquitin-protein ligase nedd-4 isoform x1	11.03	1.05E-10	7.80E-09
TR45475-c0_g2_i1	Ae-AP Head	ras-related protein m-ras-like	11	3.97E-13	6.57E-11
TR16826-c0_g1_i1	Ae-AP Head	prenylcysteine oxidase-like	10.48	9.78E-11	1.26E-08
TR53868-c5_g2_i3	Ae-AP Head	caax prenyl protease 1 homolog	10.37	4.92E-08	3.92E-06

ID	Library	Sequence description	Log2-fold change	P-Value	FDR-adjusted p-value
TR53728-c7_g1_i11	Ae-SA Head	sodium channel protein para isoform x10	13.95	4.98E-21	6.31E-18
TR49009-c9_g1_i5	Ae-SA Head	voltage-dependent calcium channel subunit alpha-2 delta-3 isoform x2	13.57	6.80E-20	6.57E-17
TR37911-c0_g2_i7	Ae-SA Head	focal adhesion kinase 1 isoform x1	12.6	4.95E-17	1.83E-14
TR43558-c0_g1_i1	Ae-SA Head	PREDICTED: uncharacterized protein LOC107045241	12.54	7.85E-17	2.77E-14
TR9014-c15_g1_i2	Ae-SA Head	a disintegrin and metalloproteinase with thrombospondin motifs 8 isoform x8	12.22	6.79E-16	1.88E-13

TR49009-c9_g1_i4	Ae-SA Head	voltage-dependent calcium channel subunit alpha-2 delta-3 isoform x1	12.01	2.64E-15	6.35E-13
TR31615-c2_g1_i1	Ae-SA Head	PREDICTED: uncharacterized protein LOC105456969	11.92	5.07E-15	1.12E-12
TR27559-c2_g1_i4	Ae-SA Head	rho gtpase-activating protein 190 isoform x1	11.36	3.46E-14	6.76E-12
TR23225-c0_g2_i6	Ae-SA Head	serine threonine-protein kinase ick-like isoform x2	11.24	7.57E-14	1.37E-11
TR42270-c3_g5_i15	Ae-SA Head	calcium-activated potassium channel slowpoke isoform x16	11.15	1.37E-13	2.39E-11
TR28916-c0_g3_i4	Ae-SA Head	liprin-beta-1 isoform x4	11.15	1.39E-13	2.42E-11
TR5686-c0_g1_i1	Ae-SA Head	rna-directed dna polymerase from mobile element jockey-like	11.09	2.08E-13	3.48E-11
TR4388-c16_g1_i1	Ae-SA Head	whirlin isoform x1	11.05	2.58E-13	4.25E-11
TR52871-c3_g1_i2	Ae-SA Head	carotenoid isomerooxygenase-like	10.91	6.65E-13	1.02E-10
TR5656-c4_g1_i1	Ae-SA Head	nuclear protein localization protein 4 homolog isoform x2	10.66	3.34E-12	4.52E-10
TR19336-c1_g1_i10	Ae-SA Head	fh1 fh2 domain-containing protein 3 isoform x4	10.26	4.36E-11	4.98E-09
TR41810-c12_g5_i10	Ae-SA Head	vinculin isoform x9	9.99	1.02E-14	2.40E-12
TR30823-c0_g1_i1	Ae-SA Head	zinc finger protein 853-like isoform x1	9.93	3.34E-10	3.24E-08
TR30823-c0_g1_i9	Ae-SA Head	zinc finger protein rotund isoform x7	9.86	5.27E-10	4.90E-08
TR53880-c6_g1_i1	Ae-SA Head	elks rab6-interacting cast family member 1 isoform x2	9.76	1.87E-07	1.27E-05

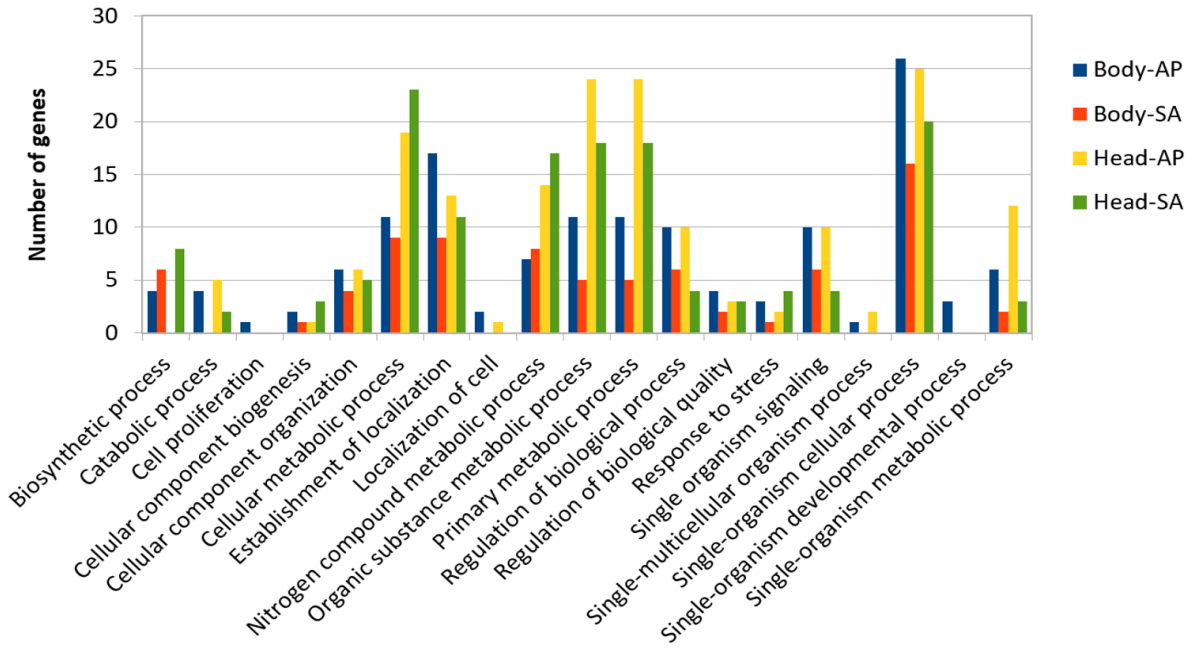

Figure 2.5. GO term distribution – Biological Process for genes with different expression patterns between *A. ervi* libraries.

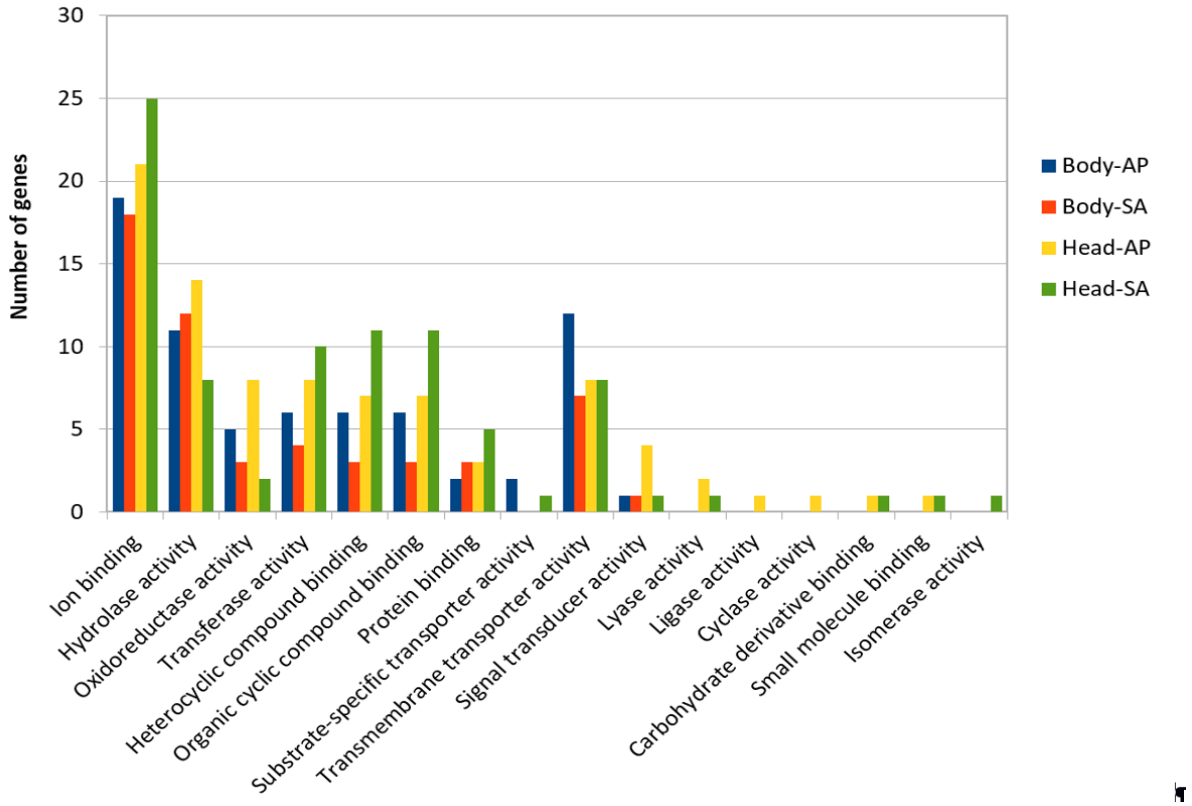

Figure 2.6. GO term distribution –Molecular function for genes with different expression patterns between *A. ervi* libraries.

Table 2.3. Expression patterns of transcripts involved in chemoperception between *A. ervi* populations reared on their natal hosts**.**

Putative annotation	Log2-fold change	FDR-adjusted p-value	Higher in
Odorant Receptor 13a like	4.13	2.26E-003	Ae – AP
Odorant Receptor 20	8.03	1.45E-005	Ae – AP
Odorant Receptor 98	3.24	3.37E-003	Ae – AP
Odorant Receptor OR2-like	8.37	3.22E-004	Ae – AP
Odorant Receptor 13 – isoform x2	3.76	5.98E-005	Ae – AP
Odorant Binding protein 83	4.40	3.78E-004	Ae – AP
Odorant Binding protein 69	3.36	2.34E-004	Ae – AP
Glutamate receptor kainate 2	3.75	2.10E-003	Ae – AP
Odorant Binding protein 56d like	7.63	2.10E-003	Ae – SA

2.5 Discussion

Our study provides the first comprehensive reference transcriptome for the aphid parasitoid wasp *Aphidius ervi* obtained from females reared on three different aphid hosts: two host-races of the pea aphid *A. pisum* and the grain aphid *S. avenae*. From the RNA sequencing of head and body of these parasitoid lines, we were able to compare expression profiles and identify putative genes involved in host fidelity (Sepúlveda et al., 2017b) Enriched GO terms in a set of significantly up-regulated genes, suggest possible gene regulatory networks responsible for the observed differences at the phenotypic level. Not surprisingly, a comparison between head and body (thorax/gaster) tissues revealed that the activity related with stimulus perception and processing (signal transduction, transmembrane transport activity among others; Figure 2.4.) are highly enriched in head tissue. On the other hand, GO terms enriched in bodies such as peptidase activity (i.e Neprilysin) are probably associated with the presence of transcripts coding for venom proteins, as *A. ervi* females have glands for venom production that is injected at oviposition and enriched in peptidases (Colinet et al., 2014; Falabella et al., 2007; Nguyen et al., 2013).

Behavioral experiments using the same laboratory populations from which the individuals of our transcriptome experiment were taken, showed that *A. ervi* collected from different aphid species (*A. pisum* and *S. avenae*) and host-races of the pea aphids (pea versus alfalfa) differed in several infectivity traits (host preference) but not in virulence (a proxy of fitness) (Zepeda-Paulo et al. 2013). This is despite the lack of any detectable genetic structuring in Chilean *A. ervi* natural populations collected from different aphid hosts species in the field (Daza-Bustamante et al., 2002; Zepeda-Paulo et al., 2015). The absence of genetic differentiation is explained by the very recent (1970ies) and single introduction event of *A. ervi* as a biological control agent for aphid pests in Chile (Zepeda-Paulo et al., 2016; Zúñiga et al., 1986). The proximal mechanisms of *A. ervi* infectivity (locating, searching and accepting an aphid host) most likely involve chemical cues from either the host-plant complex such as blends of host-induced volatiles produced by parasitized plants (Sasso et al., 2007; Takemoto & Takabayashi, 2015) or directly from the host (e.g. cuticle, cornicle secretions, faeces, exuviae, sex pheromones) (Powell & Wright, 1988). These chemical cues are most likely learned during parasitoid larval development inside the host (e.g. preimaginal conditioning; Gutiérrez-Ibáñez et al., 2007), during parasitoid emergency (early adult learning of olfactory cues; Villagra et al., 2007) or imprinted, rather than based on genetic differences in preference (i.e. innate genetic preference; Rodriguez et al., 2002; Antolin et al., 2006). Although the influence of possible genotypic variation between *A. ervi* populations can currently not be ruled out, as there could be a genetic basis accounting for variation in olfactory behavior as is the case of *D. melanogaster* (Arya et al., 2015), further studies need to be carried out to determine whether variations in olfactory behavior are inherited in *A. ervi* or they are just learned based on phenotypic plasticity and differential gene expression.

We know from many examples that the exposure of insects to different environments during juvenile development can lead to substantial differences in the transcriptome of adult individuals (Berens et al., 2015; Schrader et al., 2015). Hence, in our comparison between head transcriptomes of *A. ervi* reared on different aphid species we focused on differential expression of key components of olfactory and learning pathways (e.g. peripheral system: olfactory receptors, odorant binding proteins, ionotropic receptors, gustatory receptors; nervous system function and development). We identified *in silico* a total of 91 unigenes possessing high-sequence identities with chemosensation-related genes, including *IR*s, *OR*s,

*OBP*s, *CSP*s, *SNMP*s and *ORco*, which is similar to what has been reported for other parasitoids species (Qi et al., 2015; Zhou et al., 2015), so our approach proved to be effective for detecting and annotating genes coding for proteins associated with olfaction. As changes in olfactory sensitivity could be driven by mutations in key genes, gene gains and losses, and/or variation in gene expression (Glaser et al. 2015), and due to the lack of genetic differences between parasitoids coming from different hosts, we focused our analysis on gene expression. We found 3 *OBP*s and 5 *OR*s that were differentially expressed between head samples of Ae-SA and Ae-AP. Interestingly, both Ae-AP populations (APA and APP) had a higher number of up-regulated chemosensory genes and neuronal-related genes compared to Ae-SA (Table 2.3 – additional Table 2.1, appendix A), which is similar to that observed in other insect species capable of using different hosts, such as in the Mediterranean corn borer *Sesamia nonagrioides* (Glaser et al. 2015).

We also studied the expression profiles for genes involved in both cellular signaling and neural development. Surprisingly, genes coding for proteins involved in neuronal morphology re-modelling (Tolias et al., 2011) had higher transcriptional levels in Ae-AP heads, including Rho-GTPase activating proteins and Rho guanine nucleotide exchange factor, both of which have been described as regulators of Rho signaling activity and have been linked to olfactory learning and long-term memory formation in *A. mellifera* (Dobrin et al., 2012), while variants in genes involved in neural development and signaling (e.g., Rho proteins) are related to variation in olfactory behavior in *D. melanogaster* (Arya et al., 2015). Regarding signal transduction, we found higher expression levels in Ae-AP heads for both the sodium channel protein 60e, which participates in processing olfactory information and olfactory acuity (Kulkarni et al., 2002; Zhang et al., 2013), and Calmodulin (CaM), which interacts with ORco through binding of Ca^{+2} ions and modulates insect OR function (Mukunda et al., 2014).

Taken together, our transcriptional evidence coincides with the observation that *A. ervi* - AP shows a higher discrimination in terms of host preference than *A. ervi* - SA (Zepeda-Paulo et al. 2013). This differential expression of candidate chemosensory genes, signaling genes and neuronal development genes may explain host-fidelity in *A. ervi*, and could be a signature of adaptive phenotypic plasticity to different host and host-plant induced

environments (Glaser et al., 2015). The genes emphasized in this study deserve special attention for future research in order to prove their role in host preference and host selection by *A. ervi* (i.e, host fidelity). Further studies should also consider detailed analysis both at gene sequence level between *A. ervi* populations, and alternative splicing of these coding genes, as in the case of the tarnished plant bug (*Lygus lineolaris*), where alternative splicing may contribute to the divergence of OBPs (Oppenheim et al, 2015).

More generally, our results provide some input for discussion on the impact of phenotypic plasticity on evolutionary changes. It is indeed puzzling that, although the population does not seem to be subdivided but rather genetically homogenous (most likely due to the relative recent introduction of a limited number of individuals into Chile), we can observe distinct and persistent phenotypic and transcriptional differences between parasitoids coming from different aphid host species and host races. It has been put forward that a developmental reorganization from ancestral phenotypes due to new environmental input or conditions (host races or host biotypes in this case) does not necessarily require new mutations to produce novel or distinct phenotypes (West-Eberhard, 2003). The *A. ervi* populations in Chile may provide an example for developmental reorganization because it seems unlikely that they evolved novel adaptive mutations, as this biocontrol agent was intentionally introduced in few numbers in Chile just about 40 years ago. The next evolutionary step towards speciation based on host races would/could be genetic accommodation, i.e. developmental variants (in our case host races). When novel genetic variants are fixed within populations, those variants could limit phenotypic plasticity, so individuals can no longer easily switch between different phenotypes due to trade-offs and constraints (i.e. reduced fitness if they are not reared on their preferred hosts). It would be interesting to see whether and how many of the differentially expressed genes underlying the observed host related differences in *A. ervi* also show fixed genetic differences.

2.6 Acknowledgements

We thank Francisca Zepeda-Paulo for providing *A. ervi* populations, Sebastián Ortiz-Martinez and Daniela Sepúlveda for help in aphids and parasitoid rearing, Cinthya Villegas for help in parasitoid rearing and sexual identification, Lucia Briones for help in laboratory RNA extractions and Ruben Palma for help in data analysis. This work was funded by

FONDECYT grant 1130483 to CC Figueroa and by Iniciativa Científica Milenio (ICM) NC120027. GI Ballesteros was funded by CONICYT PhD fellowship 21120371. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Chapter III

Chemosensory genes may be involved in the formation of host fidelity in the parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae)

3.1 Abstract

To find host patches before oviposition, parasitoids of herbivorous insects utilize plant volatiles and host-derived cues. This is the case of *Aphidius ervi*, a parasitic wasp and natural enemy of several aphids, including two important global pests, the grain aphid (*Sitobion avenae*) and the pea aphid (*Acyrthosiphon pisum*). Although it has been widely used as a biological control agent of these aphid species, no studies have been conducted in order to explore the molecular basis of the olfactory mechanism underlying the significant differences in terms of host preference observed in *A. ervi*. Hence, we studied the expression profiles of selected chemosensory and olfaction-related genes in this parasitoid wasp in order to elucidate what genes are responding to aphid-host complex. This was done by developing parasitoids on two different host species (*A. pisum* and *S. avenae*) and the effect of host switching (from a natal to a non-natal host) studied. Gene expression differences were determined in heads of two outbred *A. ervi* populations originated from pea aphids (*A. pisum*) and grain aphids (*S. avenae*), and measured by quantitative real time-PCR. Parasitoids were kept on their natal (i.e., the aphid species on which parasitoids were collected in the field) or non-natal hosts for two generations. Additionally, we studied the expression profiles of the same genes but in inbred populations that have lost their host fidelity and were compared with individuals from an outbred *A. ervi* population. As expected, expression profiles of chemosensory genes in outbred populations only showed variation depending on the host used for rearing and development, thus suggesting that differences in olfactory sensitivity may participate in parasitoid adaptation to a novel host. Furthermore, we observed an overall reduction in the expression patterns of these chemosensory genes between the inbred and outbred parasitoids, which did not depend on the aphid host used for rearing/development. Hence, this reduction in the chemosensory gene expression levels would be a consequence of inbreeding under laboratory conditions, and might also be related to a reduction in terms

of the observed host fidelity, which has been previously reported for inbred *A. ervi* parasitoids.

3.2 Introduction

Insects have evolved sophisticated olfactory systems, as environmental chemical signals are critical for insect behavior, such as mate recognition, foraging, host finding and discrimination, or for finding shelter in complex environments (Suh et al., 2014). Olfaction plays a crucial role in ecological adaptations, as the sense of smell directly interfaces with the environment and neither background odors nor specific host-indicating cues are fixed in nature (Hilker & McNeil., 2008). Insects presumably modulate their olfactory system to encompass the composition of novel odorants and kairomones in response to biotic (plant or host phenotype/genotype) and abiotic factors (e.g., wind speed, temperature, humidity) influencing environmental odor profiles (Linz et al., 2013). Hence, it is expected that the insect olfactory system would adapt to different environmental conditions and/or volatiles (i.e., scent environment; Claudianos et al., 2014). This would allow insects to maintain high fitness even under changing conditions and could be a signature of adaptive phenotypic plasticity to different host and plant-host complexes (Glaser et al., 2015), ultimately providing novel targets for evolutionary processes (Arya et al., 2015; Hilker & McNeil, 2008; Moczek, 2010). Given that olfactory recognition and associated behavioral responses rely on specific sets of olfactory proteins, it is crucial to understand the molecular mechanisms involved in the perception of these cues.

The mechanism of olfaction is initiated when odorants and other semiochemicals reach the sensillar lymph through pore tubules located in the antenna and other insect tissues, and bind to the odorant binding proteins (OBPs). Then, the odorant-OBP complex is transported through the sensillar lymph and released to activate odorant-gated ion channels formed by heteromeres of odorant receptors (ORs) with a conserved odorant receptor coreceptor (ORco), which are membrane-bound proteins in the peripheral olfactory sensory neurons (ORNs; also called olfactory sensory neurons) (Leal 2013). After that the intracellular signal transduction pathway is activated, odorant molecules are then inactivated by odorant-degrading enzymes (ODEs) (Leal, 2013; Sánchez-Gracia et al., 2009;

Vandermoten et al., 2011). As ORs are the first point of neural contact for odorant molecules and chemical cues, it has been proposed that any changes in the expression levels of these olfactory genes would have a direct effect on downstream odor processing/propagation (Kopp et al., 2008; Claudianos et al., 2014), accounting for variations in the insects' host range, especially in insects with intimate relationships with their hosts, such as parasitoids (Simon et al., 2015).

Parasitoid wasps (parasitoids) are a hyper-diverse and geographically widespread insect group whose larvae feed on other arthropods (usually insects). As in host-parasitoid systems, feeding and reproduction usually ends with the death of the host (Godfray 1994) parasitoids have been widely used in biological control programs, significantly contributing to huge savings in agriculture and food security (Simpson et al., 2011). However, for successful reproduction and progeny survival, parasitoids must locate and recognize a suitable host, usually in chemically complex environments. This process strongly depends on the strategies used by the mother to exploit her environment and available hosts; Hence, parasitism involves well-developed foraging behaviors such as habitat and host finding, and accepting a suitable aphid host (parasitoid's infectivity) (Godfray, 1994). Infectivity most likely involves parasitoid's behavioral responses to semiochemical cues from either the plant-host complex such as blends of host-induced volatiles (volatile organic compounds, VOCs) produced by plants upon herbivory damage (Sasso et al., 2007; Takemoto & Takabayashi, 2015) or directly from the host (e.g., cuticular hydrocarbons, cornicle secretions, faeces, exuviae, sex pheromones) (Powell & Wright, 1988). Host recognition at species level is achieved only after antennal contact (Le Ralec et al., 2005) and involves perception of host cues which are more reliable but less detectable over longer ranges (Vet and Dicke 1992; Takemoto et al., 2011). Parasitoid females seem to use cuticular contact kairomones to discriminate between host and non-host species at very short range (less than 4mm; Le Ralec et al., 2005) or on contact (Battaglia et al., 2000), while recognition of these kairomones trigger female attack behavior (Muratori et al., 2006).

One of the most successful biological control programs is based on the use of the wasp *Aphidius ervi*, a worldwide distributed koinobiont endoparasitoid of several Macrosiphinae aphid species, including the control of the pea aphid *Acyrthosiphon pisum* on

legumes (Henry et al., 2010; Stilmant et al., 2008) and the grain aphid *Sitobion avenae* on cereals (Cameron et al., 1984). In Chile, *A. ervi* wasps were introduced from Europe during the late 70s as part of an aphid biological control program in cereals. Shortly after their introduction, *A. ervi* wasps were successfully parasitizing both *A. pisum* on legumes (e.g., alfalfa) and *S. avenae* on cereals (e.g., wheat) (Starý, 1993; Zepeda-Paulo et al., 2013). Remarkably, these two aphid species differ in several biological aspects (e.g., host range usage, body size and color, the composition of semiochemicals in the cuticle, cornicular secretions, defensive behaviors, etc.) (Daza-Bustamante et al., 2003), which opens a number of questions regarding the specificity of olfaction and their consequences on parasitism.

A recent study showed that host preference and acceptance (i.e., infectivity) depend on the host where *A. ervi* parasitoids were reared on (i.e., previous experience); however, no effects on the virulence (a proxy for fitness) were found when parasitoids were forced to oviposit on non-natal hosts (Zepeda-Paulo et al., 2013). This strongly indicates that these parasitoids can retain (i.e. through associative learning; Poppy et al., 1997) their ability to develop successfully on non-preferred hosts (Kaiser et al., 2017). This response has been interpreted as adaptive phenotypic plasticity, an evolutionary process that should lead to the formation of host fidelity (i.e., the preference for the aphid/plant system from which females emerged) (Daza-Bustamante et al., 2002; Henry et al., 2008; Zepeda-Paulo et al., 2013). Interestingly, inbred populations of *A. ervi* seem to lose their host fidelity due to a lack of host preference for any aphid host in female parasitoids, regardless of the natal host on which parasitoids developed (Sepúlveda et al., 2017b). However, it is unknown how inbreeding could modify the molecular mechanisms of olfactory perception at the chemosensory gene expression levels, which in turn could cause variations in the olfactory responses to odors (for example, by causing the degradation of sensory sensitivity and/or changes in the olfactory receptor neurons) in laboratory-reared populations compared to their "wild" counterparts from fields (Nielsen et al., 2015).

Consequently, in this Chapter we studied to what extent the exposure to a novel environment (non-natal plant-host complex) has an impact on the expression levels of selected chemosensory genes, in order to identify the molecular mechanisms of host-fidelity formation. This was done by addressing the phenotypic plasticity that two lines of *A. ervi*

parasitoids naturally parasitizing different aphid species (*A. pisum* and *S. avenae*) can display, comparing the effects of rearing parasitoids on their own host (natal host) or transplanted to other non-natal host on the expression levels of chemosensory genes. Additionally, expression of chemosensory genes was profiled and compared between *A. ervi* parasitoids collected from field (exogamic) and caged (endogamic) populations.

3.3 Materials and Methods

Parasitoid collection and rearing

Parasitized individuals of *Acyrthosiphon pisum* and *Sitobion avenae* were collected as aphid mummies from fields of alfalfa (*Medicago sativa* L.) and wheat (*Triticum aestivum* L.), respectively, in Region del Maule, Chile (S 35° 24´, W 71° 40´). Aphid mummies were isolated in Petri dishes until adult parasitoid emergence. The emerged naïve parasitoids were then determined as *Aphidius ervi* and sexed following standard taxonomic keys (Starý, 1995) under an Optika ST-155 (10x) compound microscope. In order to establish stock laboratory *A. ervi* parasitoid populations from each aphid host (*A. pisum* and *S. avenae*), five single, isolated naïve *A. ervi* virgin females were randomly selected and mated with a naïve virgin male isolated from the same population. Female and male individuals were left to mate in a petri dish for 24h with diluted honey and water for sustenance. Mated females then were transferred to a cage containing aphids *ad libitum* from the same species from which they emerged (thereafter natal host) was offered, with diluted honey and water for sustenance. The establishment of *A. ervi* parasitoids on their natal host for a single generation was used to erase any previous field experience (see Henry et al., 2008). Thus, two different stock *A. ervi* laboratory populations (20°C, D16/N8 photoperiod) were established: (i) *A. ervi* population from *A. pisum* (Ae-AP; natal host AP) and (ii) *A. ervi* population from *S. avenae* (Ae-SA; natal host SA).

Two different aphid hosts were used for parasitoid rearing: *A. pisum* (alfalfa race) maintained on broad bean (*Vicia faba* L.) and *S. avenae* maintained on barley (*Hordeum vulgare* L.). These aphids and their host plants have been used successfully for *A. ervi* rearing in previous studies (Ballesteros et al., 2017; Sepúlveda et al., 2017b; Zepeda-Paulo et al., 2013). Aphids were reared under laboratory conditions that allowed the continuous

parthenogenetic reproduction of aphids (20°C, D16/N8 photoperiod) (Zepeda-Paulo et al., 2013). All aphid populations used in this study were free of facultative endosymbiont bacteria, as they are well-known to naturally occur in aphid populations (Dennis et al., 2017; Sepulveda et al., 2017a), including the defensive endosymbiont *Hamiltonella defensa*, which confers protection against parasitoids (Oliver et al., 2014; Vorburger 2014). The presence of different endosymbionts previously reported in aphids was tested using the specific amplification of 16S rDNA from whole-body aphid DNA as described in Peccoud et al. (2014).

Reciprocal transplant experiments

To study the plasticity of chemosensory genes expression in *A. ervi*, a reciprocal transplant experiment was conducted to determine the effects of the rearing host (natal host, control condition vs non-natal host, transplanted condition) (Figures 3.1A to 3.1D). Eight chemosensory-related genes (five *OR*s and three *OBP*s; Table 3.1) were selected as differences in expression levels for these eight genes were detected in previous transcriptional studies of two *A. ervi* parasitoid populations reared on two different aphid hosts (*S. avenae* and *A. pisum*; Chapter II; Ballesteros et al., 2017).

From the *A. ervi* stock populations, aphid mummies were isolated in petri dishes until parasitoid emergence. Virgin adult female parasitoids were left to mate with a male from the same population for 24h. Mated females were transferred and maintained for two generations in a rearing cage where non-natal host aphids were offered *ad libitum.* Previous studies have shown this procedure suitable for addressing the formation of host fidelity in *A. ervi* (Zepeda-Paulo et al., 2013; Sepúlveda et al., 2017b).

Additionally, the expression levels of the same set of chemosensory genes were compared in a reciprocal transplant experiment between *A. ervi* parasitoids collected from field and inbred laboratory populations (Figures 3.1E, 3.1F). *A. ervi* field populations were obtained from aphid mummies sampled on alfalfa or wheat, and were left to acclimate on the natal and non-natal hosts under laboratory conditions for two generations (exogamic population). Inbred populations were generated after maintaining parasitoids in the same natal plant-host complex for approximately seventy-five generations (endogamic population). The experimental individuals sampled from the inbred population corresponded

to the same parasitoids studied in Sepúlveda et al. (2017b), which were preserved appropriately (see below) after preference measurements (Sepúlveda et al., 2017b).

From each rearing cage, aphid mummies were isolated in petri dishes until parasitoid emergence. Virgin adult female parasitoids were left to mate with a male from the same lineage for 24h, as after mating females display higher attraction to oviposition-site cues (Jin et al., 2017). Each mated female was then transferred to an experimental arena (a modified 2-cm-diamenter petri dish) containing a single wingless aphid and a small piece of leaf from the plant where the aphid was feeding (i.e., broad bean for *A. pisum* and barley for *S. avenae*) (Sepúlveda et al., 2017b). Immediately after successful oviposition, each parasitoid was stored separately in 1.5 ml microcentrifuge tubes containing RNALater (QIAGEN, Hilden, Germany) at -20°C for storage until parasitoid dissection and RNA extraction.

RNA extraction and cDNA synthesis

Female heads were dissected from bodies on ice using a sterile scalpel and pooled in a 1.5 ml microcentrifuge tube (N=5 per pool). We chose to study heads as most of the olfactory-associated proteins are abundant in heads and it contain most of the organs involved in chemosensory function, sensory and feeding centers (Leal 2013; Wang et al., 2017). Total RNA was extracted from each pool of heads by using the RNEasy Plant Mini Kit (QIAGEN, Hilden, Germany) and eluted in 50 µl of RNAse free water. Integrity of RNA samples was assessed using a 1.1% denaturing formaldehyde agarose gel electrophoresis, and concentration was estimated by spectrophotometry at 260nm (Epoch Microplate Spectrophotometer, Biotek) with a resulting range of 4.26 - 8.17 ng/µl of total RNA for all samples. DNA traces were removed from the samples by DNase treatment using Turbo DNase (Ambion). Single-stranded cDNAs were synthesized using the SuperScript III Reverse Transcriptase System (Invitrogen). All procedures were conducted following manufacturer's instructions.

Bioinformatic and gene expression analysis of OBPs and ORs

Relative transcript abundances of genes coding for five *OR*s and three *OBP*s (Tables 3.1 & 3.2) were assessed through Real-Time PCR (qPCR) in *A. ervi* female heads maintained on their natal or transplanted to their non-natal hosts (Figure 3.1). These genes were selected

because they show a differential expression when *A. ervi* females are reared in different plant-host complexes (Ballesteros et al., 2017; Chapter 2). The annotation of the genes was manually verified using BLASTx ver 2.7.0 against NCBI NR database (September 2017) for homology analysis with genes from other insect species such as *D. melanogaster,* which have been functionally characterized and for which odor response data have been published (Table 3.1). For each selected target gene, specific primer pairs were designed with Beacon Designer 8 software (Premier Biosoft) using our recently published *A. ervi* transcriptomic as template sequence (Ballesteros et al., 2017). All primers are listed in Table 3.2. Each PCR mixture contained 2 µl of diluted cDNA (2ng; 1ng/µl), 10 µl Maxima SYBR Green PCR Master Mix (ThermoFisher Scientific), 6.4µl of nuclease free water and 0.8 µl of each specific primer (1.6 µl for both forward and reverse primer; 10mM concentration). Negative controls (nuclease-free water) were included for detecting any cross-contamination; positive controls for qPCR reactions were also included (*A. ervi* genomic DNA). All PCR reactions were carried out in triplicate using the Mx3000P qPCR system (Stratagene) under the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 30s, 56°C for 45s and 72°C for 40s. A dissociation curve was included immediately after each qPCR using a ramp of 55-95°C to confirm the absence of non-specific amplifications. All amplicons were sequenced to confirm the specific amplification of target genes.

Expression data for each target gene was normalized using published primers which amplify Ribosomal Protein L19 of *A. ervi* (Colinet et al., 2014; primers in Table 3.2). Data from all *A. ervi* populations and rearing conditions were analyzed manually and relative transcript levels for each target gene calculated using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Each PCR reaction was performed in triplicate (three technical replicates) and the mean of three biological replicates calculated. Data were analyzed statistically by two-way ANOVA using GraphPad Prism version 6.01. The expression for a given gene was compared between parasitoids reared on natal and non-natal hosts considering the natal condition as control for which a relative expression value of 1 was assigned. In the case of gene expression comparison between outbred and inbred *A. ervi* populations, a relative expression value of 1 was assigned to the outbred condition.

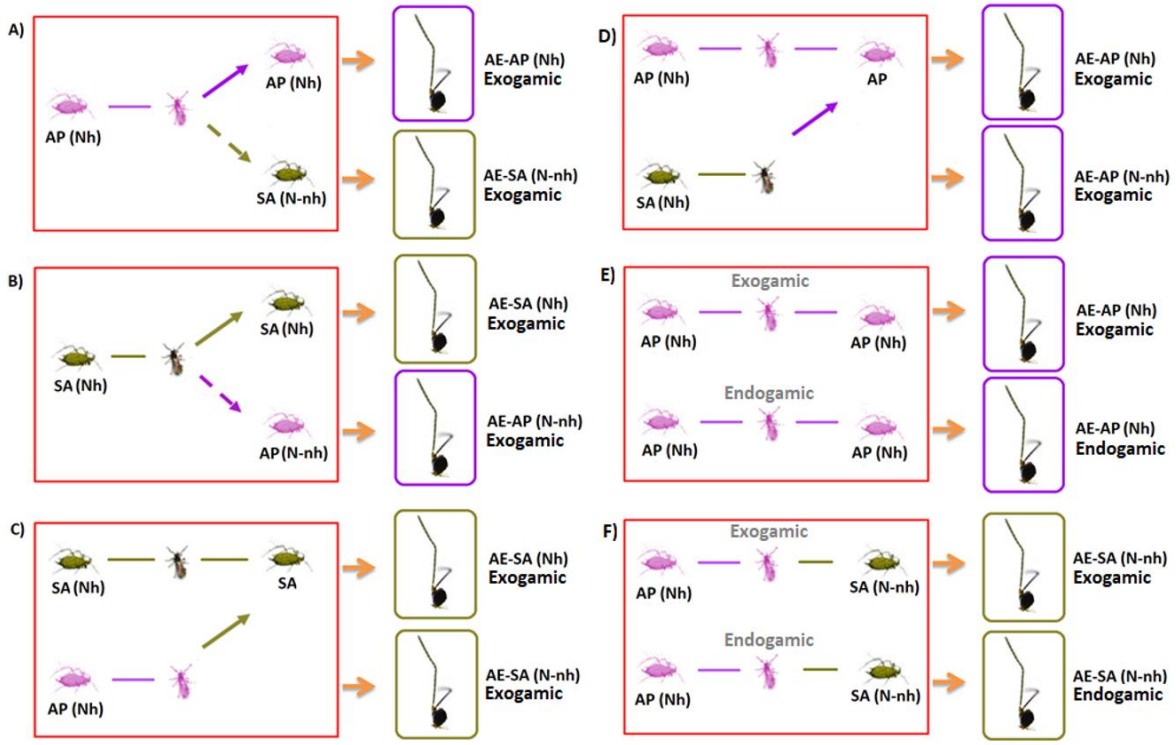

Figure 3.1. Reciprocal transplant experiment of exogamic populations of *Aphidius ervi* (panels A, B, C, D) and comparison with endogamic populations on the same hosts (Sepúlveda et al., 2017b; panels E, F). **AE:** *A. ervi;* **AP:** *A. pisum*; **SA:** *S. avenae*; **Nh**: Natal host; **N-nh**: non-natal host. **A)** AE–AP maintained in natal host AP (AE-AP Nh) and switched to non-natal host SA (AE-SA N-nh). **B)** AE-SA *S. avenae* maintained in natal host SA (AE-SA Nh) and switched to non-natal host AP (AE-AP N-nh). **C)** AE-AP maintained in natal host SA (AE-SA Nh) and AE–SA switched to non-natal host AP (AE-AP N-nh). **D)** AE-AP maintained in natal host AP (AE-AP Nh) and AE-SA switched to non-natal host AP (AE-AP N-nh). **E) AE-AP** (natal host) comparison between exogamic and endogamic (75+ generations) populations. **F) AE-SA** (non-natal host) comparison between exogamic and endogamic (75+ generations) populations.

3.4 Results

OBPs and ORs gene expression differences between parasitoids reared on natal and non-natal hosts

The expression of target chemosensory related genes was assessed when parasitoid females from the same natal host were reared on their natal host (control condition) and non-natal hosts (experimental condition). As expected (Ballesteros et al., 2017), variation in the expression levels was observed when comparing parasitoids from both *A. pisum* and *S. avenae* that were reared on their natal host or transplanted to non-natal hosts (Figure 3.2 and 3.3).

Our results indicate that *OR-H* and *OBP-F* genes are up-regulated when *A. ervi* is reared on *A. pisum* (AP) compared to *S. avenae* (SA) (Figure 3.2 and 3.3) regardless the natal host; hence it seems that the rearing on *A. pisum* increases the abundance of transcripts for both *OR-H* and *OBP-F* genes compared to *A. ervi* reared on *S. avenae*. In contrast, the *OR-B* gene showed a reduction in its expression when parasitoids were switched from their natal to non-natal hosts; this down-regulation seems to be independent of the natal aphid species. A down-regulation was detected for *OR-J* and *OR-E* when *A. ervi* is switched from AP (natal host) to SA (non-natal host), although no differences were observed for the reciprocal (SA to AP). Finally, *OBP-C* was down-regulated when *A. ervi* was switched from SA to AP but not when parasitoids were transplanted from AP to SA.

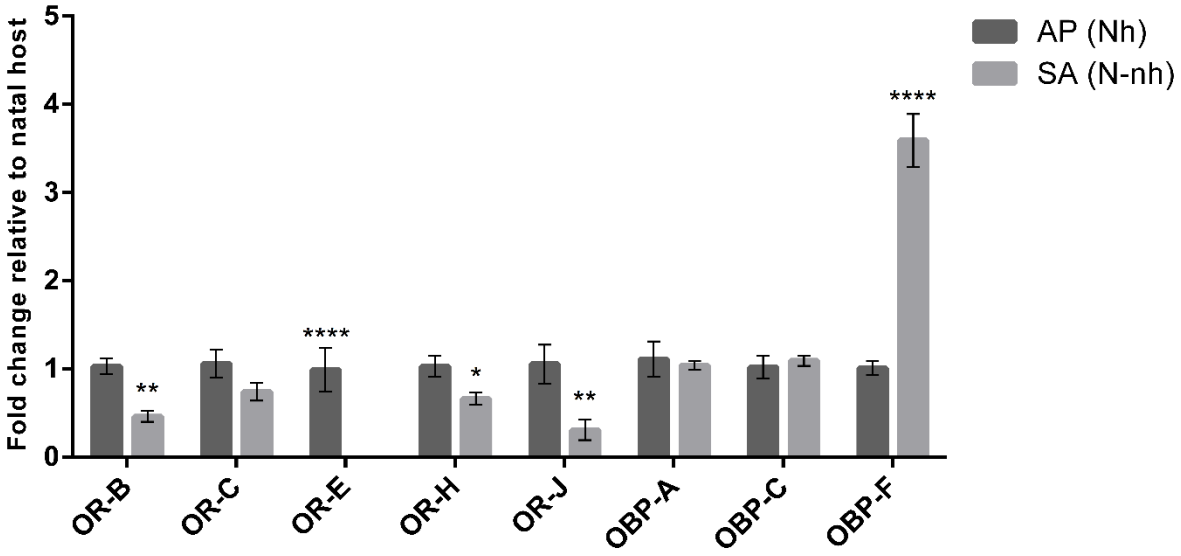

Figure 3.2. Mean mRNA expression levels of *OR*s and *OBP*s from heads of *A. ervi* maintained on natal host *A. pisum* (AP; Nh) or on the non-natal host *S. avenae* (SA; N-nh) measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the mean. * Over the bars indicate significant differences according to two-way ANOVA ($P < 0.05$).

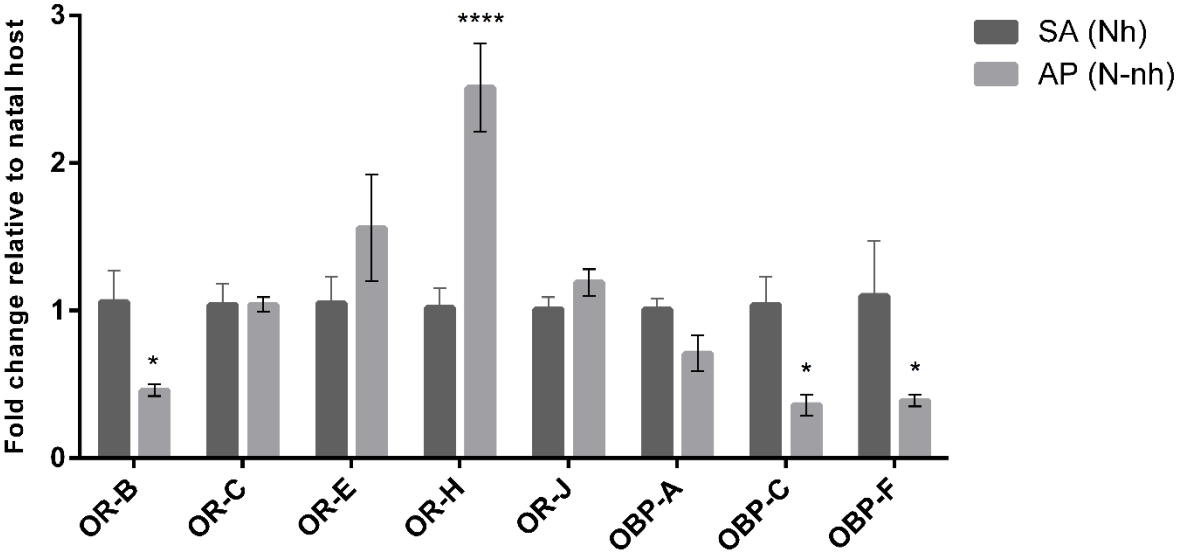

Figure 3.3. Mean expression levels of *OR*s and *OBP*s from heads of *A. ervi* maintained on the natal host SA (*S. avenae*; Nh) or on the non-natal host AP (*A. pisum*; N-nh) measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the mean. * Over the bars indicate significant differences according to two-way ANOVA ($P < 0.05$).

OBPs and ORs expression differences between parasitoids reared on different natal hosts but transplanted to the same aphid host

The expression of target chemosensory related genes was assessed when parasitoid females from different natal hosts (AP and SA) were reared on the non-natal aphid host species (SA, Figure 3.1C and AP, Figure 3.1D). Those comparisons aim to determine whether rearing on the same aphid host species alter gene expression levels of chemosensory genes.

Our results indicate that all but one odorant receptor gene (*OR-E*) had similar expression levels between parasitoids reared on SA (SA-Natal) compared to parasitoids transplanted to SA (originally from AP; Figure 3.4). In the case of females of *A. ervi* transplanted to AP (originally from SA; Figure 3.5) and compared to females maintained in AP (AP-Natal), two *OR*s showed up-regulation (*OR-E* and *OR-J),* while three *OR*s were down-regulated (*OR-B, OR-C* and *OR-J*). Hence, it seems that switching parasitoids from *S. avenae* to *A. pisum* has a higher effect on the expression levels for *OR*s, while *OBP*s expression levels remained similar between both conditions (Figure 3.5).

OBPs and ORs expression differences between field and inbred parasitoids reared on natal and non-natal hosts

Using the same set of target chemosensory related genes as above, we next compared gene expression between field (exogamic) and inbred (endogamic) populations of the parasitoid wasp *A. ervi*.

When gene expression levels were compared between parasitoids from field and inbred populations reared on AP, slightly but not statistically significant lower expression levels were observed for *OBP*s (Figure 3.6). In the case of *OR*s, lower expression levels were observed for 4 out of 5 odorant receptors (3 of them being statistically significant), while only *OR-E* in the inbred population showed a significant higher expression when compared to the field population (Figure 3.6).

Comparisons between outbred and inbred parasitoid populations switched to non-natal host SA, displayed lower expression levels for the studied genes under the inbred condition compared to field population for both *OR*s and *OBP*s (Figure 3.7). The significantly lower amount of transcripts measured for these genes in both *A. ervi* inbred populations might

be caused by long-time rearing under laboratory conditions (i.e., absence of signals), and may explain the loss of host fidelity observed previously (Sepúlveda et al., 2017b).

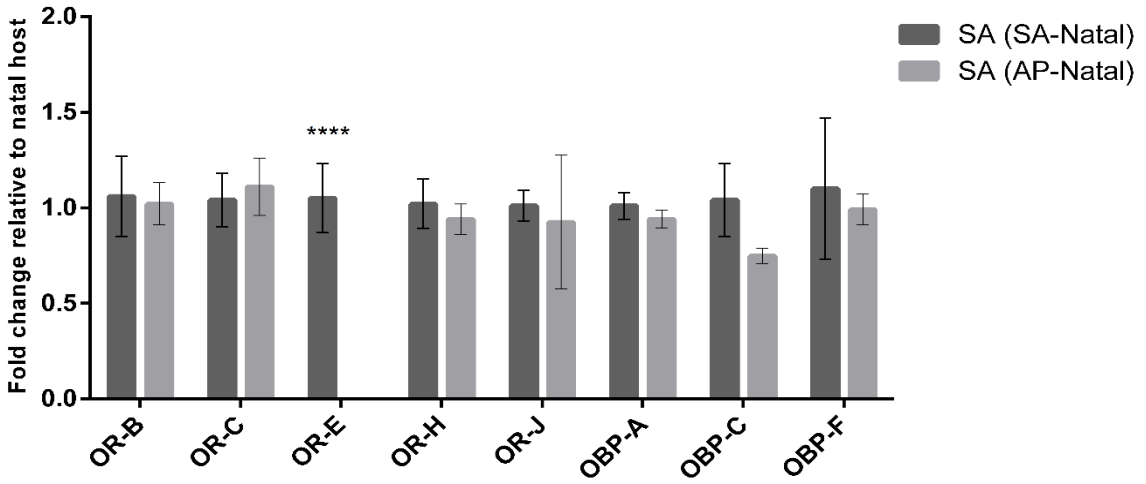

Figure 3.4. Mean mRNA expression levels of *OR*s and *OBP*s from heads of *A. ervi* maintained on the natal host *S. avenae* (SA-Natal) or switched from *A. pisum* to the non-natal host *S. avenae* (AP-Natal) measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the mean. * Over the bars indicate significant differences according to two-way ANOVA ($P < 0.05$).

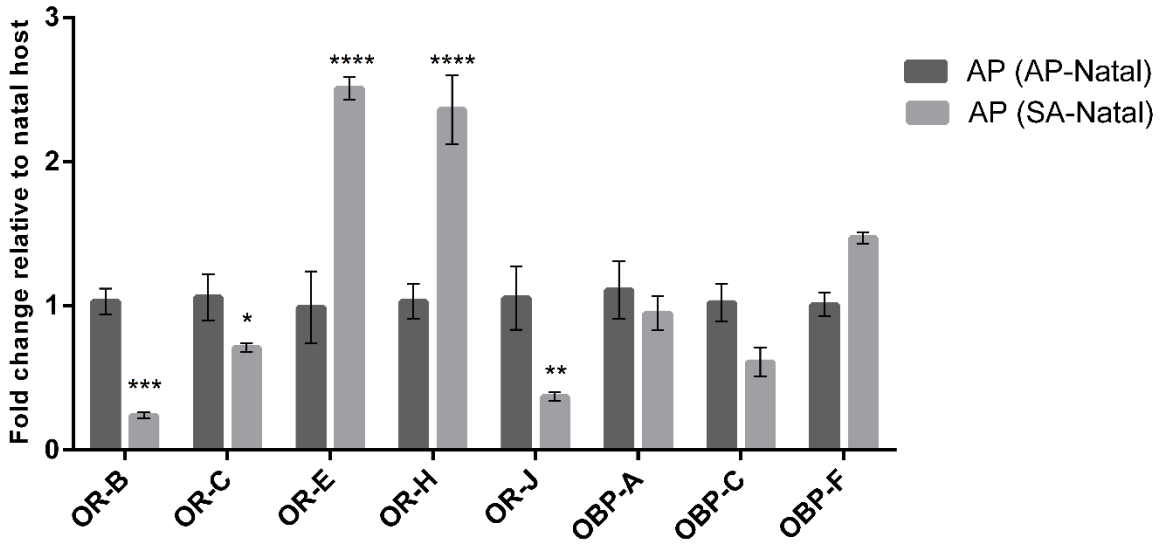

Figure 3.5. Mean mRNA expression levels of *OR*s and *OBP*s from heads of *A. ervi* maintained on the natal host *A. pisum* (AP-Natal) or switched from *S. avenae* to the non-natal host *A. pisum* (SA-Natal) measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the

mean. * Over the bars indicate significant differences according to two-way ANOVA (P < 0.05).

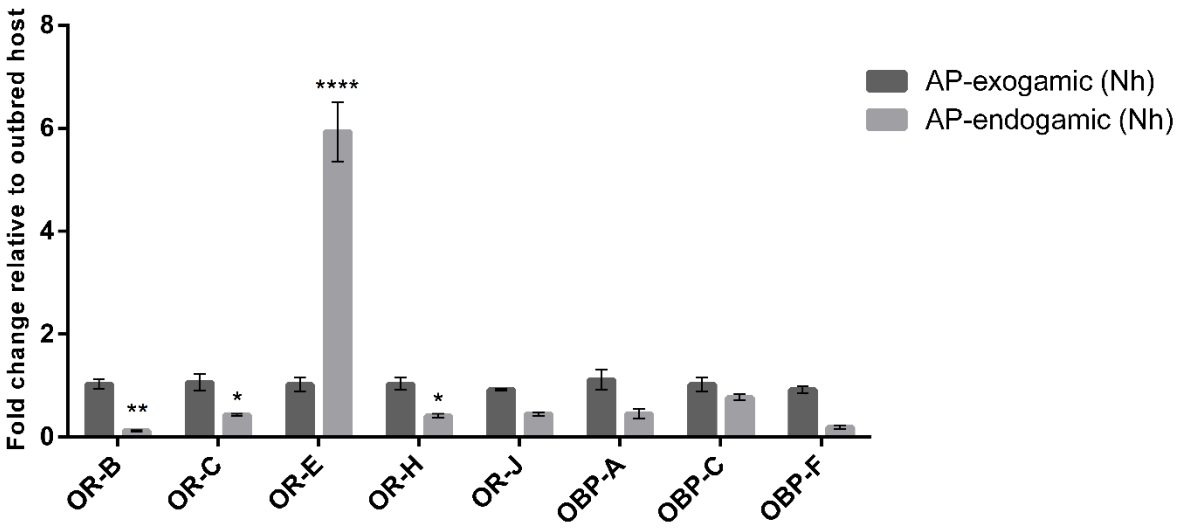

Figure 3.6. Mean expression levels of *OR*s and *OBP*s from heads of outbred (AP-exogamic) and inbred (AP-endogamic) *A. ervi* maintained on their natal host *A. pisum* (AP), measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the mean. * Over the bars indicate significant differences according to two-way ANOVA (P < 0.05).

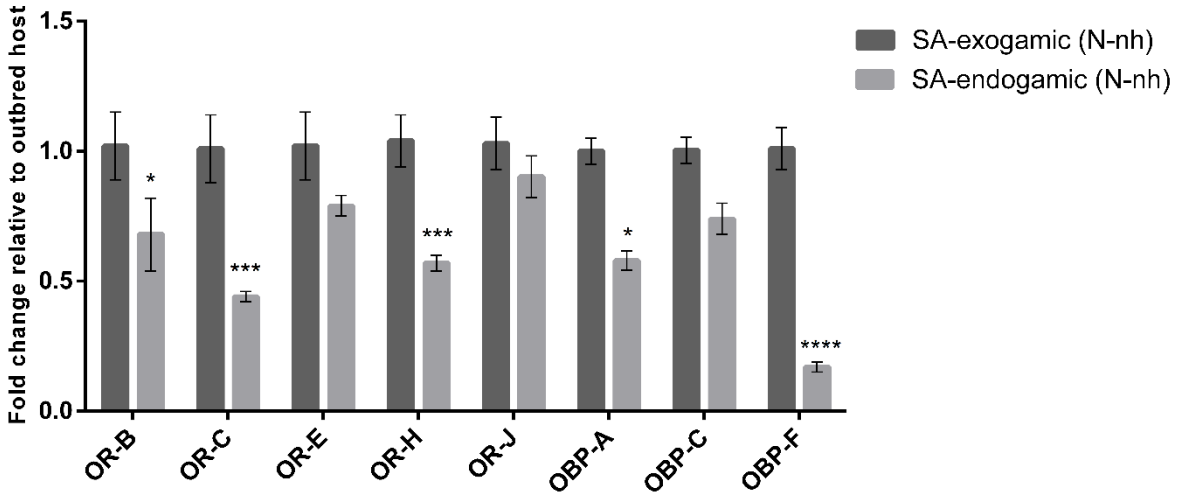

Figure 3.7. Mean expression levels of *OR*s and *OBP*s from heads of outbred (SA- exogamic) and inbred (SA-endogamic) *A. ervi* transplanted from the natal host A. pisum onto the non-natal host *S. avenae* (SA), measured by RT-qPCR. RT-qPCRs were performed using specific primers for each gene. Normalizer gene: *RPL19*. Error bars indicate standard error of the mean. * Over the bars indicate significant differences according to two-way ANOVA (P < 0.05).

Table 3.1. OR and OBP homologs from *Drosophila spp* found in *A. ervi* using BLAST, and odorants eliciting responses. † indicates transcript with higher expression levels in *A. ervi* – SA as indicated by previous transcriptomic analysis.

Transcript ID	Amplicon ID	Best *Drosophila* hit	Response / tuning to	Reference
TR10701|c0_g1_i1	***OBP-A***	Odorant binding protein *Lush*	(*Z*)-11-octadecenyl acetate; 11-cis vaccenyl acetate	Fan et al., 2011
TR39104|c3_g3_i1	***OBP-C***	Odorant binding protein 83a	l-carvone; citral	Swarup et al., 2011
TR46958|c0_g1_i1	***OBP-F †***	Odorant binding protein 56e	octanoic acid; hexanoic acid	Dworkin & Jones 2009
TR2742|c0_g1_i2	***OR-B***	Odorant receptor 9a	3-hydroxy-2-butanone; 2,3-butadeniol; 2-pentanol	Sabery & Seyerd-allaei 2016
TR48683|c0_g1_i1	***OR-C***	Odorant receptor 82a	geranyl acetate; (2R)-hexan-2-ol; citral	Münch & Galizia 2016
TR48968|c0_g1_i2	***OR-E***	Odorant receptor 43a	Z3-hexenol; 1-hexanol; cyclohexanol; 1-octen-3-ol; 2-pentanol	Münch & Galizia 2016
TR7457|c0_g1_i1	***OR-H***	Odorant receptor 13a	1-octen-3-ol; 2-heptanol; 2-exanol; 3-octanol	Münch & Galizia 2016
TR52641|c0_g1_i3	***OR-J***	Odorant receptor 85d	Ethyl pentanoate; 2-heptanone-6-methyl-5-hepten-2-none	Münch & Galizia 2016

Table 3.2. Nucleotide sequences of primers employed in qPCR in this study. * indicates sequence coding for *A. ervi* ribosomal protein L19 available in NCBI GenBank; listed primers for RPL19 are the same primers used by Colinet et al., 2014 as normalizer for qPCR analysis in *A. ervi*

Transcript ID	Amplicon ID	Forward Primer (5'-3')	Reverse Primer (5'-3')	TM (°C)
TR10701|c0_g1_i1	***OBP-A***	AGCAGTTCAATCAATTCAAG	TTCAAGTAGTCATATAGTTGGT	58.3
TR39104|c3_g3_i1	***OBP-C***	TTGAAGTTGAAATGTTGGTT	CACATATCAGGTCTTGTTTG	58.0
TR46958|c0_g1_i1	***OBP-F***	TACGATATTTACCATACAGCAT	TAGTGGAACAATTTGAAGAAC	58.7
TR2742|c0_g1_i2	***OR-B***	ACAACAGACAATGTGTATTC	AGTATAAATGGTCCTGCTAAT	57.8
TR48683|c0_g1_i1	***OR-C***	GCAATTTGTTACGGACTATT	GTTGTTTACTGTCACACATT	58.1
TR48968|c0_g1_i2	***OR-E***	TCAACAAATTCCTCCTTACA	ATACAATATGGTGGCGATAA	58.1
TR7457|c0_g1_i1	***OR-H***	GTCATTATTCACAGTTGGATT	GTATCAAGAGCAACAACAATA	58.0
TR52641|c0_g1_i3	***OR-J***	TTGATGGTGATAATGGTAAGA	CACTTGACGATATAATGACAA	57.8
JAC59129.1 *****	***RPL19***	ATCAAGCTGAAGCTCGTCGT	TGCAGCTGCTTCATCTTCAC	56.6

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3.5 Discussion

Parasitoids represent one of the most used natural enemies for biological control of pest species, as they are considered highly host-specific (Godfray 1994). This is the case of *Aphidius ervi*, an endoparasitoid wasp described as a moderate host specialist that has been successfully used for the biocontrol of economically relevant aphid species that attack different crops (e.g., cereals, legumes, potatoes) (Zepeda-Paulo et al., 2013). However, within the range of potential aphid host species that can be found in those crops, not all are equally preferred (Stilmant et al., 2008). Reciprocal transplants experiments conducted using *A. ervi* females showed that they are able to discriminate between host aphid species and choose the most suitable for oviposition. Intriguingly, no evidence of local host adaptation was found for *A. ervi*, neither on the pea or the cereal aphid, thus suggesting that the most parsimonious explanation for the presence of distinct phenotypes on different aphid hosts is the ability of parasitoid wasps to display a high phenotypic plasticity rather than a host-related genetic differentiation (Zepeda-Paulo et al., 2013). Furthermore, the lack of any detectable genetic structuring in Chilean *A. ervi* natural populations is explained by a high gene flow driven by male dispersion (Daza-Bustamante et al., 2002; Zepeda-Paulo et al., 2015).

During host-seeking, parasitoids use a group of mechanisms that account for the infectivity of these wasps (locating, searching and accepting an aphid host), most of them based on the detection of chemical cues. In fact, the parasitoid foraging involves the integration of multiple chemical cues emitted from the plant-host complex (long-range orientation), which triggers specific behaviors such as oriented flight behavior and landing on infested plants (Du et al., 1996; Du et al., 1998), and from the host itself (short range signals), which elicit several behaviors that ultimately lead to the oviposition into the aphid host (Powell & Wright, 1988), Odorants are probably handled in a combinatorial fashion, with different quantitative combinations of odorants triggering different responses; thus, minor changes in blend composition might seem insignificant but could contribute greatly to the specificity of parasitoid response (Pareja et al., 2009). For example, plants infested with aphids carrying specific symbiont species/strains are less attractive to *A. ervi* through changes

in the blends of herbivore-induced plant volatiles, although the same volatile compounds were emitted (Frago et al., 2017)

Hence, the perception of chemical cues that occurs during foraging is crucial for host finding and host recognition, but no research involving molecular mechanisms of chemical perception has been conducted on *A. ervi* so far. Our study provides the first comprehensive expression analysis for genes involved in peripheral olfactory mechanisms (*OBP*s and *OR*s) using quantitative PCR. Reciprocal transplant experiments conducted in this study showed that switching *A. ervi* females from the same population to a novel plant-host complex (non-natal host) has significant effects on how a group of chemosensory related genes (*OR*s and *OBP*s) are expressed in terms of transcript abundances in their offspring. This could be the result of exposure to both different blends of volatiles (or novel volatiles) emitted by the plant-host complex, and to the host cuticular hydrocarbons produced by the aphid host, and to which the parasitoid is exposed during its embryonic and larval development and emergence (Villagra et al., 2007). Also, differences in the expression profiles of *OR*s and *OBP*s were observed when comparing field and inbred parasitoids populations, suggesting that inbreeding under laboratory conditions disrupts the highly coordinated mechanisms of olfaction.

Currently, the knowledge on *OR*s and *OBP*s specificity in parasitoid wasps is quite limited, while the best model system studied to date is *Drosophila melanogaster*, for which odorant-response profiles for *OR*s are well characterized (Münch & Galizia, 2016). Although the complexity of olfactory sensory systems differs across species, they show striking similarities in their neurocomputational logic (Eisthen, 2002; Hildebrand & Shepherd, 1997; Leal, 2013). Next, we discuss the involvement of *OR*s and *OBP*s during host-recognition in *A. ervi*, using a comparative biology approach, as this could give insights and clues about the putative odorant responses for specific *OR*s and *OBP*s displaying differences in expression levels in the parasitoid wasp *A. ervi*.

Putative role of odorant binding proteins in parasitoid wasps host recognition

In insects, the mechanism of olfaction is initiated when odorants and other semiochemicals reach the sensillar lymph through pore tubules located in the antenna and other insect tissues and bind to the odorant binding proteins (OBPs). Then, the odorant-OBP

complex is transported through the sensillium lymph to the receptors and olfactory neurons (Leal 2013). OBPs are a large family of small, soluble and highly abundant proteins secreted into the sensory lymph, and are thought to provide a first filtering mechanism for semiochemicals as they are the main proteins involved in the interaction between odorants and the membrane-bound ORs (Tunstall & Warr, 2012). In insects such as *D. melanogaster*, *OBP*s have shown to be differentially expressed in subsets of olfactory sensilla (Larter et al., 2016), and therefore could contribute to the sensitivity or selectivity of different sensilla types (Tunstall & Warr, 2012). This has been explained in terms of different affinities to odorants displayed by OBPs, so that distinct expression patterns for *OBP* genes suggest odorant selection and triggering of specific olfactory and behavioral responses in insects that impact on host preference (Fan et al., 2011; Glaser et al., 2015).

Odorant-binding properties of OBPs have been determined for different insect species (Fan et al., 2011), including the solitary endoparasitoid wasp *Microplitis mediator* (Li et al., 2014). Interestingly, homology searches based on sequences from endoparasitoid wasps such as *M. demolitor* showed that two of the *OBP*s analyzed in our study (*OBP-A* and *OBP-F*) had high identity values (> 40%) with *OBP8* and *OBP10* from *M. mediator*, respectively, while the top BLAST hit for *OBP-C* is pheromone-binding protein 1 *M. mediator*. Functional analysis of *OBP8* and *OBP10* in *M. mediator* has shown that these genes are mainly expressed in antennae of adult wasps and can bind a broad range of odorant molecules with different binding affinities, including nonane, farnesol, nerolidol, nonanal, β-ionone, acetic ether and farnesene (Li et al., 2014). Additionally, adult parasitoids show behavioral responses (either attraction or repellence) to these volatiles (Li et al., 2014). The higher expression differences found for *OBP-F* in parasitoids maintained on *S. avenae* compared to parasitoids maintained on *A. pisum* (regardless the natal host) may be related to the developmental exposure of *A. ervi* larva to the plant-host complex, as aphid mummies were taken straight from their rearing cages and isolated in petri dishes. Moreover, the exposure to host-plant volatiles from infested plants during larval stages of *A. ervi* would induce olfactory responses in the adults (Gutiérrez-Ibáñez et al., 2007; Takemoto et al., 2011).

Putative role of odorant receptors during aphid host recognition by parasitoid wasps

Parasitoid females are attracted to volatiles emitted by aphids and may use these volatiles as a host-species recognition mechanism (Poppy et al., 1997). The E-*β*-farnesene (EBF) is an alarm pheromone produced by many aphid species that is released when aphids are attacked or irritated; this alarm pheromone is known to attracts natural enemies, including the parasitoid wasp *A. ervi* (Cui et al., 2012). However, it is unlikely that EBF participates during the host acceptance behavior in *A. ervi* due to its lack of specificity, as the EBF has been reported in both *S. avenae* and *A. pisum* among other aphid species (Francis et al., 2005).

Instead, parasitoids would rely on insect cuticular hydrocarbons (CHCs) present in the aphid exoskeleton. These non-polar lipids serve as species-specific communication cues, among other functions (Howard & Blomquist 2004), and can be a mixture of a few to more than hundreds of components of 21-50+ carbon alkanes, alkenes and branches derivatives (Chen et al., 2017). This variation would make CHCs composition highly diverse in a given insect species (Lockey 1988), while CHC profiles would be species-specific (Muratori et al., 2008; Chen et al., 2017). Hence, these qualitative differences between CHCs from different aphid species would confer parasitoids with the ability to discriminate between hosts at the species level, and adjust its parasitism strategy accordingly (Hatano et al., 2008; Muratori et al., 2006). For instance, CHCs from the cereal aphid *S. avenae* could trigger attack responses from the aphid parasitoid *A. rhopalosiphi* (Muratori et al., 2006).

In aphids, *n*-alkanes have been found to be the predominant components of CHCs and may also include alkenes and their methyl branches derivatives (Brey et al., 1985; Muratori et al., 2008). For example, *n*-alkanes range from C_{23} to C_{33} in *S. avenae*, with three predominant compounds: n-Heptacosane (*n*-C_{27}, 29%), n-Nonacosane (n-C29; 27%) and n-Hentriacontane (*n*-C_{31}, 10%) (Muratori et al., 2008). These three *n*-alkanes have different concentrations in *A. pisum* compared to *S. avenae* (*n*-C_{27}, 14%; *n*-C_{29}, 48%; *n*-C_{31}, 21%) (Brey et al., 1985). However, because of the variety of extraction/chemical analysis methods used, studies regarding chemical composition of the aphid cuticles may not be strictly comparable (Muratori et al., 2008).

As recognition is achieved after antennal contact with the contact kairomones and chemical cues in the insects' cuticle (Le Ralec et al., 2005), it is expected that *OR*s must contribute to the detection and discrimination of different CHCs. This is the case of the Indian

jumping ant *Harpegnathos saltator*, where functional analysis has shown that several ORs are narrowly tuned to specific CHCs (Pask et al., 2017). As current evidence suggests that *OR* expression is amenable to modulation by scent conditioning (Kang et al., 2017), then *A. ervi* parasitoids reared on their natal host are not expected to respond to volatiles to which they have not been previously exposed nor experienced (i.e., to volatiles derived from the non-natal plant-host complex). This is because *OR* coding genes might change their regulation/expression levels as a response to long exposures to specific environments, for example during the developmental time into the aphid's host body, which could modify the oviposition behavior of their offspring (Wang et al., 2017).

In the case of *A. ervi* parasitoids transplanted from *A. pisum* to *S. avenae* (Figure 3.2), a significant downregulation was observed for four out of five odorant receptors (*OR-B, OR-E, OR-H* and *OR-J*) in parasitoids transplanted to *S. avenae* compared to parasitoids that were kept on *A. pisum*. Furthermore, homology searches using these four *A. ervi OR*s as queries in BLASTx alignments against *Drosophila* spp. found their corresponding homolog sequences (*OR9a, OR43a, OR13a* and *OR85d*, respectively; Table 3.1) and their odorant-response profiles (Münch & Galizia, 2016; Table 3.1). *OR9a* shows a high response to 3-hydroxy-2-butanone, a volatile that has been identified in the excreted honeydew of *A. pisum* when feeding on the fababean *Vicia faba* (Leroy et al., 2011). *OR43a* shows response to (Z)-3-hexen-1-ol, among other C6-alcohol aromatic volatiles (Münch & Galizia 2016), and it is the most abundant compound found in the volatile blends emitted by *V. faba* plants when infested with *A. pisum* (Webster et al., 2008) and which is known to play a role as attractant of aphid natural enemies including the parasitoid wasp *A. ervi*, eliciting both electrophysiological and flight oriented responses (Du et al., 1998; Sasso et al., 2009). *OR13a* responds to 1-Octen-3-ol, a volatile that has also been reported in *V. faba* plants which is emitted in response to herbivory walking activity (Frati et al., 2017). Finally, *OR85d* shows response to 6-methyl-5-hepten-2-one (Münch & Galizia 2016), which is one of the most attractive volatiles for *A. ervi* females and found in the headspace of *V. faba* plants infested with *A. pisum*. Interestingly, the release of this compound is not induced by other aphids that feed on broad bean, as the case of the black bean aphid *Aphis fabae*, which is not a suitable aphid host for the parasitoid *A. ervi* (Du et al., 1998). Hence, it seems that *A. ervi* reared on *A. pisum* are able to display plasticity in their expression of *OR*s when transplanted to a new

aphid-plant complex (e.g., *S. avenae* – barley). The downregulation observed for this *A. ervi* population (from *A. pisum*, transplanted to *S. avenae*) could be a consequence of a reduced exposure to volatiles from *A. pisum* – broad bean, and suggests that gene expression of chemosensory genes is affected by exposure to plant volatiles as reported for other insect species (Wan et al., 2015). In the case of *A. ervi* from *S. avenae* transplanted to *A. pisum*-pea, only *OR-H* showed a significant upregulation, while the other *OR*s showed no variation. This implies that only *OR-H* display changes in gene expression in response to transplanting *A. ervi* parasitoids from *S. avenae* to *A. pisum*, thus suggesting that these parasitoids display a narrow plasticity in terms of ORs gene expression compared to parasitoids from *A. pisum*.

Expression levels for OBPs and ORs between field and inbred A. ervi populations

Chemosensory mechanisms play a key role in insect behaviors such as host location and host discrimination (Suh et al., 2014). However, under laboratory rearing conditions, a degradation of sensory sensitivity and variation in olfactory responses toward host volatiles may occur (Nielsen et al., 2015). This might have a significant impact on host fidelity, as the task of discriminating between blends of volatiles may be difficult for insects with an even somewhat-impaired olfactory sense (Webster et al., 2013). In *A. ervi*, previous results from behavioral experiments indicate that inbred parasitoids rapidly accept aphids with no true decision making among hosts, regardless the natal host on which parasitoids were reared (Sepúlveda et al., 2017b). Hence, it is possible that the inbreeding under laboratory conditions might have an impact on the expression levels of chemosensory genes, even if parasitoids are kept on the same natal aphid plant-host complex (*A. pisum*) or when they are transplanted to a novel aphid plant-host complex (*S. avenae*), compared to their "wild" counterparts sampled from the field (Figures 3.6 & 3.7).

While field populations were collected from alfalfa fields and maintained in *A. pisum* – *Vicia faba* system for two generations, inbred populations of *A. ervi* collected originally from alfalfa were also maintained in the same aphid-plant system for over 2 years (approximately 75 generations) (Sepúlveda et al., 2017). As these highly inbred laboratory *A. ervi* populations were reared in the same *A. pisum – V. faba* complex, they have not been exposed to the variety of volatiles emitted by different plants and animals, nor have they had to cope with chemically complex environments that may affect olfactory orientation

(Wäschke et al., 2013). This may lead to a simpler sensory processing in the inbred populations of *A. ervi*, as their expression is modulated by scent conditioning (Kang et al., 2017). This long-time exposure to *Vicia faba* could explain the higher *OR-E* expression and the reduction in expression levels for the other chemosensory genes observed between field and inbred *A. ervi* populations.

The reduced expression of *OR*s and *OBP*s might also explain the reduction of host fidelity observed in endogamic *A. ervi* populations. Behavioral changes (e.g., weaker attraction to host-plant volatiles) have also been reported for other predatory insects reared under confined laboratory conditions (Dicke et al., 2000). It is also noteworthy that the synthesis of extremely high concentrations of OBPs/ORs requires the use of large amounts of energy, which cannot be explained without a fitness gain. This is particularly true in insects, which often live on a very critical energy balance (Pelosi et al., 2005; Zhou, 2010). Thus, olfaction plays an important physiological role, both for the survival of the individuals and conservation of the species (Gu et al., 2015), and this trait must involve a trade-off with other traits (such as reproduction) (Desouhant et al., 2005; Schuker, 2014). However, rearing under highly homogenous and stable laboratory conditions for several generations with plentiful resources (food and hosts readily available) may relax the mechanisms in charge of keeping an optimal, "ready-to-use" sensory olfactory system. Hence, under these homogenous conditions, parasitoids would shut-down the expression of *OBP*s/*OR*s and lose their ability to discriminate among potential aphid hosts, making the search for a suitable host a more complex task under heterogeneous environments (Olsson et al., 2006; Wäschke et al., 2013). Hence, the lower expression of *OR*s and *OBP*s observed in inbred populations compared to wasps recently sampled from the field and established in laboratory, would explain the changes in host preference behavior (a more rapid aphid acceptance and the lack of host discrimination) observed by Sepúlveda et al. (2017) in inbred populations, which contrasts to what was observed in field populations by Daza-Bustamante et al. (2002) and Zepeda-Paulo et al. (2013). Interestingly, independent of whether a reduction in host fidelity was observed, fitness (in terms of reproductive success) was low regardless of the assayed host, mostly due to a biased production of males in the offspring compared to females (Sepúlveda et al., 2017b).

Long-term rearing under laboratory conditions of a relatively small caged population that are forced to use the same aphid-plant complex for many generations can also cause significant changes in behavior, physiology and life-history traits of individuals because of the negative impact of founder effects, genetic drift and inbreeding depression (Nielsen et al., 2015). Random drift under continued laboratory conditions may lead to either the random loss of genetic variation or unpredictable changes in ecologically important traits (Boivin et al., 2012; Vet et al., 2003), which are known to include olfactory responses (Nielsen et al., 2015). As the endogamic populations used in our study arose from a single couple randomly chosen from the populations used by Zepeda-Paulo et al. (2013), then they could have been more prone to loss their host fidelity (Sepúlveda et al., 2017b). Hence, inbreeding has deleterious effects on host recognition and acceptance, and may negatively impact their effectiveness for biological control, particularly because parasitoids are frequently mass-reared under laboratory conditions before their release at the farm scale (Zaviezo et al., 2017).

3.6 Concluding remarks

Further studies on the molecular basis of host fidelity can shed light on whether changes in expression levels of candidate *OBP*s and *OR*s are effectively involved or could be related to changes in olfactory sensory sensitivity and to variations in terms of host preference and host fidelity for both field and laboratory-reared *A. ervi* parasitoid populations. These future studies may involve for instance assessing *A. ervi* specific electrophysiological EAG responses to specific volatile compounds when parasitoids are faced to natal and non-natal aphids-plant complex. Also, silencing specific *OR* genes by RNA interference for further observing parasitoid behavioral responses seem as good predictors of host preference (Antolin et al., 2006; Sepúlveda et al., 2017b). It would be interesting to see whether the observed expression differences in this study effectively have an impact on the host-selection process in *A. ervi* parasitoid wasps.

3.7 Acknowledgements

We thank Daniela Sepúlveda for providing *A. ervi* inbred parasitoids, Cinthya Villegas for help in fieldwork and sexual identification, Lucia Briones for help in laboratory RNA extractions, Karen Balboa and Samuel Morales for help in qPCR and data analysis. This work

was funded by FONDECYT grants 1130483 and 1170943 to CC Figueroa, 1140632 to Blas Lavandero, and by Iniciativa Científica Milenio (ICM) NC120027. GI Ballesteros was funded by CONICYT PhD fellowship 21120371. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Chapter IV

Draft genome of the endoparasitoid wasp *Aphidius ervi* and its utility to provide insights into the dynamics of biological control

4.1 Abstract

The endoparasitoid wasp *Aphidius ervi* is an important biological control agent of several pest aphids, including two important global pests, the English grain aphid *Sitobion avenae* on cereals, and the pea aphid *Acyrthosiphon pisum* on legumes. Although *A. ervi* play a crucial role on the biological control of those aphid pests, and has been used both for physiological and molecular studies, no genomic sequences are available at all for this species yet. Hence, the goal of this study was to develop the foundational genomic resources for *A. ervi* alongside with a predicted, annotated gene dataset, in order to shed light on the mechanisms underpinning the biological control. Here, we present a whole genome assembly of *A. ervi* constructed using genomic DNA from a pool of adults that are the offspring of one mated female. We followed a hybrid sequencing approach of both short Illumina HiSeq reads combined with long reads from a PacBio RS II platform to obtain a genome assembly (138.9 Mb), which shows both substantial continuity and a high completeness rate (91%). Using transcriptional evidence, we predicted 20,226 genes in the *A. ervi* genome, with 10,492 (51.9%) successfully annotated using an automatic annotation pipeline. Furthermore, we describe within this dataset some gene families putatively involved in host-parasitoid interactions, such as olfaction-related and venom-coding genes. Strikingly, the analysis of this genome revealed a major reduction in one of the major epigenetic mechanisms that have been previously suggested to be important in Hymenoptera, DNA methylation; these wasps possess greatly reduced genome-wide methylation capabilities (almost zero), which raises questions on the role of DNA methylation in this parasitoid species. This *A. ervi* draft genome sequence will provide valuable information that can be used to address the dynamics of its host-parasitoid association, in addition to other biological questions regarding this insect species.

4.2 Introduction

Parasitic Hymenoptera are a diverse group of parasitoid wasps that are natural enemies of a broad range of arthropods, including those of agricultural significance (Godfray, 1994). Adult parasitoids are free-living insects that lay their eggs inside (endoparasitoids) or outside (exoparasitoids) their host, subsequently killing the host and regulating the arthropods' population sizes in nature (Godfray, 1994). This feature rapidly made parasitoid wasps to gain attention as they can be used in different biological control programs worldwide, and contribute to saving billions of dollars annually in crop losses due to pest species and the reduction of insecticide applications (Simpson et al., 2011).

One of the most widely used species in biological control programs of pest aphids is the parasitoid *Aphidius ervi*, a worldwide distributed endoparasitoid wasp of several aphid species, such as the pea aphid *Acyrthosiphon pisum* (Henry et al., 2010; Stilmant et al., 2008) and the grain aphid *Sitobion avenae* (Zepeda-Paulo et al., 2013). This species has the ability to attack many related host species (e.g., from the same aphid family) (Loxdale & Harvey, 2016) and is compatible with both mass-rearing and storage (Frère et al., 2011; Zuazúa et al., 2000), and made *A. ervi* a prime candidate for biological control of aphids through massive releases of parasitoid wasps in crop fields around the world (Boivin et al., 2012). This is also the case in Chile, where *A. ervi* was introduced in the late 70's to minimize the damages caused by the invasive grain aphid (*S. avenae*) on cereals (Zúñiga et al., 1986). Currently, *A. ervi* is the predominant parasitoid species controlling *A. pisum* on legumes (e.g., alfalfa) and *S. avenae* on cereals (e.g., wheat) (Zepeda-Paulo et al., 2013; Peñalver-Cruz et al., 2017), although these two aphid species differ in several ecologically important traits (Daza-Bustamante et al., 2003). Interestingly, the Chilean *A. ervi* populations parasitizing the two aphid species does not seem to be subdivided but rather genetically homogenous, most likely due to both its relative recent introduction and a high gene flow among populations (Zepeda-Paulo et al., 2013; Zepeda-Paulo et al., 2015).

We found differences in terms of gene expression between *A. ervi* females parasitizing different aphid host species. Hence, the phenotypic plasticity observed at transcriptomic level in *A. ervi* naturally parasitizing different hosts (see Chapter II), the gene expression differences observed for chemosensory genes when parasitoids emerge from an

aphid host different than their natal-host (see Chapter III), and the distinct and persistent phenotypic differences observed between *A. ervi* parasitoids despite being genetically homogeneous at the field (regardless they come from *S. avenae* or *A. pisum*) (Zepeda-Paulo et al., 2013), pose a challenge to understand the adaptive evolution of introduced populations of *A. ervi*. This evidence points towards the existence of molecular mechanisms that allow for the production of several phenotypes from a single genotype/genome, such as epigenetic marks and modifications (Hunt et al., 2013), which are particularly common in insects (Glastad et al., 2014), and which may be underpinning the host-preference in this parasitoid wasp.

As a response to either different ecological interactions and/or different environmental cues, epigenetic marks and modifications are involved in the modulation of gene expression that produce distinct phenotypes from the same genotype/genome, but without changing the underlying DNA sequence (Lockett et al., 2014; Hunt et al., 2013). These epigenetic mechanisms include chromatin remodeling through methylation and acetylation of histones, and DNA methylation (DNM), the latter being extensively studied (Glastad et al., 2014). DNM is an epigenetic modification of a DNA strand and corresponds to the covalent addition of methyl groups to certain cytosines in the DNA (MacDonald, 2012) that is catalyzed by evolutionary conserved enzymes known as DNA methyltransferases (DNMTs) (Bewick et al., 2016). DNMs are widely found in all three domains of life (Suzuki & Bird, 2008; Glastad et al., 2014). In insects, DNMs are mostly restricted to CG sites found in the transcribed regions of genes (Feng et al., 2010; Lyko et al., 2010; Bonasio et al., 2012), and it has been described as a key mechanism involved in phenotypic variation such as behavioral plasticity and social behavior, especially eusociality in Hymenoptera (e.g., ants, bees, wasps and sawflies) (Yan et al., 2014; Yan et al., 2015). However, no studies have been conducted in *A. ervi* regarding this epigenetic mechanism.

Although many studies have been conducted regarding the biology of *A. ervi* (He & Wang, 2006; He et al., 2005; Sasso et al., 2009), their efficacy in biocontrol programs (Boivin et al., 2012; Starý, 1993), its ability to be mass-reared and stored before release (Frere et al., 2011; Ismail et al., 2014), and its preference for the natal host (i.e., host fidelity) (Zepeda-Paulo et al., 2013; Sepúlveda et al., 2017b), there are no genomic resources available so far,

nor evidence supporting the existence of DNM as an epigenetic mechanism for this parasitoid species, although it is widespread in several Hymenoptera (Bewick et al., 2016).

Hence, this study presents the first *de novo* genome sequence for *A. ervi*, which was obtained by using a hybrid assembly strategy that combined both Illumina libraries (short reads) and a Pacific Biosciences library (long reads) into a high-quality genome draft (Utturkar et al., 2014). This genome assembly, and its resulting predicted and annotated gene sets, constitutes a powerful and valuable foundational dataset for gene and protein discovery, which will contribute to provide insights into mechanisms underlying several aspects of *A. ervi* biology, such as host selection and host-parasitoid interactions (Geib et al., 2017).

4.3 Material and Methods

Aphid and parasitoid rearing

In a previous study, Zepeda-Paulo et al. (2013) described significant differences in host preference and host acceptance depending on the host *A. ervi* were reared on. In that study, *A. ervi* parasitoids were obtained from parasitized aphids sampled from field populations of the *A. pisum* complex, which includes two host races specialized on alfalfa and pea, as well as a population sampled from *S. avenae* on wheat. Pea aphids (from both alfalfa and pea races) were maintained in the laboratory on broad bean (*Vicia faba* L.) while grain aphids were maintained on barley (*Hordeum vulgare* L.). These species have been successfully used as host plants both in this thesis (Chapters II & III) and in previous studies for aphid and *A. ervi* rearing (Sepúlveda et al., 2017b; Zepeda-Paulo et al., 2013). Parasitized aphids (recognizable as mummies) were isolated in petri dishes until adult parasitoid emergence. Species and sex determinations were performed for each emerged parasitoid using a standard taxonomic key (Starý, 1995). Parasitoid wasps were caged in the same aphid/host race plant system from which they emerged in the laboratory after being sampled in the field (*A. pisum* alfalfa, APA; *A. pisum* pea, APP; *Sitobion avenae*, SA), and new aphid hosts were offered *ad libitum*, with diluted honey and water for sustenance. Each week, new aphid infested plants were introduced into the *A. ervi* rearing cages for parasitoid population maintenance; both aphids and parasitoids were reared under laboratory conditions that allowed continuous reproduction (20°C, D16/N8 photoperiod) (Zepeda-Paulo et al., 2013). These parasitoid

populations were propagated for approximately 75 generations in the same aphid-plant system; thus, three different, highly inbred *A. ervi* laboratory populations were established. Further reduction of genetic differences among parasitoids was accomplished by randomly choosing a single pair from each population (one male and one female) to initiate new inbred populations that were kept isolated in separated cages with the same aphid-plant system. These experimental *A. ervi* populations exhibited a lower mean observed heterozygosity than founder populations at nine microsatellite loci on diploid females (Zepeda-Paulo et al., 2015; Sepúlveda et al., 2017b). All aphid populations used in this study were free of facultative endosymbionts, as aphids can carry endosymbiont bacteria such as *Hamiltonella defensa*, which may confer protection against parasitoid larvae development in most aphid species (Oliver et al., 2010; Vorburger, 2014; Zepeda-Paulo et al., 2017). Facultative endosymbiont presence was evaluated using the amplification of specific 16S rDNA from whole-body aphid DNA based on the set of known primers described by Peccoud et al., 2014. This method allows the screening and detection of facultative endosymbionts occurring naturally in aphid populations (Dennis et al., 2017; Sepúlveda et al., 2017).

Parasitoid collection, sample preparation and sequencing

As Hymenoptera species (such as *A. ervi*) have a haplodiploid sex determination, a sequencing strategy involving haploid males was used in this thesis, in order to reduce any possible genome assembly issues, which could lead to a poor-quality genome assembly (Branstetter et al., 2018). Hence, male haploid parasitoids were separately collected alive as adults from each one of the three caged parasitoid populations and stored in 1.5 ml centrifuge tubes containing ethanol 95% at -20°C until whole body DNA extraction. Males from each population were pooled before each DNA extraction (120 individuals/pool; 14 pooled samples). Pooling was required as *A. ervi* wasps are quite small organisms, so very small amounts of DNA can be obtained from a single individual; similar strategies have been used for other parasitoid wasp genomic sequencing projects such as the genome of *Leptopilina clavipes* (Kraaijeveld et al., 2016) and the genome of *Fopius arisanus* (Geib et al., 2017). For each pooled sample, total genomic DNA was extracted using the DNEasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions, treated with RNAse (QIAGEN) to remove any RNA traces, and eluted in 200 µl of DNAse free water. Total DNA was

quantified both by spectrophotometry (Epoch Microplate Spectrophotometer, Biotek) and by fluorometry (Qubit 3.0; Qubit DNA High Sensitivity Assay Kit, Invitrogen), and integrity checked via electrophoresis on a agarose gel (2ul DNA loaded in TAE 1X, 0.8% p/v agarose gel). Recovered total DNA samples were shipped in sealed 1.5 ml centrifuge tubes with dry ice to Macrogen Korea for preparation of two Illumina Paired-End (PE) libraries and six Mate-Paired libraries (MP; insert sizes of 3,000, 5,000 and 8,000 bp, respectively). For Paired-End sequencing, 120 parasitoids were used (DNA concentration > 1μg), while 720 parasitoids were used for mate-pair library construction and sequencing. Briefly, 1μg of DNA was sheared by Covaris ultrasonication (average insert size of 350bp) for paired-end reads libraries, size-selected using purification beads and ligated to indexed adapters for cluster generation and sequenced using Illumina HiSeq 2000 platform (Illumina, CA). For mate-pair libraries, the Nextera Mate Pair Sample Preparation Kit was used following manufacturer instructions; genomic DNA was fragmented and tagged in both ends with biotinylated junction adapters, purified with AMPure beads, size-selected and circularized using the biotynilated adapters. These circularized molecules are fragmented and all sub-fragments carrying the biotin tags are used for fragment enrichment, which are end repaired, A-tailed and ligated to TruSeq DNA adapters. Samples for PacBio RS II (Pacific Biosciences, CA) genome sequencing came from *A. ervi* male wasps collected from a highly inbred APP population, which was initiated as described above (Zepeda-Paulo et al., 2013). PacBio RSII platform produces long reads that read across repetitive sequences, helping to fill in missing base-pair information and perform gap closing (regions filled with the uninformative base-pair character N) in assembled genome scaffolds (Ekblom & Wolf, 2014). Total DNA from *A. ervi* female wasps was extracted at INRA Sophia laboratories and shipped to GenoScreen France for PacBio RSII sequencing using manufacturer's protocols. Briefly, genomic DNA was sheared using a Covaris g-TUBE and fragments were end-repaired and ligated with SMRTbell adapters. Fragments were then size selected with a cutoff value of 8kb by using a BluePippin size-selection system; no amplification step was performed nor required. This library was quality-checked with an Agilent DNA 12000 kit (Agilent, Santa Clara, CA, USA), and was sequenced in 4 SMRTcells RSII using P6-C4 chemistry.

Library processing and genome assembly

Genomic Illumina libraries were quality checked with FastQC ver. 0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc) to assess the presence of adapters derived from sequencing, overrepresented kmers, read length and overall read quality scores. Paired-end libraries were processed with Trimmomatic ver. 0.356 (Bolger et al., 2014) to remove any remaining TruSeq adapter sequences and eliminate of low quality bases (Q < 3) from reads, while NextClip ver. 1.3 was used to remove Nextera adapters from Mate-Pair libraries (Leggett et al., 2014). Filtered Illumina libraries were then used in genome assembly and scaffolding with Platanus ver. 1.2.1 (Kajitani et al., 2014) using default parameters. Gap closing was performed using SOAP *de novo* gap closing tool. Scaffolds with length >1000bp were selected and SSPACE-LongRead ver 1.1 tool was run in order to perform scaffolding using PacBio RS continuous long reads (with both circular consensus sequence reads and uncorrected raw reads) with default settings, allowing for correct placement of scaffolds in super-scaffolds (Boetzer & Pirovano, 2014). Assembly statistics (number of contigs, total length, N50, largest contig, %GC, etc.) were obtained with QUAST software v2.3 (Gurevich et al., 2013), while genome completeness was assessed by benchmarking the assembled genome using BUSCO (benchmarking universal single-copy orthologs) v3.0 (Simao et al., 2015). To determine whether this genome has putative genes encoding for one or more set of core genes conserved across of the Order Hymenoptera, a "completeness score" was calculated (Chapter II), using a total of 4,415 near-universal single-copy orthologs from Hymenopteran species as reference core genes (available at busco.ezlab.org; Simao et al., 2015). The whole genome assembly (v3.0) of *A. ervi* is currently hosted at the BioInformatics Platform for Agroecosystem Arthropods and publicly available upon request at the following website: http://bipaa.genouest.org/sp/aphidius_ervi/.

Gene prediction, annotation and comparative functional analysis

Gene prediction from our assembled genome was performed using the MAKER2 annotation pipeline (Holt & Yandell., 2011). The MAKER2 pipeline, using both evidence-based and *ab-initio* model algorithms for gene prediction, collapsed the results from each process into a consensus gene model and a set of predicted genes. For a more accurate gene prediction, evidence-based algorithms used both protein homology and transcriptomic RNA-seq data

obtained from a previous *A. ervi* study involving female parasitoids from the same laboratory populations (Chapter II). Protein homology was inferred by mapping protein sets from *Acromyrmex echinator*, *Drosophila melanogaster*, *Nasonia vitripennis*, *Apis mellifera*, *Hyposother didymator, Aphidius ervi* putative protein dataset (obtained previously from genome draft version 1.0) and all proteins from Uniprot-Swissprot database (June 2016) (Wenger et al., 2016).

The SNAP2 algorithm (Hecht & Rost, 2015) and AUGUSTUS ver 3.2.2 (Keller et al., 2011) were implemented for *ab initio* gene prediction. Final MAKER2 gene predictions were collected after three iterative rounds of training. This iterative approach allows the gene model to improve predictions from previous runs to train the hidden Markov model used by SNAP2. Results from RepeatMasker (Smit et al., 2015) were used to mask low-complexity repetitive regions of the genome in order to avoid false gene prediction. This used the arthropod repeat library from RepBase (Jurka et al., 2005) as well as the custom *A. ervi* repeat library created by RepeatModeler (RepeatScout, RECON and TRF). Predicted official gene (OGS 3.0) for *A. ervi* is also available at the BIPAA website. Additionally, an Apollo instance with the latest *A. ervi* genome draft and predicted gene dataset is available on the same website for performing manual gene model curation and annotation. Also, a BLAST server was implemented on BIPAA website, which allows users to perform alignments of query sequences using as subject either the *A. ervi* reference genome or CDS/transcript datasets. Annotation and functional gene classification of the entire set of *A. ervi* CDS obtained from MAKER2 pipeline was performed by local homology searches with BLASTp (version 2.5.0) using the NR database (NCBI) as reference (2016 version), setting an *e-value* of 1^{e-5} as threshold. Functional annotation was performed by loading BLASTp alignment results into Blast2GO (October 2016 database; Conesa & Götz, 2008). We also performed InterPro annotation, Gene Ontology (GO) term assignment, enzyme code and pathway annotation using Kyoto Encyclopedia of Genes and Genomes (KEGG) terms integrated into Blast2GO. Successfully annotated transcripts were categorized and assigned to GO terms from different GO categories (molecular function, cellular component and biological process). Genome annotation statistics (i.e., number of predicted genes, genome coverage) were calculated using Genome Annotation Generator tool (GAG version 2.0.1).

DNA Methylation patterns analysis and detection of annotated DNA methyltransferases

We tested for presence/absence of DNA methylation in *A. ervi* using three different approaches. First, we used the predicted CDS fasta file to analyze presence/absence of DNA methylation using CpG $_{O/E}$, which is a metric of CpG dinucleotides normalized by G and C content (GC content) and the length of protein coding genes. This was done because DNA methylation is selectively located in transcribed regions in insect genomes and highly biased into exons, while being primarily localized to the 5'-region following the translation start site of genes (Lyko et al., 2010; Bonasio et al., 2012; Hunt et al., 2013). This CpG $_{O/E}$ metric relies on the natural, spontaneous deamination of methylated cytosines to thymines; hence, genes that are hypermethylated are expected to have a lower CpG $_{O/E}$ value than hypomethylated genes. Thus, a bimodal distribution of CpG $_{O/E}$ values is expected in a mixture of methylated and low to un-methylated genes. Conversely, a unimodal distribution is indicative of a set of genes that are mostly low to un-methylated (Bewick et al., 2016). CpG $_{O/E}$ values for each gene was defined as:

$$CpG_{O/e} = \left(\frac{l^2}{l}\right) * \left(\frac{P_{CpG}}{P_c * P_g}\right)$$

Where P_{CpG}, P_C and P_G are the frequencies of CpG dinucleotides, C nucleotides and G nucleotides, respectively, each estimated from gene length (*l*) in bp. Only CDS were considered when estimating CpG $_{O/E}$ (Bewick et al., 2016). The modality of *A. ervi* CpG $_{O/E}$ distribution was tested using Gaussian mixture modeling (mixtools v1.0.4 R package). This approach has been used successfully as a robust and accurate predictor of DNA methylation (Bewick et al., 2016).

Additionally, we estimated levels of DNA methylation in both the *A. ervi* genome and CDS, using a whole genome bisulfite sequencing of bisulfite-treated DNA (MethylC-seq) library as reference, which was published recently for *A. ervi* and available at NCBI SRA database (accession GSM2204507) (Bewick et al., 2016). This Single-end, Illumina library were separately mapped to both the *A. ervi* predicted genome fasta file and CDS fasta file,

using the Bismark software version 0.19.0 (an aligner and methylation caller tool) with default parameters (Krueger & Andrews, 2011). Briefly, MethylC-seq reads were mapped to the *A. ervi* CDS sequences using Bowtie2 with a seed length of 31bp and 1 mismatch allowed. Using the resulting Bowtie2 alignments, Bismark inferred the methylation state of all cytosine positions from the WGBS reads, and methylated cytosines in CpG content, CHG context and CHH context were automatically counted.

Finally, we performed a search for homologs of genes encoding the two essential DNA methyltransferases enzymes (DNMTs) involved in DNA methylation, DNMT1 and DNMT3 (maintenance and *de novo* DNA methyltransferases, respectively) on both the *A. ervi* assembled genome and annotated gene datasets, using *DNMT*s homologs from other insect species as reference sequences (obtained from GenBank).

4.4 Results

Aphidius ervi reference genome sequencing and assembly

We sequenced eight gDNA libraries of pooled *A. ervi* males using Illumina technology and one gDNA library of pooled *A. ervi* females using PacBio RS technology. Using this approach, we obtained 1.3 billion short reads (eight Illumina paired-end libraries, 2x100bp) and 848,224 long reads from a PacBio library (mean length 5,900bp; N_{50} 9,699bp), which allowed us to generate the first, fairly contiguous and high-quality *A. ervi* genome assembly. This assembly (codenamed *A. ervi* genome assembly v3.0) is distributed in 5,778 scaffolds (N_{50} = 0.58 Mb; supplementary Table 4.1, appendix B) with an estimated GC content of 25.85% and has an estimated length of 138.90 Mb, which is quite close to the genome size reported by flow cytometry for a pool of males and females of *A. ervi* (136.92Mb; Ardila-Garcia et al., 2010). Hence, the evidence currently available indicates that this *A. ervi* genome would be the smallest known sequenced genome among the Order Hymenoptera so far. Although parasitoids have small-sized genomes, current published genome length ranges from 153.6 Mb (*F. arisanus*) to 388.8 Mb (*D. alloeum*) (Geib et al., 2017).

Assembly completeness analysis using BUSCO (benchmarking universal single-copy orthologs) showed that 90.7% complete conserved genes were present in our assembly, 4.1% corresponded to fragmented conserved genes while only 5.2% single-copy ortholog genes

were missing; the observed genome completeness is similar to other braconid genomes (available in NCBI). Additionally, assembly completeness is higher when compared to the previously published *A. ervi* transcriptome assembly (90.7% vs 70.9%) using the same Hymenoptera reference dataset (Chapter II), which was expected as this genome assembly is less fragmented compared to the *de novo* transcriptome assembly published previously (Chapter II) and which was *de novo* assembled based on short reads libraries without a reference genome. Furthermore, sequencing of one continuous long-read library (PacBio) and long-range scaffolding into super-scaffolds using SSPACE-LongRead certainly helped in filling N regions in scaffolds (thus reducing assembly gaps and genome fragmentation) while increasing assembled' genome completeness (Richards & Murali, 2015). Previous assemblies using only the eight Illumina libraries sequenced during this study (but before scaffolding using PacBio reads) showed differences in terms of scaffolds numbers and N50 values compared to this assembly, among other statistics (Supplementary table 4.1, appendix B).

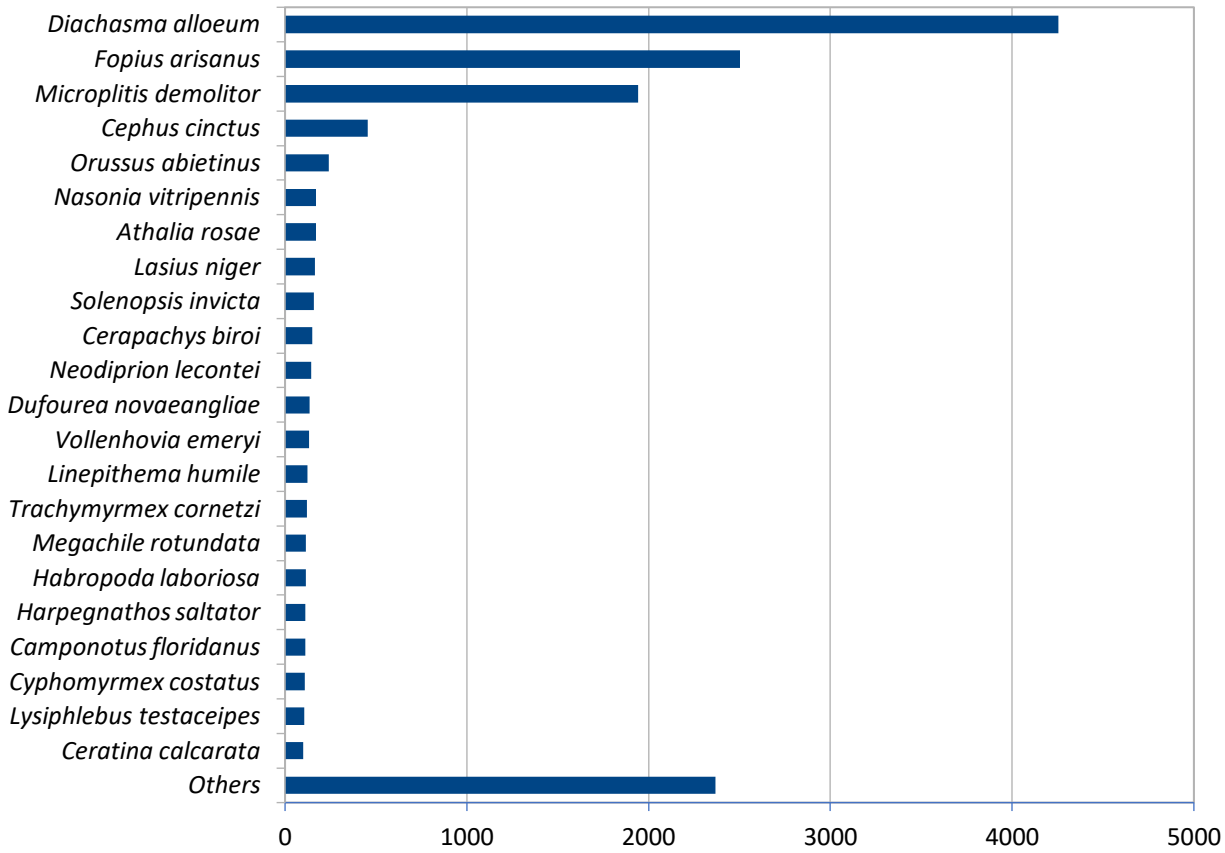

Figure 4.1. Top-Hits species distribution (calculated from BLAST hits from NR database)

Table 4.1. *A. ervi* gene annotation statistics derived from MAKER2. All sequence lengths reported in basepairs.

Feature	Count	Mean Length	Minimum Length	Maximum length	Total length
Genes	20,226	2919	6	96,195	59,030,501
mRNA	20,344	2905	6	96,195	59,094,665
CDS	20,344	1216	6	43,731	24,743,928
Exons	95,322	311	1	13,754	29,659,279
Introns	74,978	395	4	27,991	29,585,342

Aphidius ervi *gene prediction and annotation*

Genome annotation through MAKER2 pipeline identified 20,226 gene models and 16,920 complete coding sequences (containing both 5' and 3' untranslated regions) within the assembly (Table 4.1). However, it should be noted that the gene estimate is conservative, as it only accounts for complete MAKER2 gene models, while not all predicted CDS are complete; in fact 3,424 CDS (16.83%) are missing the start, stop or both codons. All predicted genes from the genome (N=20,226) from the genome were locally aligned to identify proteins within the NR database using BLASTp, which revealed that 13,571 predicted *A. ervi* genes (67.09%) were aligned with at least one protein within the NR database. Most of the sequences with BLASTp hits matched proteins from other braconid endoparasitoids such as *Diachasma alloeum* (parasitoid of the apple maggot *Rhagoletis pomonella*), *Fopius arisanus* (parasitoid of Tephritid fruit flies) and *Microplitis demolitor* (parasitoid of noctuid larvae) (Figure 4.1).

From this aligned subset, 10,492 genes were successfully categorized and assigned to Gene Ontology (GO) terms from three different GO categories (molecular function, cellular component and biological process), summarized in Table 4.2. A total of 14,614 sequences were assigned to biological process categories (Figure 4.2), 7,945 genes were classified under molecular function categories (Figure 4.3), and 5,976 were classified in cellular component categories (Figure 4.4), as genes can be assigned to more than one GO category. Genomic assembly, official gene sets, predicted CDS/transcripts, and associated annotated datasets are available upon request at http://bipaa.genouest.org/sp/aphidius_ervi.

Table 4.2. Statistics from functional annotation of *A. ervi* protein coding sequences

Total genes predicted from genome	**20,226**
With BLAST match to NR	13,571
With GO annotations	5,870
With Interproscan result	14,901
With signal peptides	1,731
With >1 Transmembrane domain	1,932

Identification of putative chemosensory and olfaction-related genes within the A. ervi genome

Olfaction plays a crucial role in insect behavior such as host location and host discrimination in complex environments (Suh et al., 2014). In the case of parasitoids wasps such as *A. ervi*, it has been reported that they are able to discriminate between hosts, and may show differences in both host preference and acceptance (Zepeda-Paulo et al., 2013). This host-discrimination ability almost certainly involves chemical signal perception (Takemoto et al., 2011). In insects, it has been described that several gene families are involved in chemical perception: Chemosensory proteins (*CSP*s), ionotropic receptors (*IR*s), gustatory receptors (*GR*s), odorant receptors (*OR*s), and odorant binding proteins (*OBP*s) (Tunstall & Warr, 2012), hence being prime genetic candidates underlying the observed differences in host-preference in *A. ervi* (Chapter II).

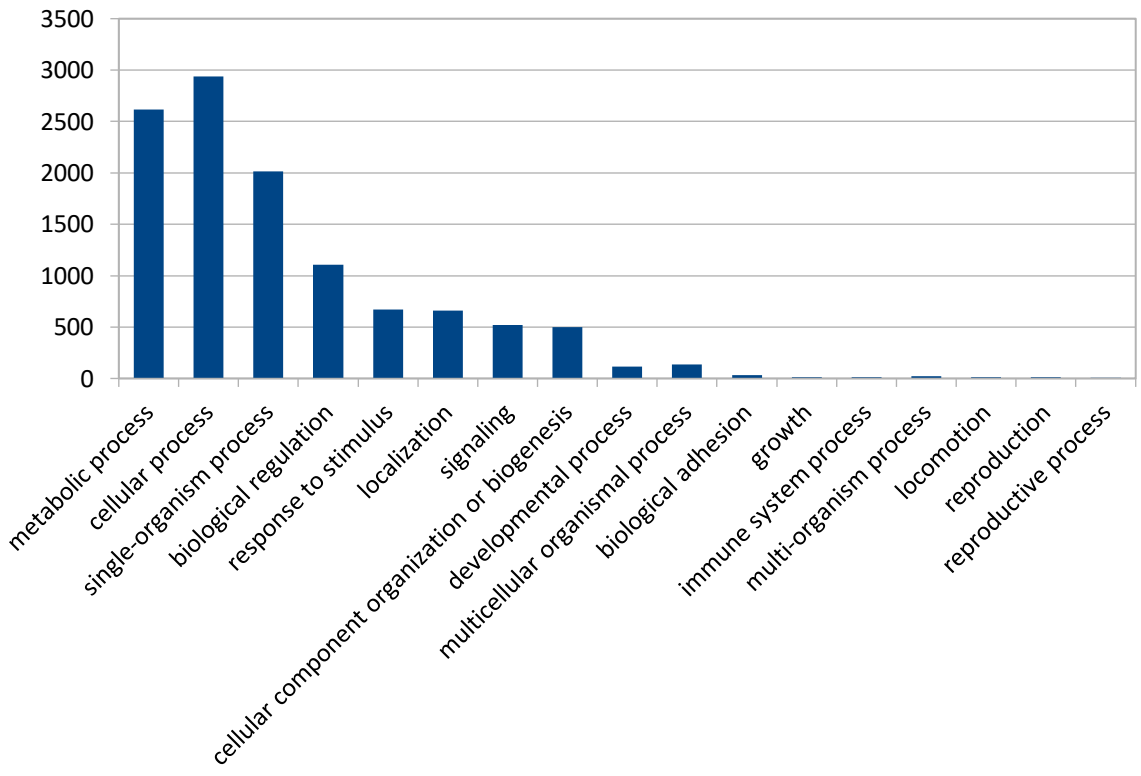

Figure 4.2. Gene ontology (GO) classification of *A. ervi* predicted genes, Biological Process. Y-axis indicates the number of genes in a main category. Specific categories are indicated on the x-axis

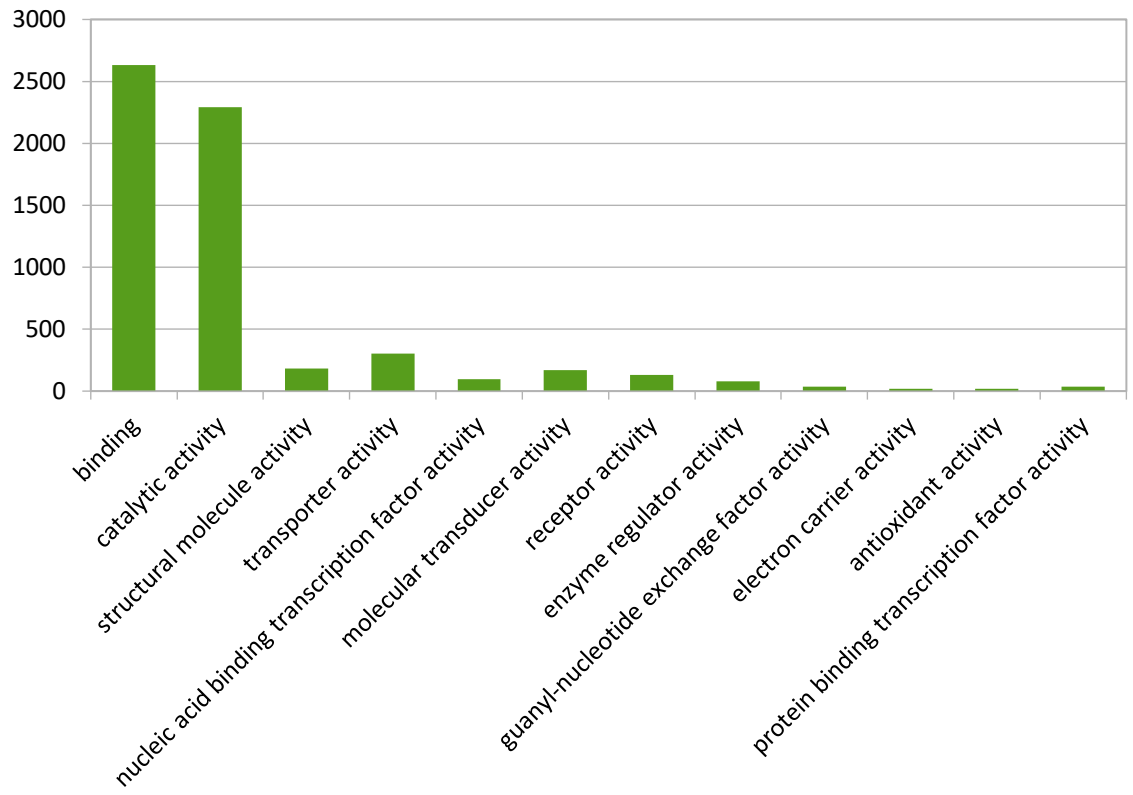

Figure 4.3. Gene ontology (GO) classification of *A. ervi* predicted genes, Molecular Function. Y-axis indicates the number of genes in a main category. Specific categories are indicated on the x-axis.

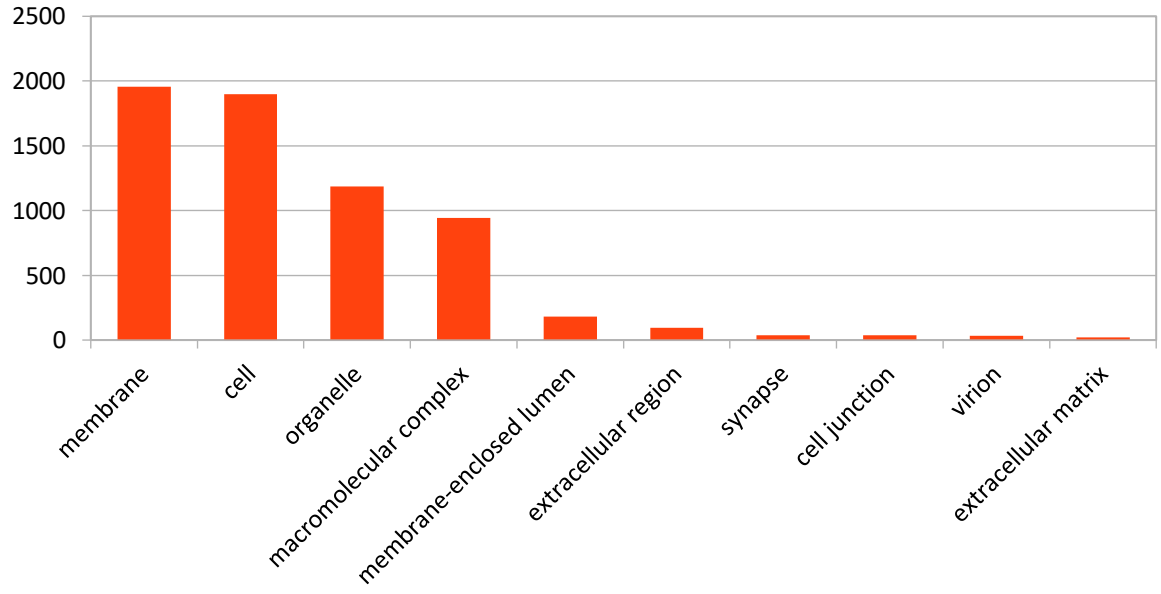

Figure 4.4. Gene ontology (GO) classification of *A. ervi* predicted genes, Cellular Component. Y-axis indicates the number of genes in a main category. Specific categories are indicated on the x-axis

Homology analysis using the annotated *A. ervi* gene set against the NR database identified 273 sequences belonging to these gene families, such as *CSP*s (15 genes), *IR*s (9 genes), *GR*s (89 genes), *OR*s (145 genes, including the conserved odorant co-receptor, *ORco*) and *OBP*s (15 genes). However, these numbers need to be taken with caution because those gene families are difficult to annotate automatically. This is because the identified genes could not represent the total number of related chemosensory genes in *A. ervi*, as some of these genes could be too evolutionary divergent to be identified using our annotation pipeline (Zhao et al., 2016).

Overall, we found more chemical perception-related genes compared to the *A. ervi de novo* transcriptome assembly (for which these gene families were also annotated), which suggest that not all these chemical perception genes were identified within the current *A. ervi* transcriptome assembly (Chapter II). An explanation is that some of these genes (such as sex-specific odorant receptors) could be expressed only in males as a result of differential sex-specific expression (Ahmed et al., 2016). It has also been described that olfactory plasticity may regulate the insect olfactory system in order to cope with various external stimuli, which occurs through changes in gene expression of several receptors (*IR*s, *GR*s and *OR*s) when the insect are in specific physiological states or conditions (i.e., feeding, age, mating status) (Jin et al., 2017). Hence, transcripts coding for genes which display differential expression upon certain specific conditions (i.e., physiological status or sex-specific) might not be detected in the recently published transcriptomic dataset, as the transcriptomic libraries used for the transcriptome *de novo* assembly were sequenced from adult *A. ervi* females (Chapter II).

Lack of DNA methylation in Aphidius ervi

We tested for presence/absence of DNA methylation in *A. ervi* using three different approaches. The first approach (based on a methylation detection pipeline combined with a bisulfite-treated genomic sequencing library; Bewick et al., 2016) showed very low amounts (0.1%) of methylated cytosines in all possible contexts (CpG, CHG and CHH) were present in both the *A. ervi* genome and coding genes. This value is almost identical to the sodium bisulfite non-conversion rate, so this very low amount of methylated cytosines could even be an artifact from the sodium bisulfite technique (Adam Bewick, pers.comm).

The second approach was based on computing the observed to expected CpG ratio (CpG $_{O/E}$) in *A. ervi*. This measure relies on the natural, spontaneous deamination of methylated cytosines to thymines, and has been considered as a robust and accurate predictor of DNA methylation. In the case of *A. ervi*, the distribution of CpG is as broad as that for other Hymenoptera, but lacking the bimodal distribution detected using this measure in coding regions of hymenopterans with DNM, such as *A. mellifera* (Figure 4.5, upper left panel). Although the Gaussian mixture modeling detected bimodality in *A. ervi* CpG $_{O/E}$ ratio, it seems that these values have a more unimodal and normal distribution, being more similar to CpG $_{O/E}$ distributions of the European paper wasp *Polistes dominula* and the parasitoid wasp *Microplitis demolitor* (Figure 4.5). Interestingly, these two Hymenoptera species also lack DNA methylation (Standage et al. 2016; Bewick et al., 2016).

Finally, we looked into *A. ervi* CDS and genome for evidence supporting the existence of the two essential DNA methyltransferases enzymes (DNMTs) involved in DNA methylation, DNMT1 and DNMT3. Only one isoform coding for the *de novo* of DNMT3 (*DNMT3B*) was found and annotated within the *A. ervi* genome (AE3006142-PA). Although *Ae-DNMT3b* retains some of the conserved domains found in other *DNMT3* orthologs, as estimated by conserved domain search tools, the identity values between orthologs ranged from 37% to 61% as calculated with BLASTp, which is explained by a high divergence detected towards the N-terminus site (which contains the conserved domains linked to interaction with chromatin), while the C-terminus site containing the catalytic domain is more conserved compared to other hymenoptera *DNMT3b* orthologs. Thus, it is possible that *Ae-DNMT3* could be a non-functional or functionally constrained protein (Adam Bewick, pers.comm). In the case of *DNMT1*, no ortholog was found within the *A. ervi* predicted coding sequences. Only a partial, incomplete ortholog was found in the genomic assembly (*Ae-DNMT1 partial*), which is missing approximately 80% in terms of sequence compared to the best BLAST hit (*DNMT1* from the alfalfa leafcutter bee *Megachile rotundata; Mr-DNMT1*). Although this particular *Ae-DNMT1* ortholog is quite fragmented and is missing the C-terminal catalytic domain, it retains the start codon and N-terminal regulatory domains (Lyko, 2017), which could also imply that it is either a non-functional or a functionally compromised protein (Adam Bewick, pers.comm). Furthermore, there is no transcriptional evidence supporting gene expression for either *Ae-DNMT1* or *Ae-DNMT3*. Overall, and

according to our results, we suggest that DNA methylation capability is either extremely low or even non-present in the *A. ervi* genome

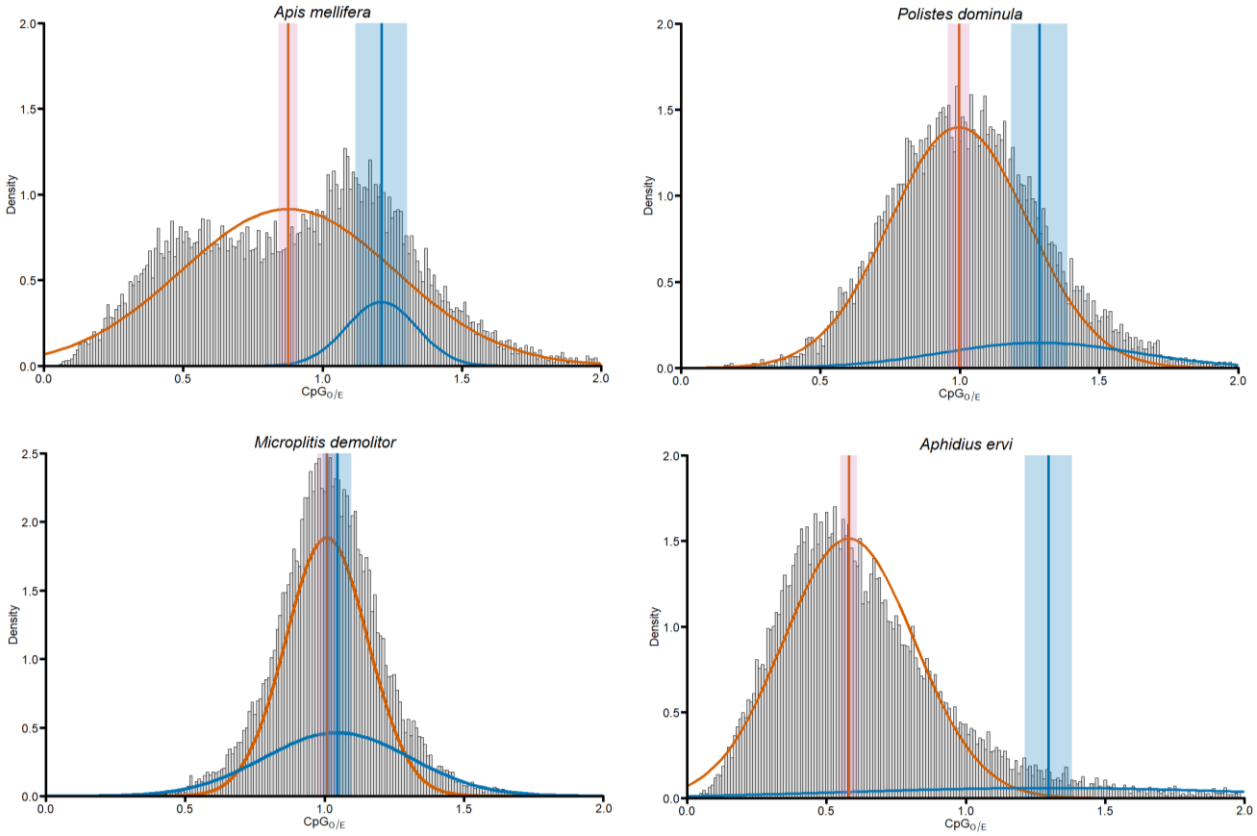

Figure 4.5. Distributions of $CpG_{O/E}$ calculated in gene coding sequences for 4 hymenopteran species with (*Apis mellifera*) or without (*Polistes dominula* and *Microplitis demolitor*) DNA methylation compared to $CpG_{O/E}$ calculated in *Aphidius ervi*. Gaussian mixture modeling was performed in R v2.3.4 using the package mixtools (v1.0.4) to estimate uni and bimodality. The solid lines line represents the mean of each distribution, and the shaded area is the 95% confidence interval around the mean.

4.5 Discussion

Despite the high amount of behavioral and physiological data derived from fundamental and applied research for *A. ervi*, no genomic information was available for this species, and the first transcriptomic assembly was published only very recently (Chapter II). Our current study provides a much-needed genomic resource for the aphid parasitoid wasp *A. ervi*, a model species widely used to understand host-parasitoid interactions. The *A. ervi* genome is a rather compact genome (~138.9 Mb) with a reduced GC content (25.85%) in comparison with other Hymenoptera. This GC content value is also lower compared to other parasitoids, which range from 30.6% (*C. vestalis*) to 40.6% (*N. vitripennis*) (Geib et al., 2017). It has been suggested that reduced genome-wide GC content in hymenopteran insects (such as parasitoids) could be linked to codon usage bias (non-uniform usage of codons during the translation); high AT content of genes favors biased usage of synonymous codons ending with A or T in hymenopteran genomes, while synonymous codon usage vary within genomes in patterns that seems to be distinct for each species (Behura & Severson, 2012). In some eukaryotes, synonymous codon usage for protein-coding genes correlates with gene expression levels: highly expressed genes exhibit more codon bias than genes expressed at average levels (Whittle & Extavour, 2015). Biases in synonymous codon usage may arise from deleterious mutational load, or from selective forces favoring translationally optimal codons ("preferred codons"). These preferred codons promote an efficient translation of genes (more rapidly and/or more accurately) than their synonymous counterparts (Sharp et al., 2010; Carlini & Makowski, 2015). Hence, variations in GC content and synonymous codon usage could affect gene prediction, especially for a new genome sequencing project (Bowman et al., 2017). The usage of transcriptomic evidence derived from RNAseq data obtained previously (*A. ervi* transcriptome sequencing project, Chapter II) allowed us to iteratively train the gene prediction algorithms within the MAKER2 pipeline and effectively improve gene prediction results both in terms of gene structure and number of genes (i.e., detection of novel genes) (Bowman et al., 2017).

Perhaps the most surprising finding from our genomic assembly was that *A. ervi* apparently lacks a functional complement of the canonical enzymes involved in DNA methylation (including a loss of the maintenance *DNMT1*), as well as extremely reduced levels of DNM in both the genome and in the predicted genes/coding sequences. Although

DNM is present in all insect orders except Diptera (Bewick et al., 2016), it has been described as a key mechanism playing an important role in the modulation of phenotypic plasticity through transcriptional regulation, genomic imprinting and silencing of repetitive DNA elements (Li-Byarlay 2016). But in some parasitic wasps such as *Microplitis demolitor* and *M. mediator,* extremely reduced or completely absent DNA methylation levels have been reported (Bewick et al., 2016). This suggests that the loss/reduction of DNA methylation would be rather species/lineage specific (Bewick et al., 2016). Hence, it could be possible that in these species/lineages, subtle mutations in *DNMT1* or *DNMT3* introduced a reduction in DNA methylation during insect evolution that was later followed by a complete loss of both DNA methylation and *DNMT* genes (Lyko, 2017). This seems to be the case of *A. ervi*, where the incomplete set of *DNMT*s found in its genome could be an evolutionary remnant of the DNA methylation enzymatic machinery (Lyko, 2017). However, the lack of a functional, canonical DNA methylation mechanism does not allow to conclude that DNA methylation is completely absent in *A. ervi*. For example, using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method, a very low DNA methylation level (0.034%) has been detected in *Drosophila melanogaster*. Although the lack of any canonical DNA methylation enzyme in *Drosophila* (Yan et al., 2014) may explain that observation, the level of methylation detected is up to two orders of magnitude below the detection limit of the bisulphite sequencing method (the standard technique for analysis of DNA methylation; Capuano et al., 2014). Thus, *D. melanogaster* may have a yet undisclosed mechanism involved in DNA methylation (Rasmussen et al., 2016). Similarly, the very low levels of DNA methylation detected in *A. ervi* could be explained following that evidence, although further studies should consider the usage of more accurate, improved and sensitive techniques such as LC/MS/MS; Rasmussen et al., 2016).

Interestingly, DNA methylation is not unique in its potential to affect gene regulation, as other epigenetic mechanisms (nucleosome positioning, histone protein variants, and histone posttranslational modifications such as acetylations or methylations) may be involved in the modulation of gene expression by altering local accessibility of chromatin to transcription factors and the basal transcriptional apparatus (Glastad et al., 2017). This is the case of *D. melanogaster*, where other epigenetic mechanisms, such as histone lysine methylation, are involved in the regulation of adults' behavior polymorphism (Holowatyj et

al., 2015; Anreiter et al., 2017). Hence, further work should also be focused on studying whether if there is evidence of these alternative epigenetic mechanisms in *A. ervi* and if they participate in the modulation of behavioral phenotypes, such as the differences in host preference and acceptance described previously from parasitoids populations reared or collected from different aphid host species, but which seem to be rather genetically homogenous (Zepeda-Paulo et al., 2013).

4.6 Concluding remarks

Having genomic resources for the parasitoid wasp *A. ervi* will allow for further research on host-parasitoid interactions and biological control at a foundational genomic level (Geib et al., 2017). Indeed, a combined collaborative international network of several research groups with different interests on the ecology and physiology of host/parasitoid interactions is currently on-going. This consortium is using the available *A. ervi* resources in order to generate a curated, solid reference dataset that will be used in future studies involving parasitoids. Hence, it is expected that future research will address the genetic basis underlying several relevant traits such as host selection/preference, specific venom-coding genes and venom protein composition influencing the hosts' physiological systems to favor parasitoid development, among others, which have not been previously described in solitary endoparasitoids of aphids (Geib et al., 2017), and which can now be addressed at the genomic level. It would also be interesting to see whether other epigenetic mechanisms rather than DNA methylation could be present in *A. ervi* and whether they are actively participating in the modulation of gene expression in this wasp used for biological control of pest aphids.

4.7 Acknowledgements

We thank Francisca Zepeda-Paulo for providing *A. ervi* populations, Daniela Sepúlveda and Cinthya Villegas for help in parasitoid rearing and sexual identification, Lucia Briones for help in laboratory DNA extractions for Illumina sequencing, Jean-Christophe Simon and Jean-Luc Gatti for support with DNA extraction and PacBio sequencing, and Fabrice Legeai for support with the assembly and annotation of the *A. ervi* genome. This work was funded by FONDECYT grants 1130483 and 1170943 to CC Figueroa, 1140632 to Blas Lavandero, and by Iniciativa Científica Milenio (ICM) NC120027. GI Ballesteros was funded by

CONICYT PhD fellowship 21120371. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Chapter V

General Discussion and Conclusions

5.1 Host fidelity in an introduced parasitoid wasp

Parasitoid wasps are among the natural enemies most used for biological control of pest insects, as they are considered host-specific (Godfray 1994). This is the case of *Aphidius ervi*, an endoparasitoid wasp successfully used in the biocontrol of economically relevant aphid species and introduced in Chile as part of an aphid biological control program in cereals (Starý, 1993). However, the natural occurrence of *A. ervi* attacking different aphid species and displaying significant variation on host preference and acceptance (Peñalver-Cruz et al., 2017; Zepeda-Paulo et al., 2013) opens interesting questions regarding the molecular basis and evolution of both host preference and host specific adaptations in this parasitoid species. Although these differences should lead to the formation of host-races and speciation, as a result of genetic differentiation among *A. ervi* populations, the available evidence suggest that the behavioural differences in terms of host preference observed in introduced *A. ervi* populations seem not to be related to specific genetic differentiation, but rather to environmental variation (e.g., host aphid, aphid-plant interaction) and to transcriptional phenotypic plasticity, which could be playing a key role in the observed host fidelity. Additionally, it has been proposed that *A. ervi* displays molecular mechanisms involved in gene regulation (such as epigenetic mechanisms), which could modify the expression of certain genes and develop distinct phenotypes in response to different environmental cues. However, and to the best of our knowledge, the lack of both genomic and transcriptomic resources makes it extremely difficult to study such molecular mechanisms in this parasitoid species.

For this reason, in this thesis we focused on addressing several questions regarding the molecular basis and mechanisms underpinning the phenotypic plasticity on host preference displayed by *A. ervi*. The results presented in chapters II and III support the hypothesis that phenotypic plasticity in host selection and preference traits displayed by different lineages of *A. ervi* parasitizing different aphid host species is characterized by a wide plasticity at the transcriptome level. First, **we present evidence of a wide phenotypic**

plasticity at transcriptional levels between *A. ervi* lineages parasitizing two different host species in Chapter II using a *de novo* transcriptomic approach and bioinformatics pipelines. **These substantial differences found in the transcriptomes of adult *A. ervi* depend on the aphid-plant complex where parasitoids develop.** Interestingly, our study found variation in the expression profiles of chemosensory genes, signaling genes and neuronal development genes. This finding suggest that host preference and host fidelity would rely on differences in terms of expression levels of chemosensory genes in *A. ervi*, while being a signature of adaptive phenotypic plasticity to different host and host-plant induced environments (Glaser et al., 2015). Furthermore, variation in the expression profiles of selected chemosensory genes (ORs and OBPs) was also detected in reciprocal transplant experiments (Chapter III). These results show that switching *A. ervi* females from the same population to a novel plant-host complex (non-natal host) has an effect on how a group of chemosensory related genes (ORs and OBPs) are expressed in terms of transcript abundances in their offspring**. Taken together, the results of chapters II and III suggest that differences in olfactory sensitivity would be related with the formation of host fidelity towards a novel host.** Thus, further research is required in order to elucidate if changes in expression levels of candidate OBPs and ORs are indeed related to changes in both olfactory sensory sensitivity and variations in terms of host preference and host fidelity in *A. ervi*.

On the other hand, the second hypothesis tested that DNA methylation would be the epigenetic mechanism underlying that phenotypic plasticity observed in *A. ervi* (chapters II and III) would not be supported by the results from this thesis (Chapter IV). Indeed, our results strongly suggest that **DNA methylation would not be an epigenetic mechanism underlying the transcriptional plasticity observed in this species**, and that this loss of DNA methylation would be rather species-specific. Thus **the results suggest that alternative epigenetic mechanisms other than DNA methylation would be explaining the transcriptional plasticity observed in this parasitoid species when parasitizing different aphid hosts**. Hence, further research is required in order to establish whether these alternative epigenetic mechanisms are present in *A. ervi* and whether they could be modulating the transcriptional differences observed. For instance, nucleosome positioning, histone protein variants, and histone posttranslational modification, among others, need to be

explored in terms of their influence on expression variation studied in this parasitoid wasp (Glastad et al., 2017).

Hence, the main conclusions drawn from this thesis are: **I)** transcriptional levels in *A. ervi* display substantial differences depending on the aphid-plant complex where parasitoid develop. **II)** Expression levels of genes involved in chemosensory perception (ORs and OBPs) also display significant differences between *A. ervi* females, depending if they were exposed to a natal host or to a novel plant-host complex (non-natal host). **III)** An alternative epigenetic mechanism, rather than DNA methylation, would be explaining the transcriptional plasticity observed in this parasitoid species, when parasitizing different aphid hosts.

5.2 Future directions

Biological control programs based on parasitoid wasps are currently saving billions of US dollars annually by virtue of their ability to control pest species, thereby also reducing insecticide applications worldwide (Simpson et al., 2011). However, rapidly growing populations of introduced parasitoids in agroecosystems may lead to undesirable effects, such as spillover into natural adjacent habitats and environmental harm in non-target species (Boivin et al., 2012), while reducing the parasitoids' efficiency as biological control agents of agricultural pests (Rand et al., 2006). Hence, enhancing the efficiency of *A. ervi* as a biocontrol agent of specific agricultural aphid pests is highly desirable. In this context, the foundational genomics/transcriptomics datasets and results obtained during this thesis should contribute to further research on the molecular basis of several key aspects and mechanisms of parasitoids' biology related to an increased efficiency of *A. ervi* controlling aphid pests. For instance, the knowledge of specific olfaction-related genes in this parasitoid species would be useful in further research regarding the characterization of specific volatile compounds involved in host location and host discrimination of a particular aphid species. The use of heterologous expression systems (Hughes et al., 2010) or the gene silencing using specific targeted RNA interference combined with electrophysiological responses in parasitoid individual wasps, should shed light on the volatiles/blends detected and learned during the formation of host-fidelity through characterizing the odorant-induced specific gene expression profiles (Sun et al., 2016). Thus, specific volatile cues and compounds could

be used in the field to enhance the atractability of parasitoids to crops infested with a certain aphid species, by using attracting plants placed around the field in a typical push-pull strategy (Sasso et al., 2007), or by priming parasitoids' host-seeking behavioral responses through the exposure to specific olfactory cues either during parasitoids' development or in adult stages.

Many parasitoids species used in biological control programs, including *A. ervi*, are reared in commercial insectaries in small caged populations under constant, artificial conditions before being released to farms and/or natural environments (Fernández & Nentwig, 1997). As this rearing protocol increases the chances of inbreeding, especially when species are kept for long time (Zaviezo et al., 2017), and the inbreeding reduces host fidelity in *A. ervi* (Sepúlveda et al., 2017b), then future research based on genomics/transcriptomics datasets presented herein could be focused on controlling the genes and molecular mechanisms underlying host fidelity. For example, the levels of expression of chemosensory genes could be manipulated in order to maintain/increase specificity and preference towards a specific aphid host, even during long-term rearing under stable conditions. Thus, the negative effects of confined mass-rearing on host fidelity could be controlled to increase the efficiency of inbred parasitoids as biocontrol agents of relevant aphid pests in the highly homogeneous agroecosystems that dominate modern agriculture.

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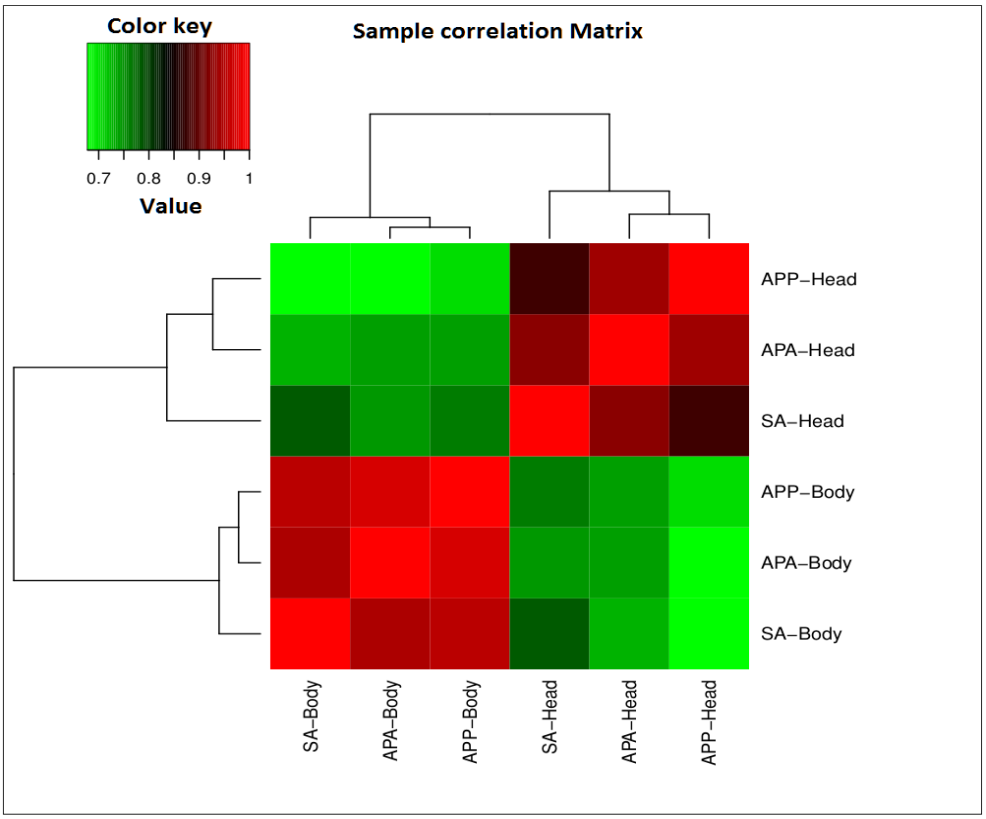
Appendix A

Supplementary material for Chapter II

Supplementary figure 2.1. Sample correlation matrix heatmap for all *A. ervi* libraries.



Supplementary table 2.1: genes up-regulated between *A. ervi* populations; available at https://peerj.com/articles/3640/ as supplementary Table 1**.**

Supplementary table 2.2: Final number of reads from each library used in DE analysis.

Population	Body	Head
A. ervi - APA	43,733,854	38,876,038
A. ervi - APP	40,189,908	38,403,840
A. ervi - SA	66,280,364	53,068,010

Appendix B

Supplementary material for Chapter IV

Supplementary table 4.1: assembly statistics for *A. ervi* genomes. § indicates genome assembly reported in Chapter IV

	Ae_genome_v1.0	**Ae_genome_V2.0**	**Ae_genome_V3.0**[§]
# contigs (>= 0 bp)	224,706	3,865	5,778
# contigs (>= 1000 bp)	7,623	3,864	5,777
Largest contig	2,849,622	4,656,333	3,671,467
Total length (>= 0 bp)	171,357,109	144,532,283	138,951,524
Total length (>= 1000 bp)	135,958,237	144,531,287	138,950,528
N50	394,255	705,903	581,355
N75	76,289	156,189	119,055
GC (%)	25.91	25.89	25,85
Assembly	Paired end & Mate-Pair (Illumina), *A. ervi* males	Ver 1.0 scaffolded with PacBio Reads	Ver 2.0 with mitochondrial DNA removed from scaffolds