Life-history evolution in hymenopteran parasitoids
the roles of host and climate

To, my family, Laleh, Kaveh and Keyvan

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Contents

Sampling points Iran

Chapter 1

General introduction and discussion

Life-history theory

Life-history theory predicts the existence of constraints and trade-offs between traits contributing to the fitness of an organism. Constraints reduce the flexibility in the evolution of particular combinations of traits (Roff 1992). A trade-off means that a trait can only increase in value at a cost to another trait. For instance, natural selection may favor an increased reproductive rate, but this may be impeded by a trade-off between reproduction and survival. Due to this trade-off any increase in reproductive rate will come at a cost to survival rate or vise versa (Roff 1992, Stearns 1992). This trade-off can be studied by measuring traits that are directly related to reproductive rate and survival such as age-specific fecundity, age and size at maturity, size at birth, number of offspring and survival rate. Traits that affect fitness indirectly, such as resource acquisition and allocation, foraging behaviour, stress tolerance, resisting parasite or predator, competitiveness are also considered life history-traits (Jervis et al. 2005; Maeda 2006; Steiner and Pfeiffer 2007). Life-history theory deals with analyzing variation in these traits and investigates relationships between them. The central aim is to establish how variation in life-history traits may lead to variation in fitness of individuals. Life-history theory views organisms from both ecological and evolutionary perspectives (Roff 1992).

Natural selection on life-history traits

Natural selection acts upon fitness-related variation in phenotypes. Given additive genetic variation, trait values that enhance fitness will increase in frequency over time (Fisher 1930). Fitness in general is defined as the ability of individuals to survive and reproduce in a given environment (Brwon 1993). Fitness thus reflects the contribution of that each individual makes to the gene pool of the next generation (King and Standsfield 1990; Daan and Tinbergen 1997; Sober, 2001; Orr 2009). Genetic variation in fitness is the raw material for natural selection without which adaptation can not occur (Orr, 2009). Two different fitness measures are used in evolutionary studies: global fitness and local fitness. Global fitness is the overall fitness of an organism that includes all the interactions between traits contributing to fitness. In other words global fitness is the balance between different fitness components. Global fitness has frequently been

assumed to be identical to lifetime reproductive success (R0) in stable populations or to the intrinsic population growth rate (r) in growing populations (Roff 1981; Charnov and Berrigan 1991; Fox and Wolf 2006). Local fitness is the fitness of each component independently and can be use instead of global fitness only if overall fitness increases with local fitness (Roff 1992).

Life-history traits can vary enormously between and within species. For example, different species of flatfishes may range in body size from 2 cm to 200 cm and in age at maturity from less than one to over 10 years (Roff 1992). Another example is variation in clutch size in birds which ranges from one egg per clutch in albatrosses to nine eggs per clutch for geese species. Some impressive variation in life-history traits at the intraspecific level has also been documented. For example, members of one population of the flatfish *Hippoglossoides platessoides* averaged 25 cm in body length and took 6 years to reach maturity, while in another population the fish measured 60 cm and took over 20 years to reach maturity (Roff 1992). Similarly, age at maturity in the European minnow, a freshwater fish, ranged from one to seven years (Mills 1988). Such variation in life-history traits may be subject to natural selection and result in genetic and phenotypic differences between populations and even speciation when populations have diverged dramatically in the long term. Such continuous spectrum of change in life-history traits is called life-history evolution.

Reproductive success: the main target of selection

In many studies reproductive success has been used to represent global fitness (e.g., Roff 1981; Charnov and Berrigan 1991; Fox and Wolf 2006). Evolution is th result of natural selection favoring individuals with high global fitness. If we equate increase in global fitness with increased reproductive success then the main product of evolution is optimization of reproductive success. Life-history theory predicts a trade-off between reproduction and survival (Roff 1992). This means that the potential increase in reproductive rate in a given environment is limited by the imposed cost on survival rate. As a consequence, a balance between reproduction and survival is inevitable. This trade-off may be observed in nature in different forms, the simplest being a negative relationship between the number of young produced and the parent's survival (Reznik 1985; Roff 1992). It can also be seen as a negative relationship between the number of offspring and the size of the offspring (Williams 1966; Smith and Fretwell 1974; Gustafsson and Suderland 1988; Roff 1992) or between time or resources spent on current versus future reproduction (Godfray 1987; Ackerman and Eadie 2003; Ellers and Jervis 2003, 2004). To maximize reproductive success, organisms must balance reproduction and survival optimally. The question of how individuals maximize reproductive success in a given

environment is thus a fundamental question in evolutionary ecology. The maximization of reproductive success is influenced by different factors. The strength and importance of each factor can be determined by the environment. I will discuss some important factors affecting reproductive success in different environments.

Resource acquisition and allocation

One of the main factors influencing reproductive success is resource acquisition and allocation (e.g., de Jong and van Noordwijk 1992; Tanaka 1996). Life-history theory also predicts that resource availability shapes life-history-traits and drives the evolution of organisms (Roff 2002). If resource acquisition and allocation can change the fitness of organisms then it is expected to be subject to natural selection. A considerable number of life-history studies have been devoted to resource acquisition, allocation and the resulting optimal balance between life-history traits (e.g., van Noordwijk and de Jong 1986; Engen and Saether 1994; Worley et al. 2003; Jervis, et al. 2008). Resource acquisition often has been studied as a component of the foraging behaviour of animals. Foraging determines resource intake by the organism. A wide variety of animal foraging strategies has been reported both inter and intraspecifically (Gray and Hodgson 1997; Caldow et al 1999; Jia et al 2002; Cooper 2005). Boggs (1992) suggested that foraging is linked with life-history and must be studied together with resource allocation to provide a better understanding of population dynamics in contrasting environments. Variation in foraging strategies may change the pattern of resource allocation dramatically in different populations or individuals within the same population (Weimerskirch, et al 1997). Resource distribution patterns in the environment and competition between individuals within species also may change foraging strategies and resource intake rates (Sutherland 1996). Efficient foraging strategies may help organisms to overcome the competition between individuals of the same species (Caldow et al 1999). Variation in foraging may affect the fitness of individuals and consequently result in natural selection and potentially the diversification of populations. Many studies reported spatial or temporal partitioning in the foraging patterns of coexisting species under resource limitation (Shargal et al 2000; Hadiprakarsa and Kinnaird 2004). Niche partitioning is the result of long term evolution of traits which allow organisms to exploit distinct ecological niches and allows the coexistence of competitor species in same habitat. For example, differences in the combination of enzymes allows species to prefer different diets (Voytek and Joyce 2009). It is obvious that the presence or absence of a coexisting competitor in different habitats can drive the evolution of populations of the same species in different directions.

Allocation of resources to different traits strongly influences the

fitness of an organism. We have already mentioned that the spatial or temporal distribution of resources influences foraging behaviour and thus resource intake rate (Roff 2002). The availability of resources is a habitat characteristic that may differ between environments. The allocation of limited obtained resources to competing traits can play a fundamental role in fitness of organisms. Many organisms are adapted to a narrow environmental range and may perform poorly in different habitats (Nagy 1970; Fenster and Galloway 2000). Different combinations of trait values may be optimal in different environments. For example, there is evidence for differences in reproduction and survival for birds in different habitats (Clark and Shutler 1999). Habitat specific resource allocation strategies may improve the fitness of individuals. One of the main challenges of evolutionary ecology is to understand the optimal strategies for resource acquisition and allocation. This optimal strategy can vary drastically depending on the habitat and resource distribution patterns. First we need to understand the role of habitat in life-history evolution. A habitat is a combination of biotic and non-biotic factors which form the environment of each organism. Interaction of the organism with both biotic (other species) and abiotic (climate factor and geographical structure) factors can shape its life-history traits. This thesis focuses on the role of hosts as the main biotic factor, and climate as the main abiotic factor in the evolution of life-histories in parasitoids.

Hymenopteran parasitoids

Parasitoids are a group of insects which lay their eggs on or inside the body of preimaginal (and rarely adult-) stages of other insects (egg, larva or pupa) and complete their development by consuming the host tissue, eventually killing their host (Godfray 1994, Quicke 1997). Juvenile parasitoids inhabit a single host from early life to maturity which results in an intimate relationship between parasitoid and host (Godfray 1994). Different groups have been defined within parasitoids, depending on their life style. Idiobionts are a group of parasitoids that start consuming their host immediately after parasitism, while koinobionts allow their host to continue feeding and increase in size. Endoparasitoids define a group of parasitoids that live and feed inside the host body incontrast ectoparasitoids that live and feed externally on the host. Parasitoids have also been categorized based on the stage of the host which they attack for example egg, larval (nymph) or pupal parasitoids (Godfray 1994, Quicke 1997). Parasitoid life styles are predominantly found in two orders of Insecta. The greatest diversity of parasitoids occurs in the Hymenoptera, with a substantial minority in the Diptera. Much smaller numbers are found in other orders, e.g. in the Coleoptera. Parasitoids can in some cases control insect populations and are often used as biological control agents of insect pests on agricultural crops in field. Studies of the cues that parasitoids use to find their host gained them prominence in the scientific study of

animal behaviour (Godfray and Shimada 1999). Using hosts- parasitoid as a model system provided an opportunity to test population dynamic models. Godfray (1994) reviewed most of these studies and many new studies have been published since then (Quicke, 1997; Tilman & Kareiva, 1997; Wilson and Hassell 1997; Turchin, 1998; Dieckmann et al., 2000; Ellers et al. 2000b; Schofield et al. 2002; Rivero and West 2002; Lett et al. 2003; Rivero and West 2005; Liu et al 2009). Studies on host-parasitoid relationships have made valuable contributions to evolutionary ecology. Examples include the optimization of foraging strategy according to different host distribution patterns and environmental factors (e. g., Vet et al. 1990; Schofield et al. 2002) and in the role of habitat stochasticity in energy allocation strategy (e. g., Ellers et al. 2000b). Furthermore, comparative studies on host-parasitoid interactions have provided a spectacular evolutionary view of this interaction and the co-evolution of these organisms. Some well known examples of host parasitoid interactions are geographical variation in parasitoid virulence and host defense systems (Kraaijeveld and van Alphen 1994; Kraaijeveld and van Alphen 1995) or superparasitism as an adaptive strategy in parasitoids (Alphen and Visser, 1990; Kraaijeveld et al.,1995). All of these aspects have made parasitoids an excellent model system to study ecology and evolution.

Life-history traits in parasitoids

There is a substantial body of literature on parasitoid life-history (e. g., Price 1973; Blackburn 1991; Godfray 1994; Kraaijeveld & van der Wel, 1994; Jervis 2001; Ellers and Jervis 2003; Jervis et al 2003 Rivero and West 2005). Hymenopteran parasitoids show huge variation in life-history traits. For instance, the ectoparasitic wasp *Sericopimpla sericata* (Ichnieumonidae) measures 12 mm, lives up to 140 days and lays only 30 relatively large eggs (2 mm) in its entire life. By contrast, the endoparasitic wasp *Trioxys complantus* (Braconidae) is only 1.3 mm long, lives 28 days and lays 180 small eggs (0.1 mm) during its lifetime (Mayhew and Blackburn 1999). Astonishing variation in reproductive strategy was also reported for two hyperparasitoids of *Cotesia glomerata*, *Gelis agilis* and *Lysibia nana.* The former has large eggs, long life-span and few progeny per day but performed with similar efficiency as the latter which is reversed in all these traits (Harvey 2008). Although intraspecific variation in life-history traits of parasitoids is much smaller in scale compared to interspecific variation, still considerable life-history variation has been reported among populations of the same species. Variation in reproductive success and allocation to early life reproductive was observed for *Asobara tabida* populations along a geographic cline (Kraaijeveld & van der Wel, 1994). A quantitative genetic study also showed heritable variation in fecundity of the parasitoid *Anagrus delicates* (Cronin and Strong 1996). Studies have shown size dependent variation in life-span and fecundity

of parasitoid individuals (Ellers and Jervis 2003; Jervis et al. 2003; Jervis and Ferns 2004; Thorne, et al. 2006).

Parasitoids should optimize their reproductive strategy by maximizing the number of eggs laid before the end of their life. This means that both time limitation, i.e. coming to the end of life with unlaid eggs left in their body, and egg limitation, i.e. finishing their eggs much earlier than the end of life, could both play a role in parasitoids (Ellers 1998). The optimization of reproduction has been the focus of many life-history studies in parasitoids in both ecological and physiological contexts. Timing of reproduction (age-specific fecundity) is an important component of the reproductive strategy of parasitoids. Parasitoids were initially divided into two groups based on their reproductive strategy. Pro-ovigenic species emerge with all eggs mature, while in synovigenic species, none or only a portion of eggs are mature at emergence (Flanders 1950). Synovigenic parasitoids are able to adjust the timing of egg maturation from early to later in life time. In fact, a continuum of strategies exists between these extremes, which is expressed as the index of ovigeny (Jervis et al. 2001). Timing of egg maturation shows a negative relationship with body size and a positive relation with habitat stochasticity (Ellers 1996; Ellers et al. 2000b; Ellers and Jervis 2003; Jervis et al. 2003; Jervis and Ferns 2004; Thorne, et al. 2006).

Resource allocation in parasitoids has been studied in both ecological and physiological contexts and has been reviewed recently by Jervis et al. (2008). Different nutrients (e.g. sugar, glycogen, lipid and protein) play different roles in locomotion or ovigenesis. Stored lipids are important in both ovigenisis and somatic function in parasitoids (Ellers 1996; Ellers et al. 1998; Pexton and Mayhew 2002; Casas et al. 2005; Rivero and West 2005). The lack of lipogenesis in adult parasitoids results in a key role for lipid allocation. A trade-off in lipid allocation to initial reproduction and fueling soma have been demonstrated both inter and intraspecificly for hymenopteran parasitoids (Ellers and van Alphen 1997; Pexton and Mayhew 2002). The cost of future reproduction on teneral lipid reserve was also shown for synovegenic parasitoids (Ellers et al. 2000b). Plasticity in resource acquisition and utilization, for example through adult host feeding or egg resorption may mask this trade-off (Jervis et al 2001, Jervis and Kidd 1986; Burger et al 2004; Heimpel and Collier 1996). The effect of host feeding on female fecundity varies between parasitoid species (Ueno and Ueno 2009). Resource allocation plays a pivotal role in intertrait correlations because of trade-offs in allocation of resources to different traits. In this thesis, I examine variation in resource allocation in hymenopteran parasitoids in relation to climate and host.

Climate

The ecological and evolutionary effects of climate on life-history

traits have taken on a special relevance in the light of recent concerns over climate change (e.g., Stenseth and Mysterud, 2002, Stenseth et al. 2002; Winkler et al. 2002; Stenseth and Mysterud, 2005). A wide range of meteorological factors contribute to form the climate. The strength of each factor may vary between climates. The Islamic Republic of Iran consists of a wide variety of climates. A study on the climatic regions of Iran analyzed the contribution of 27 factors to the variance in climate and identified six as the main climatic factors: temperature, humidity, precipitation, cloudiness, wind and thunder (Masoodian 2003). Among these, temperature and humidity are widely regarded as the most important components of climatic variation (Gorshkov and Makarieva 2002). In life-history studies too, temperature has been mentioned as a key factor affecting the fitness of a wide range of organisms (Roff, 2002). The effect of temperature on life history traits of different organisms has been well documented in many studies (e.g., Johnston and Benneth, 1996; Dahlgaard et al. 2001; Clarke, 2006). The role of temperature on insect life history traits include the increase of some traits with increasing temperature (Nespolo et al. 2007; Stelgenga and Fischer 2007; Karl and Fischer, 2008) or reduction of the traits with increasing temperature during development (Bazzocchi at al. 2003; Karl and Fischer, 2008; Nilssen 1997; Dhileepan at al., 2005; Colinet at al. 2007; Fischer et al. 2003). Climate may affect parasitoid life-history directly or indirectly by altering the life-history traits of the host. Both of these will be addressed in this thesis. Among the climatic factors that will be discussed are frost and drought. Frost can play a crucial role in shaping life-history traits of organisms (Inouye 2000). Dramatic declines in nutrient reserves (mainly in lipid content) under desiccation and cold stress have been documented for insects (Downer and Matthews 1976, Udonsi 1984, Djawdan et al. 1997, Minois and Le Bourg 1999, Colinet et al 2006). In nature, adult hymenopteran parasitoids can obtain carbohydrates from nectar. Carbohydrates and moisture are essential determinants of lifespan for many hymenopteran species (Lewis et al 1998). The availability of food sources in nature may not only affect the longevity of parasitoids but also their fecundity (Hagley and Barber 1992; Olson, D. L. and Nechols 1995). The patterns of nectar availability can be highly diverse in nature due to climate. A lack of a carbohydrate sources in nature can result in starvation of parasitoids (Jones and Jackson 1990; Jervis et al. 1996;Williams and Roane 2007). The evolution of life-history traits in hymenopteran parasitoids is affected by climate in many different ways. It therefore requires broad-scale research to understand the evolutionary aspects of climate and climate change in hymenopteran parasitoids.

Host

The life histories of parasitoids and that of their hosts are intimately linked. Direct host-parasitoid interactions may result in co-evolution between the two species. Second, climate may affect the

host distribution pattern and host quality, which may then affect the parasitoids. An important component of host-parasitoid interactions is the immune system of the host and the ability of parasitoids to suppress it. This has been well studied in hymenopteran parasitoids and their hosts. Encapsulation is a common reaction in insects host to escape parasitism. It operates by aggregation of haemocytes around the parasitoid egg which results in melanization of the egg (Nappi 1975; Rizki and Rizki 1984). *Asobara tabida* populations show geographic variation in their ability to resist encapsulation which is correlated with variation in encapsulation ability of the host, *Drosophila melanogaster* (Kraaijeveld and van Alphen 1994, Kraaijeveld and van Alphen 1995). Interactions between hosts and parasitoids are not limited to the immune system of the host and virulence of the parasitoids. Manipulation of host behaviour by the parasite in such a way that it increases the fitness of parasite has been termed the "*extended phenotype*" (Dawkins 1982). There are many examples in which parasites manipulate their host behaviour in wide range of host, including snails (Miura *et al.* 2006), birds (Holmstad *et al.* 2006) and hymenopteran parasitoids (Brodeur and Vet 1994; Tanaka and Ohsaki 2006; Grosman et. al 2008). Examining variation in the manipulation behaviour of closely related parasitoid species may help to understand its fitness value. The potential effect of climate in shaping these manipulative interactions has never been addressed. Two other important host effects that may be affected by climate are host quality and host distribution pattern. The effect of climate on vegetation and fruit availability may contribute to variation in host quality through differences in food sources and competition. For example, the performance of parasitoids on herbivorous insects has been shown to vary depending on plant cultivars or soil types (Moon et al. 2000; Moreau et al. 2009; Sarfraz et al. 2009). Immature parasitoids are limited to a single host and the amount of resources that they can exploit is thus dependent on the nutrients gained by the host, which in turn is affected by food availability and competition. Host availability in nature is highly dependent on temperature and precipitation. Both temporal and spatial distribution patterns of the host are dictated by climate and are important sources of selection on life history traits of parasitoids. In particular, the stochasticity of the climate has a strong effect on the evolution of life history traits in parasitoids (Ellers et al. 2000b; Ellers and Jervis 2003). In stable climates the probability of finding hosts may be constant in any season, while in more stochastic climates parasitoids may face a lack of hosts at certain times, because of host population crashes. Parasitoids are expected to adapt their life history decisions, such as foraging behaviour, in relation to host distribution pattern (Vos & Vet, 2004; Kraaijeveld & van der Wel, 1994; Kraaijeveld et al., 1995; Dubuffet et al., 2006). Overall, host quality and availability are crucial for parasitoids and are likely to impose strong selection on life-history traits of parasitoids.

Aims and scope the thesis

This thesis explores the relationships between parasitoids, hosts and climate from an evolutionary point of view. I chose hymenopteran parasitoids as a model system. The Hymenoptera are one of the largest orders of Insecta and include the largest group of parasitoids with about 50000 described species (Godfray 1994). An additional motivation for this choice was the role of hymenoteran parasitoid as biological control agents in pest control in agriculture crops. Studying the interplay between parasitoids, hosts and climate may contribute to our understanding of processes involved in the establishment of parasitoids in a new habitat. To study these questions, I adopted a comparative approach. Variation in life-history traits has studied for species or populations from different habitats. First, I looked at closely related species which have evolved from a common ancestor, but occupy different habitats. Second, I studied genetic and life-history trait variation in different populations of a single species. These populations were geographically isolated in contrasting climates.

This thesis examines the following five predictions:

1. Closely related species manipulate their host's behaviour in different ways, depending on their habitat.

2. Different strategies of parasitoid species in spending their limited resource budget result in a lack of correlation between developmental time and life span.

3. The trade-off between initial egg load and lipid reserve is influenced by body size at both the inter- and the intraspecific level.

4. Strong selection on life-history traits of parasitoids induced by climate and host is expected to promote genetic divergence of parasitoid populations along a geographical cline.

5. Natural selection in geographically isolated populations should favour different life history strategies. Variation in life-history traits is thus expected to correlate with climatic factors.

Chapter 2: Closely related parasitoids induce different pupation and foraging responses in *Drosophila* larvae

In this chapter I tested the first hypothesis by comparing pupation site of hosts parasitized by different species of *Asobara* (Braconidae). The central goal of this chapter was to show variation in adaptive host manipulation behaviour by parasitoids. The species originated from highly divergent habitats with very wide differences in climate, host and

competitor species assemblages. The results not only revealed variation in strength of host manipulation but also in the direction of the induced changes. At least for one species (*A. tabida*) the induced change in pupation site was shown to be adaptive for the parasitoid.

Chapter 3: The lack of correlation between developmental time and adult life span in parasitoids: the role of metabolic rate and fat reserves

This chapter explores the relationship between developmental time and life span in presence and absence of food in five species of *Asobara* cultured on *Drosophila melanogaster.* The lack of lipogenesis during adult life in parasitoids places severe constraints on allocation of the limited amount of lipids that can be sequestered from a single host. This is expected to result in different life-history strategies and variation in metabolic rate, lipid storage and lifespan.

Chapter 4: Adult size and early investment in reproduction in five species of *Asobara* parasitoid wasps

Life-history theory predicts a trade-off between initial egg load and lipid content (Roff 1992). Furthermore, large individuals are expected to postpone maturation of more of their eggs than small individuals, while habitat stochasticity should select for higher initial egg loads (Ellers and Jervis 2003). I tested these predictions at both inter- and intraspecific levels for five species of *Asobara*. The results provided strong support for these predictions at both levels.

Chapter 5: Genetic structure of *Leptopilina boulardi* populations from different climatic zones of Iran

I documented genetic variation of 11 populations of *Leptopilina boulardi* along a geographic transect in Iran to test whether populations from different climatic zones had diverged genetically. Nine populations could be divided in groups clustering together based on their zone of origin. One population differed from all others and was probably imported from elsewhere. A montane population was found to be genetically similar to those from the nearby lowlands. Both distance and physical barriers contributed to the genetic structure of these populations.

Chapter 6: Local adaptation in life history traits of *Leptopilina boulardi* populations from different climate zones of Iran

I investigated the energy reserves (lipid, glycogen and sugar) and reproductive effort (egg load and egg size) of 11 populations of *Leptopilina boulardi* originating from contrasting climate zones of Iran. Most traits showed significant divergence according to climate. Two main climatic

factors, precipitation and frost, explained significant amounts of the variance in life-history traits. This finding suggests that natural selection on life-history traits imposed by climate can result in rapid evolution of these traits.

General conclusion

The results in chapter two demonstrate significant manipulation of host behaviour by two out of five *Asobara* species affecting both pupation height of the host and foraging behaviour of host larvae. Manipulation of aspects of host behaviour by hymenopteran parasitoids have been reported previously (Brodeur and Vet 1994, Tanaka and Ohsaki 2006; Grosman et al. 2008). However, these studies only demonstrated a host manipulation by a single parasitoid species. Comparison of closely related species originating from different habitats revealed different manipulation strategies when interacting with a common host. Our empirical evidence demonstrated the adaptive value of the induced change by *A. tabida* but not for *A. citri*. As host manipulation by *A. tabida* and *A. citri* occurred in different directions, it is likely that the optimal strategy for host manipulation is species-specific, because these species live in climatically contrasting habitats.

The results of chapter three showed a lack of correlation between developmental time and adult life span of five *Asobara* species. This is consistent with previous findings for hymenopteran parasitoids (Blackburn 1991; Eijs & van Alphen, 1999). The novelty of this chapter is in revealing the role of lipid reserves and metabolic rate in adjusting species-specific life span. Much of the variation could be explained by the habitat of origin of the species. Variation in host distribution pattern results in differences in foraging behaviour among hymenopteran parasitoids (Vos & Vet, 2004; Kraaijeveld & van der Wel, 1994; Kraaijeveld et al., 1995; Dubuffet et al., 2006). It is obvious that different of variation in foraging behaviours require different activity patterns and different metabolic rates. As a consequence of the lack of adult lipogenesis in hymenopteran parasitoids (Ellers 1996; Rivero and West 2002; Giron and Casas 2003; Visser and Ellers 2008) and variation in metabolic rate, parasitoids will vary in lifespan due to exhausting lipid resources at different rates.

In chapter four I show a high initial reproductive effort in smaller *Asobara* species and low initial reproductive effort in larger species. Consistent with this interspecific pattern I found a negative correlation between size and initial reproductive effort on an intraspecific level. These patterns are in agreement with the Ellers and Jervis (2003) model on timing of reproduction in hymenopteran parasitoids. Furthermore, I found a trade-off between initial reproductive effort and lipid reserves in four out of five species, which is consistent with life-history theory (Roff 1992).

In chapter five I established the genetic structure of 11 populations of *Leptopilina boulardi* from different climatic zones of Iran by employing the Amplified Fragment Length Polymorphism (AFLP) technique. Except for an unexpected pattern for one population and an identical genetic structure for another population which is geographically close to the next zone, a clear genetic separation based on geography was observed. This is consistent with the results for AFLP patterns in other insect populations (Reineke et al., 1999; Ravel et al., 2001; Pannebakker et al. 2004; Alamalakala et al. 2009; Tao et al. 2009) and shows that local adaptation to climate is possible.

In the last chapter I found substantial variation in energy reserves (lipid, glycogen and sugar) and reproductive effort of Iranian *L. boulardi* populations. Similarly to genetic structure, the pattern of life-history traits separated populations clearly for most traits. This suggests that divergent natural selection may contribute to the differentiation of populations (Endler, 1986). Most traits showed a correlation with one or both of two climate factors, the number of rainy days and the number of frost days per year. These two climatic factors impose strong selective pressures in the form of drought and frost stress.

To conclude, I suggest that life-history traits of parasitoids are subject to natural selection pressures from both host and climate. These factors are likely to interact through the effect of climate on hosts. The intimate relationship of host and parasitoid and the dependency of parasitoids on their hosts for crucial resources like lipids, result in specific recourse allocation strategies to fit habitat requirements. Resource allocation in parasitoids is species specific and highly diverged among populations.

Chapter 2

Closely related parasitoids induce different pupation and foraging responses in *Drosophila* larvae

Seyahooei, M. A., F. J. L. Kraaijeveld-Smit, Kraaijeveld, K., Crooijmans, J. B. M., Van Dooren, T. J. M. and van Alphen, J. J. M. 2009. Closely related parasitoids induce different pupation and foraging responses in Drosophila larvae. Oikos 118: 1148-1157

Abstract:

Few examples exist where parasites manipulate host behaviour not to increase their transmission rate, but their own survival. Here we investigate fitness effects of parasitism by *Asobara* species in relation to the pupation behaviour of the host, *Drosophila melanogaster*. We found that *Asobara citri* parasitized larvae pupate higher in rearing jars compared to unparasitized controls, while *A. tabida* pupated on or near the medium. No change in pupation site was found for three other species. A follow-up experiment showed a non-random distribution of parasitized and unparasitized pupae over the different jar parts. To test the adaptiveness of these findings, we performed pupal transfer experiments. Optimum pupation sites were found to be different between host individuals; wall individuals survived better than bottom individuals, but bottom individuals did worse at the wall. Two parasitoid species that alter pupation site significantly showed high rates of diapause at their 'preferred' pupation site. For one of them, *A. citri*, pupation occurred at the optimal site for highest survival (emergence plus diapause). From literature we know that pupation height and foraging activity are genetically positively linked. Therefore, we implement a short assay for rover/sitter behavioural expression by measuring distance travelled during foraging after parasitism. For one out of three species, foraging activity was reduced, suggesting that this species supresses gene expression in the *for* pathway and thereby reduces pupation height. The parasitoid species used here, naturally inhabit widely different environments and our results are partly consistent with a role for ecology in shaping the direction of parasite-induced changes

to host pupation behaviour. More parasitoids are found on the wall of the rearing jar when they originate from dry climates, while parasitoids from wet climates pupate on the humid bottom.

Introduction:

Parasite infection often induces changes in host behaviour (Combes 1991, 1998, Poluin 1998, Moore 2002). These changes can result from resistance adaptations of the host, pathological side-effects of the infection or from the parasite manipulating host behaviour in order to enhance its own transmission rate. The latter is known as the 'extended phenotype' (Dawkins 1982). Manipulation of host behaviour by a parasite will be selected for when the normal host behaviour is suboptimal for parasite transmission. Many examples of such manipulation exist. Snails change habitat preference when infected with a trematode parasite (Miura et al. 2006). Birds infected with a vector-borne parasite have decreased mobility, which increases predation risk and therefore stimulates parasite dispersal (Holmstad et al. 2006). *Leptopilina boulardi*, a hymenopteran parasitoid, has an increased tendency to lay eggs in already parasitized hosts when infected with a virus, which promotes horizontal transmission of the virus (Varaldi et al. 2003). Clearly, parasite-induced changes in host behaviour can have profound impacts on parasite fitness.

In addition to increasing their own transmission rate, parasites may also manipulate the behaviour of their host to enhance their own survival directly. Some examples exist, especially in relation to host and parasitoids (parasites that always kill their host), a common threat to insects. They lay their egg on (ecto) or inside (endo) the body of preimaginal (egg, larva or pupa) or adult stages of the host and complete their development on or inside the host's puparium or body (Godfray 1994, Quicke 1997). An example of host behavioural changes comes from the famous caterpillars infected with the ecto-parasitoid *Cotesia glomerata* which defends their parasitoid against natural enemies by behaving aggressively towards them and also via spinning a web around the pupae (Brodeur and Vet 1994, Tanaka and Ohsaki 2006). Similarly, the braconid parasitoid, *Glyptapanteles sp*. induces a behaviour in its host, *Thyrinteina leucocera* to guard parasitoid pupae and this results in increased parasitoid survival (Grosman et al. 2008). In insects, one trait that has large consequences for survival, is pupation site selection behaviour. During the immobile stage, insects are vulnerable to many dangers, including desiccation, fungal infection, predation / parasitism and super or hyperparasitism. Insects are thus under strong selection to optimize the timing and location of pupation in a way that minimizes such risks. Pupation site selection occurs at the late larval stage and is affected by moisture, temperature, density, phenotype/genotype, sex, species and the presence of predators or (super and/or hyper) parasitoids (Sameoto and Miller 1968,

Barker 1971, Sokolowski and Hansell 1983, Sokolowski et al. 1986, Schnebel and Grossfield 1992, Pivnick 1993, Tanaka and Ohsaki 2006, Riedl et al. 2007).

Parasitoids and hosts may often differ in optimal pupation strategy. For example, when the parasitoid spends more time in the puparium than the host (which they often do) it will be at risk of desiccation, predation and hyperparasitism by pupal parasitoids for longer and may prefer a more humid and concealed pupation site. Furthermore, differences in body size between parasitoid and host results in differences in surface/body mass ratio, which may also influence the optimal pupation site (Carton et al. 1986, Ellers 1998). Because it is the host that pupates, the parasitoid has to manipulate the host if it is to achieve its own optimal pupation strategy. In spite of the considerable interest in parasite-induced changes in host behaviour, little is known of the effects of parasitism on pupation site selection behaviour. One example that we do know of is that of the Bertha armyworms, *Mamestra configurate* which pupate in the leaf litter around the host plant when parasitized by *Microplitis mediator*. Those not parasitized all pupate on the host plant (Pivnick 1993). Although these observations are made, little experimental evidence is present to show that these behavioural changes actually increase parasitoid survival.

Here we investigate whether the pupation behaviour of Drosophila changes as a result of attack by *Asobara* parasitoid wasps. Species in the genus *Asobara* cover a wide range of ecological niches, including wet tropical conditions, dry deserts and cool temperate conditions. They further differ markedly in generation time and hence in the time spent in the host puparium. Thus, the different species of *Asobara* are expected to differ in the degree to which they are in conflict with their host over pupation behaviour. This provides an ideal system to test if parasitoids alter pupation behaviour of their host in an adaptive manner.

We use five species of *Asobara* that span a range of environments and that are all cultured on *Drosophila melanogaster*. Two species are naturally adapted to dry climates, while the other three originate from wet and humid climatic conditions (Table 1). We hypothesize that the former are better adapted to lower humidity conditions than the latter. The conditions in our rearing jars show a steep gradient in humidity. Close to the medium at the bottom of the jar, conditions are much more moist than on the glass higher up. Thus, if our expectation is correct and if parasitoids are able to manipulate their host's pupation behaviour, we expect species from the dry areas to pupate higher in the jars compared to larvae parasitized by wasps from wet areas. Therefore, we compare the pupation behaviour of *D. melanogaster* larvae parasitized by different species of *Asobara* to that of unparasitized larvae. Finding such differences, we then ask whether these might be adaptive. In order to test this, we performed a series of translocation experiments in which hosts pupae were transferred from their selected (high or low in the jar) to the un-

selected site. In the first experiment, we use unparasitized hosts only, to examine the adaptive significance of pupation site selection in individual larvae. This is important, since although we know that this behavioural trait has large consequences for survival (Sokolowski et al. 1986, Rodriguez-del-Bosque and Smith 1996) there is no experimental evidence to show that individually selected sites are the sites which yield optimal survival. In the second translocation experiment, we used parasitized hosts to assess the adaptive value of parasite-induced alterations in pupation behaviour. For parasitoid translocations we not only investigate the effect of translocation on emergence, but also on diapause and total survival (emergence + diapause). These translocations enable us to examine three possible sources of variation which may address survival variance:

1. different effect of pupation sites --> transferred from wall to wall (WW) ≠ bottom to bottom (BB).
2. variation of individuals which is caused by differences between individuals in optimal pupation site --> WW > WB and/or BB > BW.
3. pre-pupal stage determines the fitness --> BB = BW and / or WW = WB.

Finnaly, we try to touch upon the genetic mechanism underlying parasitoid-induced changes in host pupation behaviour. Foraging behaviour is regulated through the foraging pathway, which is largely controlled by the *for* gene. Alleles at the *for* gene induce two behavioural phenotypes, known as 'rovers' and 'sitters'. Rovers move more during foraging and pupate higher compared to sitters (Graf and Sokolowski 1989, Sokolowski 1980). The pleiotropic effects of foraging on pupation behaviour suggest that pupation behaviour is at least partly regulated through the *for* pathway (Sokolowski and Hansell 1983). Thus parasitoids could achieve changes in pupation site by influencing the expression level of the *for* gene which results in rover/sitter phenotypic behaviour. As the first step to test this hypothesis we did a short assay for rover/sitter phenotype behaviour by observing foraging behaviour of parasitized versus unparasitized larvae.

Material and methods

Strain of parasitoids and hosts

Parasitoids and host

We used the five species of *Asobara* described in Table 1. The host used in all experiments was *Drosophila melanogaster* from a laboratory stock that has been maintained in our lab since 1966 at 20918C and 16:8 h day:night regime. This stock is polymorphic for the rover and sitter alleles of the *for* gene.

Table 1. Origin, collection and rearing summary of the *Asobara* species used in this study. All of the parasitoid species have been reared on *D. melanogaster* since collection in our lab at 20918C and 16:8 h L:D.

Parasitoid	Origin	Climate at origin	Collected	Rearing condition in lab
A. citri	Africa, sub-Saharan Africa	Dry & hot	Lamto, Côte d'Ivoire, 1995	20±1ºC&16hL: 8hD
A. persimilis	Australia	Mediterranean hot & dry summer	Sydney, 1997	20±1ºC&16hL: 8hD
A. tabida	Holarctic, Europe & north America	Temperate & wet with cold winter	Leiden, 2006	20±1ºC&16hL: 8hD
A. pleuralis	South-east Asia, Oriental tropic	Tropical wet forest	Manado, Sulawesi Indonesia, 2005	25±1ºC&16hL: 8hD
A. japonica	Japan Tokyo	Temperate & wet	Tokyo, 1995	20±1ºC&16hL: 8hD

Pupation height

To test whether different species of *Asobara* have different effects on host pupation behaviour, we set up an experiment using the five species of *Drosophila* parasitoids (Table 1) and a control group (non-parasitized hosts). Each group consisted of 10-12 replicates. The experiment was performed in small glass tubes (2.2 × 8.0 cm) containing a layer of agar medium (3 ml $vial^{-1}$) and a thin layer of yeast suspension (25 g dry yeast per 100 ml H2O) as food source (0.3 ml $vial^{-1}$). To each tube we added 20 second instar host larvae and two mated female wasps. The control vials were left without parasitoids. Tubes were placed in a climate room at 25ºC, 65% relative humidity and a 16:8 h light:dark regime. These condition resulted in very low parasitation rates for *A. tabida* and we repeated the experiment for this species at 20ºC to provide optimum conditions for *A. tabida* parasitism rate. Furthermore, vials were filled with food medium (recipe: <http: // www.utm.utoronto.ca / ~ w3for age // >), instead of agar medium. Wasps were removed from the treatment vials after 2 h. The vials were then left in the climate room for five days, by which time all hosts had pupated. We measured pupation height as the distance between the surface of the medium and the pupa (Sokolowski and Hansell 1983). We also recorded whether the pupa was parasitized or not. Those that were not parasitized were discarded from the analyses.

Pupal translocation experiment

Translocation experiments were performed to test whether the

choice of pupation site affected fitness of *D. melanogaster*. Forty jars (3.5 × 8.5 cm) containing agar medium and one ml yeast suspension (32 g yeast per 100 ml water) were prepared. To each we added ~ 100 2nd instar host larvae. When the larvae had pupated, 40 pupae were collected from each jar: 20 from the medium on the bottom of the jar and 20 from the side of the jar. These pupae were then placed in new jars (10 pupae per jar) containing standard agar medium. Ten pupae that came from the side were placed back onto the side and ten pupae that came from the side were placed on the medium. Ten pupae from the medium were placed onto the side and ten back on the medium. After emergence, the number of flies per jar was scored. Per treatment group ten jars were prepared and the experiment was performed twice. The jars were kept at 20ºC.

A similar translocation experiment was performed for parasitized larvae. This time, 3 female parasitoids were added to the jars containing ~ 100 2nd instar host larvae. All five parasitoid species were used. Just after pupation, per species, twenty jars were prepared for translocation side (bottom - bottom, bottom - wall, wall - wall, wall - bottom). Jars were placed at 25ºC, apart from the *A. tabida* parasitized group, which was kept at 20ºC. After 4 - 5 weeks we scored the status for each translocated pupae: parasitized or unparasitized, and emerged, dead or in diapauze.

Foraging path length

The pupation height experiment revealed significant differences in pupation height of larvae parasitized by two out of the five species (*A. tabida* and *A. citri*; Results). To test whether these differences could be caused by effects of parasitism on expression of the *for* gene, as the first step to check the expression level of this gene we tested for differences in foraging path length by a short assay for rover / sitter behaviour, which is regulated by the same genetic pathway. As a control, we also measured path lengths of *A. pleuralis* parasitized larvae, where a change in pupation site was not significant in the pupation height experiment. The maximum individual variation in foraging behaviour of *D. melanogaster* larvae occurs at the mid-third instar larval stage (Sokolowski 1980, Sokolowski and Hansell 1983). At this stage the larvae leave a clear trail on the yeast which can be measured. This path length is routinely used as a measure of foraging intensity (Sokolowski 1980, Sokolowski and Hansell 1983). We measured foraging path lengths of parasitized larvae and compared them to those of unparasitized control larvae. In each experiment 30 mid-2nd instar larvae were placed in a jar (3.5 × 8.5 cm) with a layer of agar and 0.3 ml of yeast solution. To the groups in the parasitized treatment, we added two mated female parasitoids for two hours. Five jars per treatment (control vs parasitized) per parasitoid species were prepared for each experiment. Treatment and control jars of *A. citri* and *A. pleuralis* were kept in a climate room at 25ºC, while *A. tabida* and its control jars were reared at 20ºC. The immune reaction of parasitized larvae results in

slightly slower growth rates and thus longer development time compared to control larvae (Wertheim et al. 2005). Therefore we designed a time table in such a way that the control jars for each treatment were started one day after those of the treatment larvae. This created mid-third instar larvae of both parasitized and control groups on the same day.

To measure foraging path length we used plastic petri dishes (Ø 8.5 cm and 1.4 cm high) containing a layer of agar on which we spread 1.5 ml of yeast solution (8.5 g per 25 ml) (Sokolowski 1980). Path lengths of six larvae per jar were measured individually by placing them in the centre of the petri dish and allowing them to forage for five minutes (Sokolowski 1980). The trail track was transferred onto a transparent sheet and its length was measured at a later time. For the parasitized group, larvae were dissected to determine whether they were parasitized or not or had encapsulated the parasitoid larvae. Encapsulation by aggregation of haemocytes around the parasitoid eggs is a common strategy in insects to escape parasitism (Nappi et al. 1975, Rizki and Rizki 1984). Host larvae in the parasitized group that contained no wasp larvae or contained an encapsulated parasitoid egg were excluded from the analyses.

Statistical analyses

Standard linear parametric models were unsuitable for the analysis of pupation height, as this measure is bounded below (the medium) as well as above (the bottom of the stopper sealing the glass vial). We applied a Weibull parametric survival regression analysis in the free statistical software R 2.5.1 to our data (Ihaka and Gentleman 1996, Therneau and Grambsch 2000). Since data distributions were similar in shape to Weiball. We included the random jar effect as frailty in this model.

The results of the translocation experiments were analysed using generalized linear models (GLM) with a quasibinomial distribution in R. For host we estimated probabilities of emergence. We then determined whether translocation side contributed to variation in the number of emerged flies by comparing the full model to one from which translocation had been dropped using a χ^2-test. We record the χ^2-value and its associated p-value. Post-hoc t-tests from this model where used to indicate which of the four translocation sides (BB, BW, WW, WB) differ from each other in emergence rate. The statistical analyses concerning translocation experiments involving parasitized pupae was similar to that of the host experiment. However, three additional tests were added per parasitoid species. In addition to emergence rate, we analysed the number of parasites in diapause, the proportion of pupae surviving (emerged plus diapause) and the number of flies versus parasitoids picked from either the bottom or the wall at the start of the translocation experiment. The latter analyses was thus an independent repeat of the pupation height experiment.

Foraging pathlengths were analysed using generalized linear mixed effect models (LMEM, Pinheiro and Bates 2000), in the R-package nlme.

Treatment (parasitized or control) was entered as a fixed effect while jar was entered as a random effect. The experiments for the three parasitoid species were not performed at the same time, and therefore analysed separately. In all three cases we searched for the minimum adequate model (Crawley 2005) by starting with the maximal model and then dropping the main factor (treatment) if non-significant. Comparing the simpler to the more complex model was done using a likelihood-ratio test. We recorded these likelihood-ratios and their associated p-value.

Results

Pupation height

First we compared pupation heights of *D. melanogaster* larvae parasitized by five different *Asobara* species and those of control larvae. Pupation height of host larvae differed significantly after parasitism by different species (likelihood ratio test, $\chi^2 = 5.16$, DF = 1, $p = 0.005$) (Fig. 1a-f). A post hoc z-test revealed that larvae parasitized by *A. citri* pupated significantly higher than control larvae ($z = 2.52$, $p = 0.01$) and larvae parasitized by *A. tabida* had a non-significant tendency to pupate lower than control larvae ($z = 1.67$, $p = 0.09$) Parameter estimates from this full model also showed that the direction of pupation height different between parasitoid species. We observed some similarities in distribution pattern of pupation site. *A. citri* and *A. persimilis* showed a similar pattern, which differed from *A. tabida*, *A. pleuralis* and *A. japonica*. (Fig. 1a-f). In the replicate experiment of *A. tabida*, performed at a lower temperature, parasitism induced a highly significant reduction in pupation height (Fig. 1g-h, likelihood ratio test, $\chi^2 = 7.34$, DF = 1, $p = 0.006$).

Pupal translocation experiments

Host - emergence (survival) success

Emergence success was significantly influenced by translocation site in the case of unparasitized host pupae (Fig. 2a and 4a which are identical, DF = 3, $\chi^2 = 69.6$, $p < 0.001$). Post-hoc t-tests (see complete overview of t- and p-values for all species in Appendix 1) revealed that wall to wall and wall to bottom transfers resulted in equally high emergence success, which was higher than bottom to bottom or bottom to wall transfers. Bottom to wall transfers resulted in even lower emergence rates compared to its control, the bottom to bottom transfers (Fig. 2a, 4a).

Parasitoids - number of flies versus wasps

In our pupation height experiment we found a significant increase in pupation height for *A. citri* parasitized larvae and a reduction in *A.*

tabida relative to control larvae. Furthermore, a similar distribution pattern to *A. citri* was observed in *A. persimilis* while distribution patterns of *A. pleuralis* and *A. japonica* more resembled those of *A. tabida*. Perhaps the effects of parasitoids on host pupation behaviour are small, but consistently present across experiments. If this is the case, we would find a different number of flies versus parasitoids between the wall and the bottom when picking pupae for the translocation experiment. For *A. citri* and *A. persimilis* we expect to find more parasitoids versus flies on the wall compared to the bottom, and vice versa for *A. tabida*, *pleuralis* and *japonica*. Table 2 shows that these expectations are met, except for *A. citri*, where the number of flies was indeed lower on the wall compared to the bottom, but this difference was not significant. Perhaps this is due to the fact that in that case parasitisation success was very high which results in very low numbers of flies (Table 2).

Parasitoids - emergence success

Emergence success was significantly influenced by translocation site for all but one species, namely *A. pleuralis* (Appendix 1, Table 3, Fig. 2). The optimal site to pupate at in terms of emergence success differed between parasitoid species. For *A. citri* most pupae emerged from the bottom location, regardless of whether this was the original site of pupation (BB) or not (WB, Fig. 2b). For *A. persimilis* the highest emergence rate was obtained if bottom pupae were translocated to the wall (BW, Fig. 2c). *A. tabida* individuals emerged at the highest rate either when relocated back to the wall (WW) or transferred from bottom to wall (BW). Those transferred from the wall to the bottom (WB) performed better compared to those from the bottom to bottom group (BB; Fig. 2d). *A. pleuralis* emergence rate did not depend on translocation site, and was very high at all sites (Fig. 2e). In the case of *A. japonica*, as in *A. citri*, most pupae emerged from the bottom location (BB and WB; Fig. 2f). For this species, a wall to wall (WW) transfer resulted in intermediate emergence success, while a bottom to wall (BW) transfer led to the lowest emergence success.

Parasitoids - diapause induction

Diapause initiation was significantly influenced by translocation in three out of five species, namely *A. citri*, *A. tabida* and *A. japonica* (Appendix 1, Table 3, Fig. 3). A high diapause induction was observed in *A. citri* at the wall position, either for those transferred from bottom or from wall to wall (Fig. 3b). Very rare cases of diapause were observed at the bottom site in this species (BB and WB; Fig. 3b). In contrast to *A. citri* we found a high proportion of wasps in diapause at the bottom for *A. tabida* (Fig. 3d). No cases of diapause were observed for this species at the wall site (WW and BW; Fig. 3d). Diapause in *A. japonica* occurred in both bottom and wall, while the frequency of diapause initiation was higher in the wall treatments (WW and BW; Fig. 3f). A few *A. persimilis*

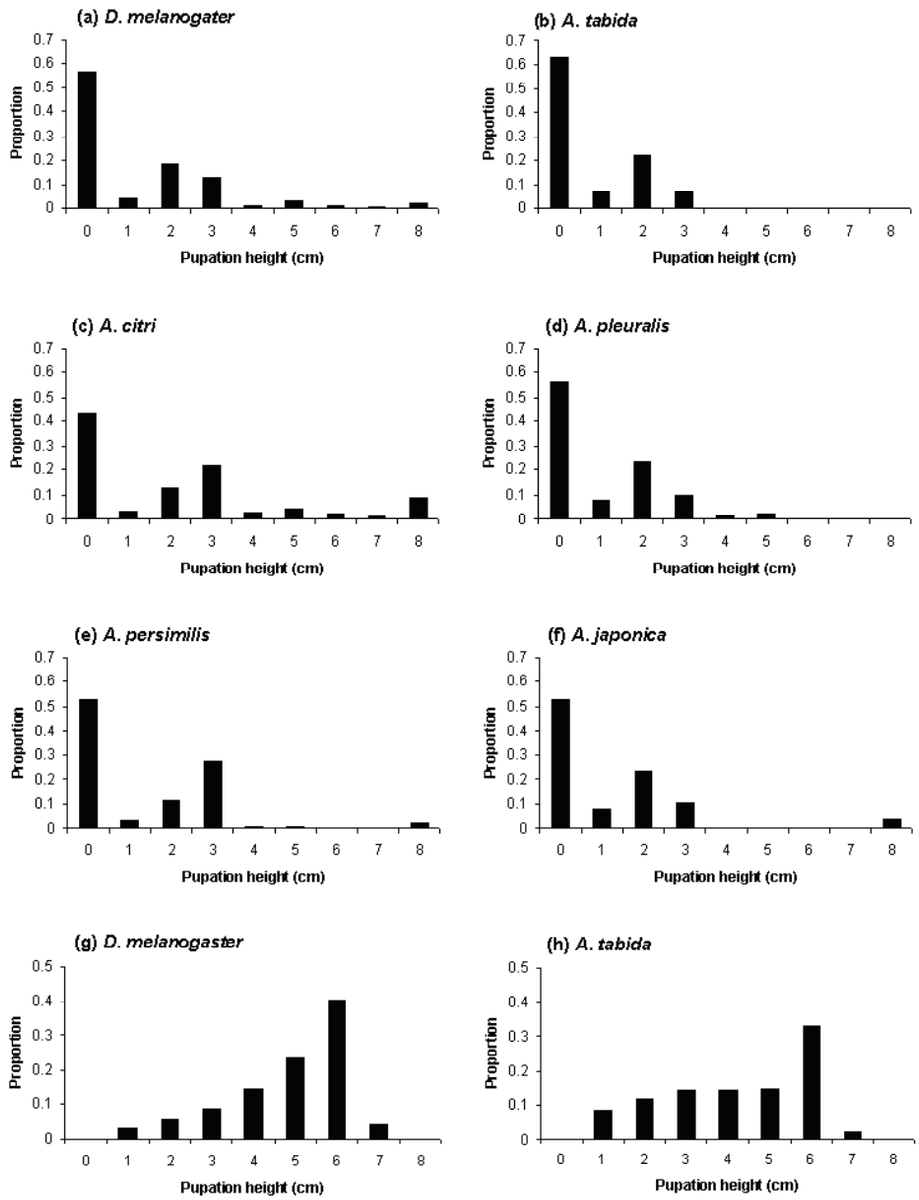

Figure 1. Pupation height variation in relation to parasitism by five species of parasitoid. (a) Pupation height of the host, *D. melanogaster* (control). (b-f) hosts parasitized by different parasitoid species. All were reared at 25ºC on a yeast medium. (g, h) Pupation height of control *D. melanogaster* hosts and hosts parasitized by *A. tabida* in the replicate experiment. This experiment was conducted at 20ºC, and on a yeast / sugar medium in order to increase parasitism rate.

individuals went into diapause. Those that did, mainly came from the bottom to bottom treatment (BB; Fig. 3c). Diapause was not observed for *A. pleuralis (Fig. 3e).*

Parasitoids - survival (emergence - diapause)

Survival was significantly influenced by translocation site for three out of five species, namely *A. citri*, *A. persimilis* and *A. tabida* (Appendix 1, Table 3, Fig. 4). For *A. citri*, a higher number of surviving pupae was found on the wall compared to the bottom (Fig. 4b). This is in contrast to the number emerging, which was highest at the bottom. This difference can be attributed to the fact that a large proportion of the pupae that were transferred from either wall or bottom to wall went into diapause (Fig. 3b). No significant differences in survival were observed for *A. persimilis* (Fig 4c). In contrast to *A. citri*, *A. tabida* individuals mostly went into diapause at the bottom (BB and WB). As a result, survival was similar for wall to wall, wall to bottom and bottom to wall transfers. Bottom to bottom transfers still had significantly lower survival rates (Fig. 4d). For *A. pleuralis* emergence rate was high at all four sites, and no diapause events were observed, thus survival rate does not differ from emergence rate (Fig. 2e and 4e are identical). Diapause in *A. japonica* mostly occurred for the wall treatments (WW and BW), where emergence was relatively low compared to the bottom (Fig. 2f). This led to a very high survival rate for all four translocation treatments which eliminated the transfer effects seen in emergence rate (Fig. 4f).

Foraging path length

In the short assay for rover/sitter phenotypic behaviour the distance travelled while foraging did not differ between parasitized larvae and unparasitized larvae for *A. citri* and *A. pleuralis* (Fig. 5a-b: *A. citri*, likelihood-ratio test; $\chi^2 = 0.09$, $p = 0.76$; *A. pleuralis*, $\chi^2 = 0.04$, $p = 0.84$). Larvae parasitized by *A. tabida* travelled less while foraging compared to unparasitized larvae (Fig. 5c: $\chi^2 = 23.55$, $p < 0.001$).

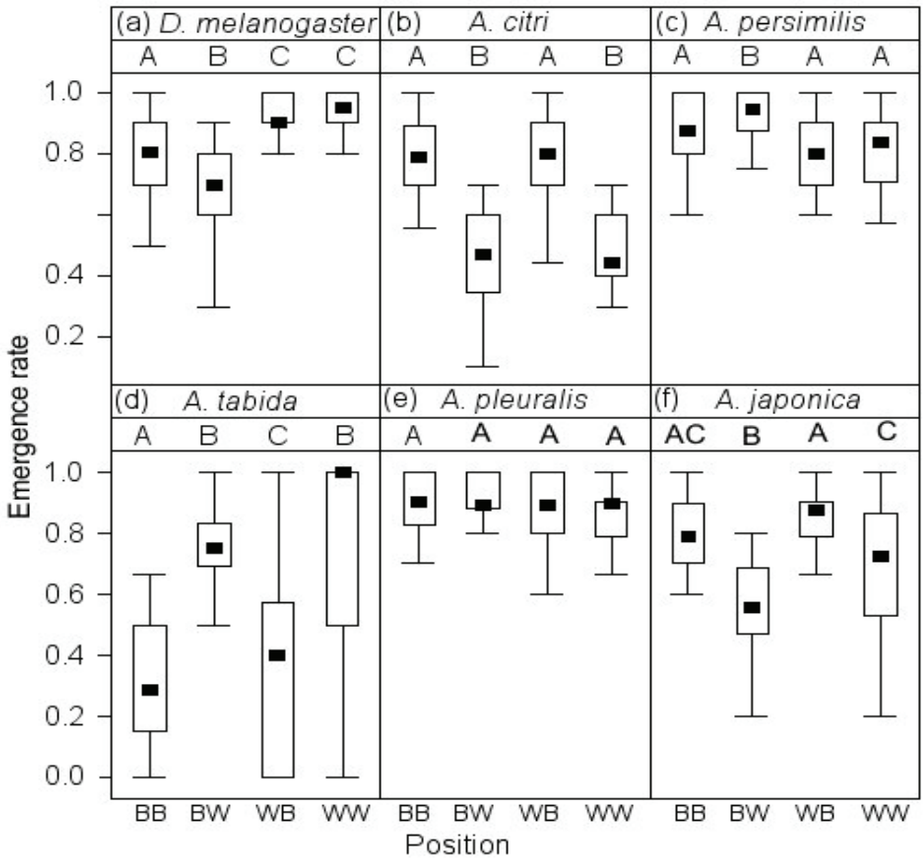

Figure 2. The effect of translocation on the emergence rate for the host and the five parasitoid species. BB-transferred from bottom to bottom, BW-transferred from bottom to wall, WB-transferred from wall to bottom and WW-transferred from wall to wall. Square symbols represent the median of all groups. The box represents 50% of all data points for that treatment (interquartile range). Error bars represent the minimum and maximum observed pupation height, unless outliers are present. When outliers are present the error bar represents 1.5 × the interquartile range. To indicate the results of post-hoc t-tests to indicate which treatments differ within species we labeled each box with a letter. Boxes with identical letters do not differ significantly from each other.

Discussion

The pupation height experiment showed that attack by five species of *Asobara* affected pupation height of *D. melanogaster* larvae in different directions. Pupation height changed significantly after attack by two out of five species of *Asobara*. For one species, *A. tabida*, first only a trend was observed. Due to low parasitism rate at high temperatures, we were not able to firmly state that parasitism changes pupation behaviour for this species. Therefore, for *A. tabida* we repeated the experiment at 20ºC resulting in a significant effect of parasitism on pupation height for this species too. Although at 25ºC the effect of *A. tabida* was not significant, the direction of induced change remained consistent with the change observed at 20ºC (Fig. 1b, 1h). *A. tabida* significantly reduced pupation height while *A. citri* increased pupation height. Further evidence for pupal site changes in response to parasitoid attack was found by collecting pupae from the wall and bottom at the start of the translocation experiment. Relatively more parasitoids compared to host pupae were found at the wall for *A. persimilis*, while for *A. tabida*, *A. pleuralis* and *A. japonica*, more parasitoids were found at the bottom. Pupal translocation experiments of the host showed that optimum pupation site differs between individuals. Overall, individuals pupating on the wall survived better than those pupating on the bottom. However, bottom individuals did worse when translocated to the wall. Similar translocation experiments were performed for parasitized larvae to investigate whether the change in pupation behaviour is adaptive for the parasitoid under our laboratory conditions. The results of the translocation experiments are summarized in Table 4. Our results showed that diapause was induced at the preferred site for both *A. citri* and *A. tabida*, the species that showed significant change in pupation height. In terms of survival, the result obtained for *A. citri* was consistent with an adaptive explanation (Table 4). Hosts attacked by *A. citri* tended to pupate on the wall and transferring pupae to that location increased their survival rate. In contrast to our expectation, survival of *A. tabida* decreased at the selected pupation site. The analysis of foraging path lengths showed a significant reduction in foraging activity of larvae parasitized by *A. tabida*. As both foraging path length and pupation height are to are large extent regulated by the *for* gene, this indicates that *A. tabida* may reduce the expression of this gene.

Pupation site in unparasitized *Drosophila* larvae is known to depend on moisture, temperature, density, phenotype / genotype (rover vs sitter) and species (Sameoto and Miller 1968, Barker 1971, Sokolowski et al. 1986, Schnebel and Grossfield 1992). The decision of where to pupate seems to be taken during the wandering stage which occurs a few hours before pupation (Riedl et al. 2007). During this stage, larvae move away from the food source and return to the food source many

Table 2. The average number of fly pupae ± SE collected from the wall or bottom at the start of the pupal translocation experiment (n = 80 pupae per species). The p- and χ^2 - values are the results of GLM models that test whether the ratio of fly to wasp pupae differ between wall and bottom.

	Average nr. of flies (out of 10)		
	Wall	Bottom	P-value (χ^2)
A. citri	0.28 ± 0.09	0.43 ± 0.13	0.41 (0.8)
A. persimilis	0.23 ± 0.08	0.83 ± 0.15	< 0.001 (15.3)
A. tabida	7.03 ± 0.34	3.45 ± 0.29	<0.001 (104.8)
A. pleuralis	1.30 ± 0.19	0.80 ± 0.15	0.04 (5.4)
A. japonica	1.28 ± 0.30	0.50 ± 0.14	0.012 (15.3)

Table 3. The effect of pupal translocation on emergence, survival and diapause rates.

	Emergence success		Survival success		Diapause induction	
	χ^2	P-value	χ^2	P-value	χ^2	P-value
A. citri	81.5	<0.001	30.4	<0.001	259.0	<0.001
A. persimilis	16.5	0.001	17.3	0.001	4.67	n.s
A. tabida	74.4	<0.001	26.4	<0.001	53.2	<0.001
A. pleuralis	2.7	n.s.	2.7	n.s.	0 .0	n.s
A. japonica	40.3	<0.001	4.2	n.s.	49.5	<0.001

times, as if testing what the conditions are like, before they choose a site to pupate. Although we know that pupation site varies with both abiotic and biotic factors, experimental evidence to show that pupal site selection increases survival of the fruit fly is limited. Sokolowksi et al. (1986) showed that adult emergence in *D. melanogaster* of larvae that pupated on fruit decreased as soil water content increased. In contrast, those pupated on the soil had lower adult emergence when soil water content decreased. We observed an overall better survival for unparasitized hosts pupated on the wall. Perhaps this can be explained by fast growing, fit, larvae being able to reach the wall, filling up this space, while the weak, slow growing larvae are forced (or not able to climb) to pupate at the bottom. Inconsistence with this explanation is the fact that survival of sitter phenotypes is reduced when moved to a rover phenotype pupation site. Thus our experiment partly supports the hypothesis that one phenotype (rover) is fitter over another phenotype (sitter). However, the fact that optimal pupation sites differ between individuals within the same population, also indicates a role for individual pupation site selection.

Finding a free enemy site for pupation could for both the host, as well as the parasitoid, be a reason for pupation site selection. Ohsaki and

Sato (1994) showed that an oligophagous butterfly, *Pieris rapae*, chose a suboptimal food resource host-plant for egg laying, which was less attractive for parasitoids. This increased its survival but came at the cost of losing component of fitness in other traits. Furthermore, parasitoids face the treat of being parasitized by hyperparasitoids. To avoid this, they can manipulate their host behaviour, for example by leaving the food patch or nest, in search for an enemy free space, in order to enhance its own survival (Stamp 1981, Fritz 1982, Brodeur and McNeil 1992, Grosman et al. 2008, Harvey et al. 2008).

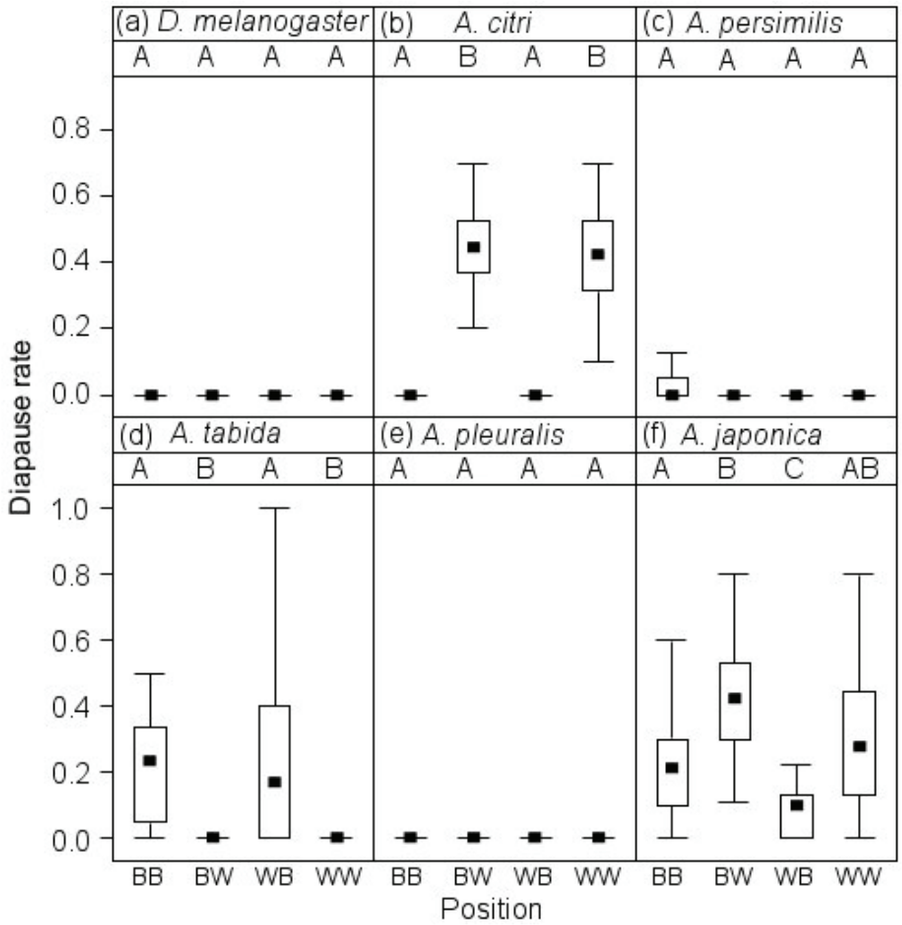

Figure 3. The effect of translocation on diapause rate for the host in the five parasitoid species. BB-transferred from bottom to bottom, BW-transferred from bottom to wall, WB-transferred from wall to bottom and WW-transferred from wall to wall. See Fig. 2 for box-plot details.

Our results suggested that hosts pupate at higher locations in the jar (where conditions are drier) when parasitized by *A. citri* and *A. persi-*

milis. Both these species inhabit arid environments (Table 1), suggesting that their survival is highest in dry conditions. Our translocation experiments support this conclusion for *A. citri*, but found no significant effect in *A. persimilis*. The three remaining species (*A. japonica*, *A. pleuralis* and *A. tabida*) are all found in relatively humid environments. Hosts parasitized by these species pupated on or near the medium in relatively moist conditions. In the field, the optimal pupation site should minimize the risk of desiccation and microbial infection (Alphen and Thunissen 1982). As mentioned before, another likely mechanism is that the behaviour change may be to reduce hyper or superparasitism rate. Apart perhaps from desiccation, none of these risks were present in our experiments. Thus, it could be that our lab stocks of these parasitoids have retained the ability to change host pupation behaviour in the absence of the other selective forces favouring these traits. To address this we need more field experiments for each species at their site of origin.

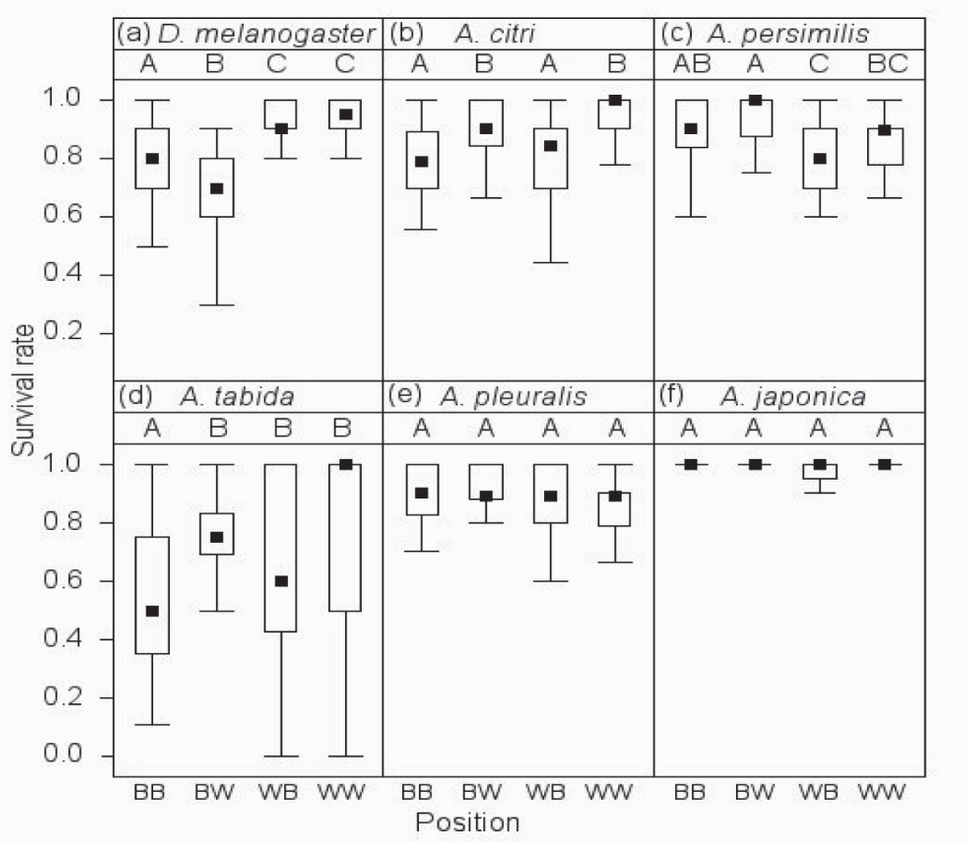

Figure 4. The effect of translocation on the survival rate (emergence plus diapause) for the host and the five parasitoid species. BB-transferred from bottom to bottom, BW transferred from bottom to wall, WB-transferred from wall to bottom and WW-transferred from wall to wall. See Fig. 2 for box-plot information.

Unexpectedly, we found that pupation site affected the probability of entering diapause in three out of five species of parasitoids. Such conditional diapause has not previously been demonstrated in Drosophila parasitoids. Diapause in insects can be induced by cold, heat and desiccation stress (Danks 1987, Denliger 2007) and is generally viewed as an adaptation to survive periods of unfavourable conditions (Masaki 2002). In *A. japonica*, diapause occurs at the wall, and this may be seen as a stress response to unfavourable conditions at this site. In contrast, in *A. citri* and *A. tabida*, diapause was most frequent at the site where the hosts tended to pupate after parasitism (wall and bottom, respectively). Perhaps natural selection favours high rates of diapause in these species, for example because successful reproduction is unlikely after normal development time. Suitable humid conditions may be short-lived in the arid environments inhabited by *A. citri*, while the habitat of *A. tabida* is strongly seasonal.

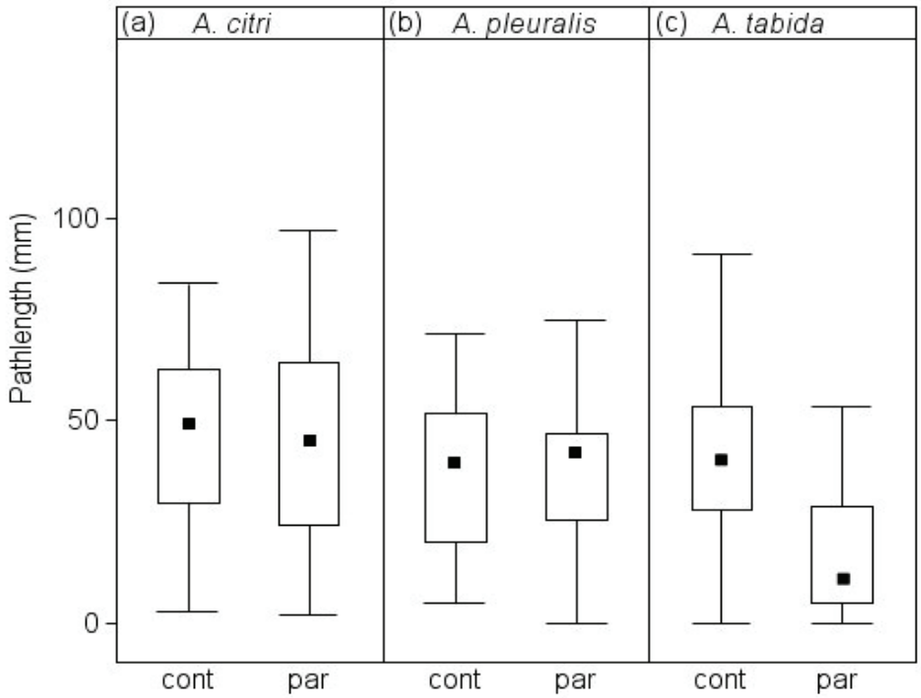

Figure 5. Foraging path lengths (distance travelled) of D. melanogaster larvae parasitized by three species of parasitoids. Cont-unparasitized control larvae, par-parasitized larvae. See Fig. 2 for boxplot information.

If diapause in our lab stocks is a genetic relic from their natural past, it is remarkable that it has been retained. Some of these species have been in culture in our lab for 13 years. During this time we certainly

selected against diapausing individuals. After a maximum of four weeks, when all non-diapausing individuals have emerged, rearing jars are discarded, along with any diapausing individuals. The genetic basis of adult diapause induction is relatively well understood in *Drosophila melanogaster*. An allele of the gene timeless known to induce diapause has recently spread through *D. melanogaster* populations in Europe, where diapause enhances winter survival (Tauber et al. 2007). This example shows that diapause alleles can become fixed in a population rapidly.

A potential mechanism behind the change in host pupation behaviour in response to parasitoid attack is a parasitoid-induced change in the expression of the *for* gene. This gene regulates foraging behaviour in the host, *D. melanogaster* (Sokolowski 1985). Rover phenotypes move more during foraging and pupate further from the food source than sitter phenotypes (Sokolowski and Hansell 1983, Graf and Sokolowski 1989). *A. citri* could simply alter their host's pupation site by stimulating for expression, while *A. tabida* could achieve pupation close to the food source by decreasing for expression For *A. tabida* it is known that alongside the egg, also venom is injected. This venom induces a reduction in behavioural activity of the larvae for the first 24 h after parasitism (Moreau et al. 2002). For *A. citri*, such venom injection is not known to occur (Prevost et al. 2005). Thus, perhaps this venom reduces activity in *A. tabida* right up until the time to pupation. To test this hypothesis, we measured path lengths of foraging *D. melanogaster* larvae that were parasitized by *A. citri, A. tabida* or *A. pleuralis* (as a control). Our results revealed no correlation between pupation and foraging behaviour by *A. citri* and *A. pleuralis* and hence *for* was not responsible for the change in pupation behaviour observed in *A. citri*. Many genes besides *for* contribute to the determination of pupation site (Riedl et al. 2007). Our result indicates that the change in pupation behaviour after attack by *A. citri* is regulated via genes other than foraging related genes. In contrast, we found a significant reduction in foraging path length of larvae parasitized by *A. tabida* compared to unparasitized control larvae. Therefore, the result for *A. tabida* is consistent with our hypothesis that parasitoids can alter host behaviour by altering the expression of the *for* gene. In order to confirm this observation, a more detailed study of host gene expression of the *for* gene and genes in this pathway in response to parasitism is underway. Checking the expression level of the other candidate genes for pupation height in the parasitized larvae by *A. citri* may provide evidence of other host gene expression manipulation in this species.

Table 4. Interpretation of the pupal translocation experiment involving parasitized larvae. The pupation manipulation column represent the direction of change in response to parasitism as indicated by the pupation height and translocation experiments (for the latter: the ratio of flies to wasps picked from the wall and bottom at the start of the experiment). In this column 2 represents strong evidence (supported by both pupation and translocation experiment) and 1 represents weak evidence (support from either pupation height or translocation experiment only). The consequence of this effect of parasitism for survival and diapause induction is shown as adaptive, maladaptive or neutral (no effect). For this assessment we assume that being able to enter the diapause stage is a positive trait, enabling parasitoids to survive for a longer period. We indicate which of the following prediction are met: 1) there is a site difference, WW-BB or BB-WW, 2) individuals chose its own optimal site, WW-WB and/or BB-BW, 3) pre-pupal stages determine success, BB-BW and/or WW-WB.

			Emergence			Survival			Diapause		
	Original condition	Effect of parasitism on pupation site	Highest	Consequence	Prediction	Highest	Consequence	Prediction	Highest	Consequence	Prediction
D. mel	Wet/Cool	--	Bottom and wall	--	1, 2 and 3	Bottom and wall	--	1, 2 and 3	Not present	--	--
A. citri	Dry /Hot	To wall (1)	Bottom	Maladaptive	1	Wall	**Adaptive**	1	Wall	**Adaptive**	1
A. persimilis	Dry/Hot	To wall (1)	Wall (BW only)	No effect	?	Bottom and wall	No effect	3	Few events	No effect	--
A. tabida	Wet/Cool	To bottom (2)	Wall	Maladaptive	1	Wall	Maladaptive	1, 3	Bottom	**Adaptive**	1
A. pleuralis	Wet/Hot	To bottom (1)	No effect	No effect	--	Bottom and wall	No effect	--	Not present	No effect	--
A. japonica	Wet/Cool	To bottom (1)	Bottom	**Adaptive**	1	Bottom and wall	No effect	--	Wall	Mal-adaptive	1

In conclusion, we have shown that *D. melanogaster* larvae alter their pupation behaviour after attack by *Asobara* parasitoids. The patterns are species-specific and may relate to the environmental conditions under which these species live in nature. While we show that individual pupation-site selection by *D. melanogaster* larvae increases survival, we were not able to show that parasitoid-induced changes were consistently adaptive. Last, the mechanistic processes underlying the change in pupation behaviour appear to differ between species. While we are currently still a long way removed from a coherent explanation for these patterns, their study will certainly enhance our understanding of the way in which parasites influence their host's behaviours.

Acknowledgements

We would like to thank Prof. M. B. Sokolowski and Dr K. D. Williams from the Univ. of Toronto at Mississauga, Canada, for their support in terms of lab use and valuable comments related to pupation height experiments.

Chapter 3

A lack of correlation between developmental time and adult life span in parasitoids: the role of metabolic rate and fat reserves

Abstract

Developmental time and body size correlate with lifespan in a wide range of taxa, but not in insect parasitoids. To explain this, we suggest that interspecific variation in intrinsic adult metabolic rates and differences in allocation of lipids to longevity and reproduction result in variation of adult lifespan, independent of development time. When the rate of development is independent of adult metabolic rate, adult lifespan is free to adapt to the adult environment. To test these ideas, we measured metabolic rate, lipid content and egg load at eclosion, developmental time, and lifespan of females with and without carbohydrate food in five species of *Asobara,* parasitoids of *Drosophila*. No relation between development time and adult longevity was found. As predicted, metabolic rates varied between species and appeared to trade off with adult longevity. We found no clear link between initial egg load and the longevity of a species, suggesting that lipid allocation may be less important in determining adult lifespan. Our results indicate that differences in metabolic rate have an important effect on adult lifespan, without affecting developmental rate in parasitoids.

Introduction

Quantifying traits affecting the fitness of organisms is one of the greatest challenges of evolutionary biology (Harvey 2005). Among these traits, special attention has been paid to those related to lifetime reproductive success as it seems the closest approximation to total fitness (Stearns 1989; Godfray 1994; Roff 1992). The transition from juvenile to adult is a crucial step in the life history of most organisms. This transition can be characterized either by the age or size of the organisms at which this transition occurs. Age at maturity influences lifetime reproductive

success and fecundity increases exponentially with body size in a wide range of animals (Stearns1992). Both body size and age at maturity have been predicted to correlate positively with lifespan (Stearns 1992). Comparative studies have confirmed such relationships in a broad range of taxa for age (Stearns & Crandel 1981; Charnov & Berrigan 1990; de Magalhaes et al. 2007) and for size (Roff 1992; Purvis and Harvey 1995). However, a comparative study of hymenopteran parasitoids revealed that the correlations between developmental time with either body size or adult life span are absent in this group (Blackburn 1991). Parasitoids thus appear to be an exception to the general rule. Eijs and van Alphen (1999) studied whether this lack of correlation could be explained by constraints imposed by the host. They measured developmental time, life span and body size in five species of *Leptopilina*, larval parasitoids of *Drosophila*, reared on different host species. Consistent with aforementioned studies they found no correlation between life span and developmental time of parasitoids. These traits also did not correlate with host developmental time. They suggested two mechanisms for this lack of relationship. First, stochasticity of the environment could select for delayed emergence and thus slower development. Second, developmental rate could be host specific. Here, we suggest a third hypothesis, i.e. that species differ in adult metabolic rate and that this causes variation in adult lifespan independent of developmental rate.

Parasitoids rely on resources obtained from a single host for their entire development. At least part of these resources must also be allocated to reproductive output and adult survival. Since solitary species often grow only marginally smaller than their host, host size may limit parasitoid body size (Sequeira and Mackauer 1992, Harvey and Strand 2002, Harvey et al. 2004). Adult parasitoids are further constrained by their lack of lipogenesis (Ellers 1996; Olson et al. 2000; Rivero and West 2002; Giron and Casas 2003; Lee et al. 2004; Visser and Ellers 2008). As a consequence, parasitoids should be strongly selected for optimal uptake and allocation of resources (Slansky 1986; Sequeira and Mackauer 1993; Jervis, et al.2008). Harvey (2005) argued that mortality risk at the developmental stage should influence the developmental strategy of parasitoids. Parasitoids in environments with high risk of larval mortality would be selected for higher growth rates and shorter developmental times compared to parasitoids in low-risk environments. Likewise, parasitoids in growing populations are under selection for faster development and shorter generation times. However, this would come at a cost to either lifespan or reproduction. Efficient uptake during the short developmental time may help to reduce this cost. Also, availability of resources rich in carbohydrate - which parasitoids can utilize as adults - may compensate for effects of faster development on longevity in nature.

Metabolic rate may influence life span in parasitoids. Metabolic

rate represents the rate of resource consumption by organisms. Fast consumption of any essential resource may result in shortened lifespan if it is not replenished from their diet. Another reason for a shorter adult lifespan in organisms with high metabolic rate is provided by Harman's (1956) theory of the role of reactive oxygen species (ROS) in ageing. As the result of respiration, free radicals are generated in the mitochondria of organisms. The ROS destroy macromolecules and so contribute to aging. Thus higher metabolic rate in parasitoids may result in shorter life span by either exhausting lipid supplies or as a side effect of ROS.

Selection in different environments could have resulted in adaptive differences in resource allocation between related parasitoid species and explain why congeneric parasitoids developing on the same host and receiving the same amount of resources, exhibit enormous variation in life-history traits. Here we test the hypothesis that variation in adult metabolic rate and in lipid allocation affect lifespan in parasitoids independently from developmental rate. We predict that (1) interspecific variation in intrinsic adult metabolic rates and (2) interspecific differences in the allocation of lipid resources to longevity and reproduction result in interspecific variation of adult lifespan.

We also suggest that variation in developmental rate in parasitoids is adaptive and uncoupled from metabolic rate in adults. We measured metabolic rate, lipid reserves, weight, egg load, developmental time and life span (with and without carbohydrate resource) of newly emerged females of five closely related species of *Asobara*, parasitoids of *Drosophila* larvae. Theses species originated from different environments, but were cultured on a common host (*Drosophila melanogaster*) under standardized laboratory conditions.

Material and methods

Parasitoids and host

Five species of *Drosophila* parasitoids of the genus *Asobara* (Ichneumonoidea: Braconidae) were used in this study (Table 1). The species originated from different climatic and geographic regions in the world. All are koinobiont endoparasitoids, which parasitize their host by laying a single egg into the host body. The larvae allow the host to reach pupation before they kill it. Parasitoids were cultured at a 20 ± 1ºC and 16:8 h day/night regime on a laboratory strain of *Drosophila melanogaster* (WW), that has been maintained in the laboratory since 1966 at 25±1ºC and 16/8 h day/night regime.

Table1. Origin, climate at origin and collection summary of the *Asobara* species used in this study. All of the parasitoid species have been reared on *D. melanogaster* since collection in our lab at 20±1ºC&16hL: 8hD.

Parasitoid	Origin	Climate at origin	Collected
A. citri	Africa, sub-saharan Africa	Dry & hot	Lamto, Côte, d'Ivoire, 1995
A. persimilis	Australia	Mediterranean hot & dry summer	Sydney, 1997
A. tabida	Holarctic, Europe& north America	Temperate & wet with cold winter	Leiden, 2006
A. pleuralis	South-east Asia, Oriental tropic	Tropical wet forest	Manado, Sulawesi Indonesia, 2005
A. japonica	Japan Tokyo	Temperate & wet	Tokyo, 1995

Test for phylogenetic independence

The phylogeny of the species was constructed by combining molecular data from three mitochondrial markers, COI, ND1 and 16S and identifying the most parsimonious tree using PAUP* 4.0-test version 4.0d63 (Swofford 1998). For further details on data collection for fat reserve, egg load and phylogeny see chapter 4.

To test whether closely related species resemble each other more closely than distantly related species in the traits under investigation we used a test for serial independence (TFSI) as implemented in the software Phylogenetic Independence version 2.0 (Reeve and Abouheif 2003). The average value for each trait was calculated for each species and placed at the tips of the phylogenetic tree (Abouheif 1999). These values were then randomly reshuffled 1000 times. The observed topology of trait values was compared to the distribution of randomly generated topologies. We report the P-value representing the chance that the observed topology could have arisen by chance, i.e. in the absence of phylogenetic autocorrelation.

Developmental time (immature life span)

Immature life span (including embryonic, larval and pupal period) of each individual was measured as the time from parasitism of the host to the emergence of the wasp. This experiment was performed in glass vials (3.5×8.5 cm) which contained an agar bottom with a layer of one ml yeast suspension (32 g yeast per 100 ml water) as food source on top. To each vial we added 100 2nd instar host larvae and 3 mated adult female wasps. After two hours the parasitoids were removed and the vials kept in the climate room at 25 ºC, 65 % relative humidity and a 16:8h light:dark regime. The vials were checked twice daily for emerging wasps over the next five weeks. The experiment was carried out with 10

replicates and repeated once, giving a total of 20 replicates per species.

Longevity (adult life span) with and without food

Longevity was measured for 80 newly emerged virgin female wasps per species in each treatment. The wasps were kept in groups of four in small glass tubes (2.2×8.0 cm). To avoid desiccation, a layer of agar was added to the bottom of each tube. The 20 tubes per species were divided in two groups. A drop of honey was added as a food source to the underside of the sponge lid of 10 of the tubes, while the other 10 tubes were left without honey. The vials were checked for dead wasps twice per day for the next five weeks. This experiment was carried out in a climate room at 25 ºC, 65 % relative humidity and a 16:8h light:dark regime.

Metabolic rate

For each replicate, virgin female wasps were assayed for CO2 production at 25ºC. The wasps were harvested within five hours after eclosion, isolated and kept at 4ºC. The day after emergence the wasps were anesthetized on ice and transferred to cylindrical glass tubes. The wasps were then assayed in a 16-channel respirometer (Li-Cor 6251 infrared CO2 detector; Sable System International, www.sablesys.com). As the amount of CO2 produced by a single wasp was too low to be detected by this respirometer, the wasps were pooled in groups of 20 individuals per container. To avoid interaction of the wasps, which may cause increased activity and consequently higher metabolic rate, the experiment was carried out in complete darkness. To reduce the effect of time of day, we performed the experiment according to a time schedule in which we measured three replicates of each species simultaneously twice per day. All of the measurements were performed in the mornings. In total, the experiment consisted of 24 replicates per species and lasted 4 days. The data were collected with ExpeData™ software (Sable System International, www.sablesys.com) and exported to an excel data sheet for further analysis.

Fat reserve and egg load

Egg load and fat content were measured on virgin female wasps harvested within five hours of eclosion. For each species 35 individuals were randomly chosen from 15 rearing jars, isolated and stored at -80ºC until further analysis. To measure egg load wasps were dissected and ovarioles opened in a drop of demineralized water. The number of eggs was counted on a photograph of whole egg batches made under a stereo-microscope. Fat reserve was measured by ether extraction (David et al. 1975, Ellers 1996).

Statistic analysis

Developmental time and longevity of females of each species were analyzed by applying a Weibull parametric survival regression in the free statistical software R 2.5.1 (Ihaka & Gentleman 1996; Therneau & Grambsch 2000). We included the random jar effect as frailty in this model. The results of the metabolic rate and fat reserves were compared between species using analysis of covariance (ANCOVA) in which dry weight was added as covariate to the models to represent body size. In the analysis of metabolic rate we first log transformed metabolic rate (the amount of CO2 produced by the wasps per hour). In each case we searched for the minimum adequate model by starting with the maximal model, then dropping the interaction term and then main factors from the model if non-significant.

Results

Phylogenetic independence

None of the traits under investigation showed significant phylogenetic autocorrelation (tests for serial independence: developmental time $P = 0.32$; life span with food $P = 0.09$; life span without food $P = 0.17$; fat content $P = 0.19$; egg load $P = 0.09$, metabolic rate $P = 0.15$). We therefore did not correct for phylogenetic effects.

Developmental time

Developmental time differed significantly among the five species of *Asobara* (Likelihood ratio test, $\chi^2 = 3254.0$, d.f. = 4, $P < 0.001$). Post-hoc z-test revealed that developmental time was shortest in *A. pleuralis* and significantly different from all other species. *A. tabida* had the longest developmental time (Fig 1, Table 2). *A. citri* also had a long developmental time, although significantly shorter than that of *A. tabida* (Fig.1, table 2, Appendix 2). *A. persimilis* showed intermediate developmental time significantly different from the other species except from *A. japonica* (Appendix 2).

Table2. Summary statistics (mean ± SE) of developmental time, longevity (with and without food), egg load (number of egg / wasp), residuals of the regressions of metabolic rate (log transformed) and lipid reserve against dry weight in five species of *Asobara*. All measurements were conducted at 25 ±1ºC and 60% RH in 16hL: 8hD except metabolic rate, which was measured under continual darkness.

Species	Developmental time / day	Longevity with food / day	Longevity with-out food day	Egg load (no. egg / Wasp)	Residuals of metabolic rate	Residuals of lipid reserve
A. tabida	21.58 ± 0.0568	20.63 ± 1.0709	7.60 ± 0.2991	127.32 ± 3.3063	-0.06724 ± 0.01271	0.00267 ± 0.00108
A. citri	19.33 ± 0.0659	18.60 ± 1.1173	10.42 ± 0.4722	144.90 ± 4.2304	-0.01741 ± 0.00756	0.00325 ± 0.00078
A. pleuralis	13.33 ± 0.0394	17.00 ± 0.8086	12.53 ± 0.4326	101.77 ± 4.3697	-0.07542 ± 0.00626	-0.00126 ± 0.00100
A. persimilis	16.18 ± 0.0612	9.58 ± 0.4471	4.70 ± 0.3063	177.97 ± 5.9298	0.01823 ± 0.00617	-0.00025 ± 0.00060
A. japonica	15.98 ± 0.0604	4.55 ± 0.2055	3.45 ± 0.1129	72.87 ± 3.6281	0.14183 ± 0.00714	-0.00614 ± 0.00159

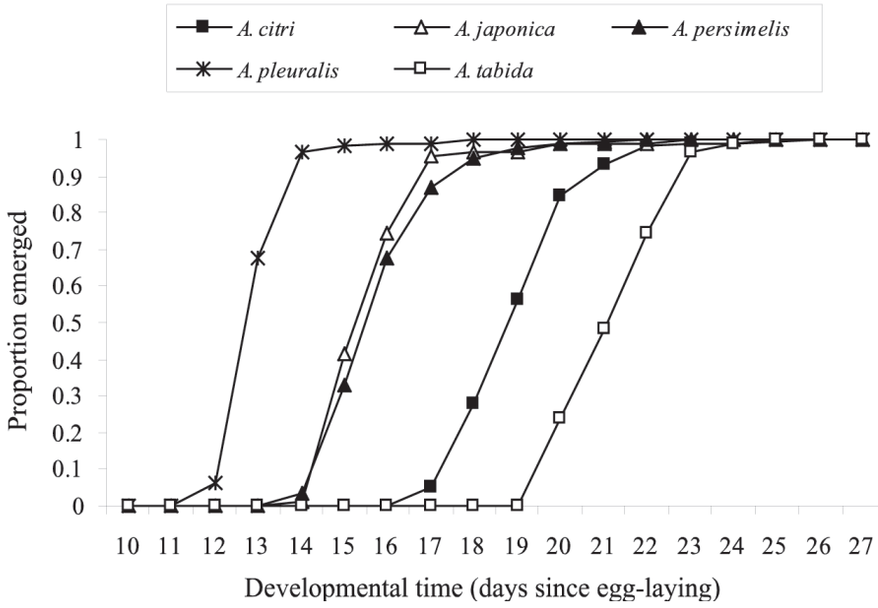

Fig.1: Developmental time in days of the five species of *Asobara* at 25ºC and 65 % relative humidity and a 16:8h light:dark regime.

Adult longevity with food

Longevity of the adult virgin female wasps in the presence of food showed significant variation among the species of *Asobara* (Likelihood ratio test, $\chi^2 = 254.98$, d.f. = 4, $P < 0.001$). Post-hoc z-test revealed that lifespan was longest for *A. tabida* and *A. citri* with no significant difference between these two species (Fig.2, Table 2, Appendix 2). The other three species differed significantly in lifespan from all others. From long to short the species ranked as follows: *A. pleuralis*, *A. persimilis* and *A. japonica* (Fig. 2, table 2, Appendix 2).

Adult longevity without food

Longevity of the adult virgin female wasps in the absence of food was significantly lower than in the presence of food (Likelihood ratio test, $\chi^2 = 299.96$, d.f. = 1, $P < 0.001$). Differences in longevity between the species remained significant (Likelihood ratio test, $\chi^2 = 266.44$, d.f. = 4, $P < 0.001$), but the pattern differed drastically for some species. The highest longevity was observed for *A. pleuralis* (Fig.3 , table 2). A post-hoc z-test revealed that longevity of this species was significantly greater than for all other species. Longevity of *A. tabida* and *A. citri*, which showed the longest survival time in the presence of food, dramatically decreased

when adults were kept without food. The decrease in longevity of *A. tabida* was greater than for *A. citri.* (Fig.3, table 2, Appendix 2). The remaining two species showed the same order for longevity without food as in the presence of food (Appendix 2).

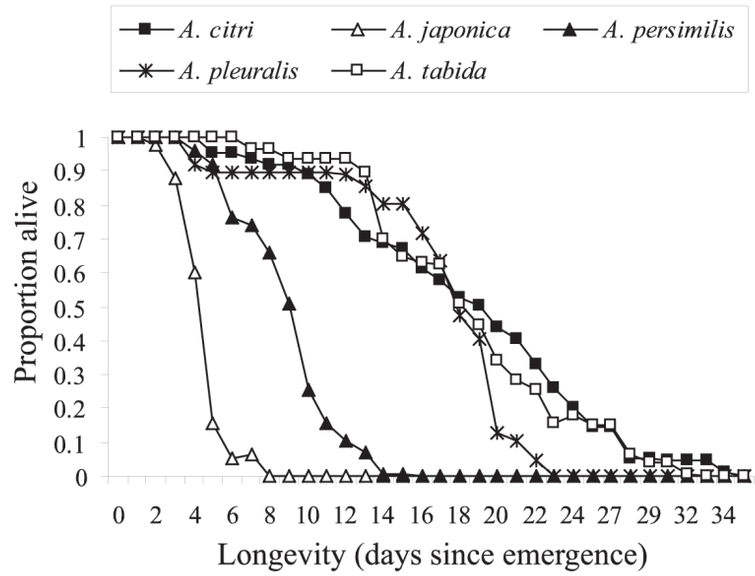

Fig.2: Longevity of adult female wasps with food (carbohydrate resource) in the five species of *Asobara* at 25ºC and 65 % relative humidity and a 16:8h light:dark regime.

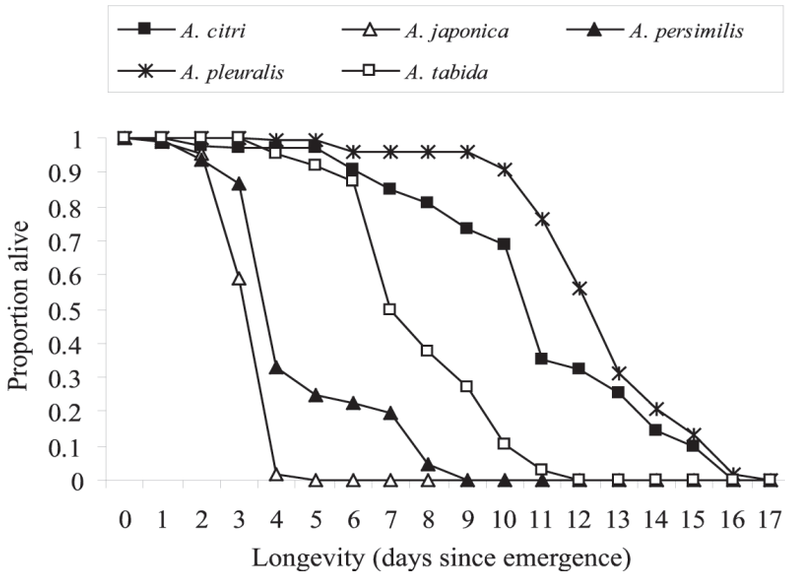

Fig.3: Longevity of adult female wasps without food (carbohydrate resource) in the five species of *Asobara* at 25ºC and 65 % relative humidity and a 16:8h light:dark regime.

Metabolic rate

We observed significant differences in metabolic rate between species ($F_{4,114}$ = 191.06, P< 0.001). Metabolic rate showed a significant allometric relationship with body size (log-transformed data $F_{1,110}$ = 90.63, P< 0.001). There was no significant interaction between body size and species, indicating that the slopes of the allometric scaling relations were consistent between species ($F_{4,110}$ = 1.69, P = 0.157). Post-hoc t-tests revealed the highest metabolic rate for *A. japonica*, which was significantly higher that all of the other species (Appendix 2). The other species ranked as follows: *A. persimilis, A. citri, A. tabida, A. pleuralis*, but the differences between the last two species were only marginally significant (Fig. 4, Appendix 2).

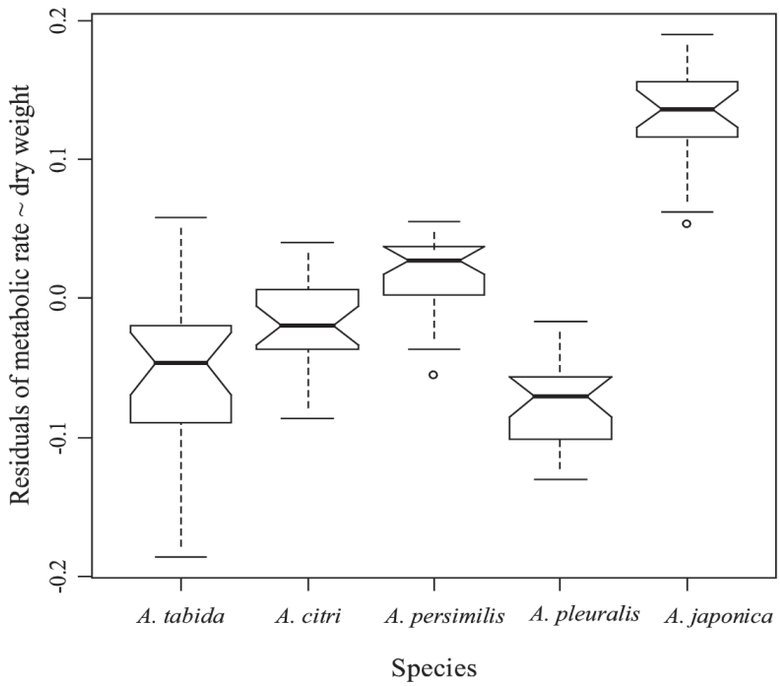

Fig.4: Variation in metabolic rate in five species of *Asobara,* values are residuals of the regression of metabolic rate against dry weight at eclosion, both dry weight and metabolic rate data are log transformed.

Fat reserve

Fat reserves differed significantly between the species (Fig.5, $F_{4,173}$ = 39.25, P< 0.001) and scaled with body size ($F_{1,173}$ = 351.65, P< 0.001). The interaction term was not significant, indicating that the slopes of the relation between fat content and body size were similar among species

($F_{1,173}$ = 1.11, P = 0.35). Post-hoc t-tests revealed the following ranking of the five species: *A. tabida/A. citri, A. persimilis, A. pleuralis, A. japonica* (Fig.5, Appendix 2).

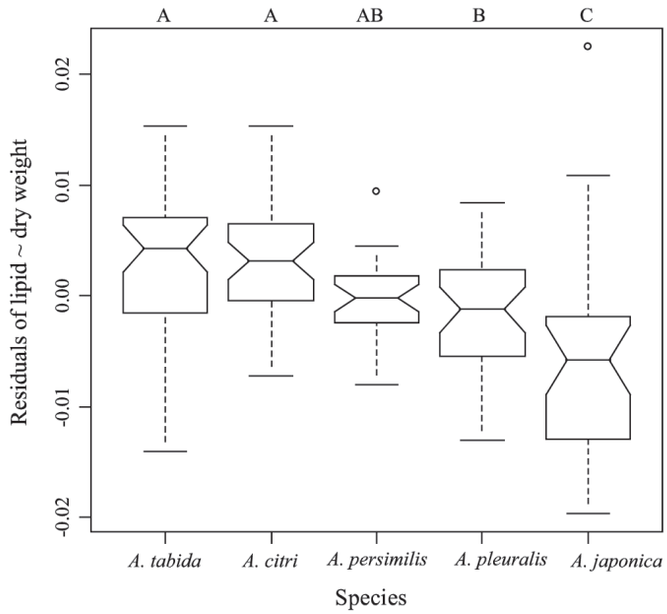

Fig.5: Variation in lipid reserve in five species of *Asobara*. Values are residuals of the regression of lipid reserve against dry weight at eclosion.

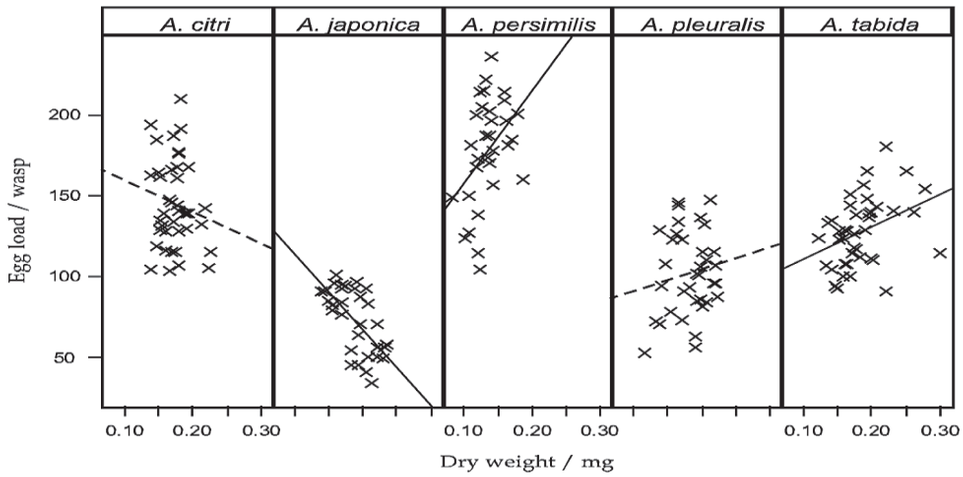

Fig. 6: Correlation between body size (dry weight) and egg load at eclosion (number of egg / wasp) in five species of Asobara, in dashed lines correlation are not significant.

Egg load

ANCOVA including body size as covariate indicated significant differences in the slope of the relationship between body size and egg load among the species (Fig.6, $F_{4,169} = 5.29$, $P < 0.001$). In two out of five species egg load was positively correlated with body size (Fig.6, *A. tabida*, $F_{1,35} = 6.33$, $P= 0.016$; *A. persimilis*, $F_{1,35} = 5.63$, $P = 0.025$). In *A. japonica* egg load showed a significant negative correlation with body size (Fig.6, $F_{1,35} = 19.29$, $P< 0.001$). No significant correlation between egg load and body size was observed for *A. citri* and *A. pleuralis*. Regardless of size, a post-hoc t-test revealed significant differences in egg load between all species. The highest egg load was observed in *A. persimilis* and it was significantly higher than that of all other species (Fig.6, table 2, Appendix 2). *A. japonica* showed the lowest egg load (Appendix 2), significantly lower than that of all other species. The other three species ranked as follows (from high to low), *A. citri*, *A. tabida* and *A. pleuralis*.

Discussion

Our results demonstrate considerable interspecific variation in most of the life-history traits we examined. All of the measured traits were found to be phylogenetically uncorrelated which indicates the potential for rapid evolution of these traits and implies that the differences are adaptations to the environment of each of the species.

Like previous studies in hymenopteran parasitoids, we found no clear relation between developmental time and adult life span. For example, the species with the shortest developmental time (*A. pleuralis*) lived the longest in the no-food treatment and also relatively long in the food treatment, while the shortest-lived species (*A. japonica*) had an intermediate developmental time. Thus, our study confirms the results of Blackburn (1991) and Eijs & van Alphen (1999), supporting the notion that development time and adult lifespan are independent in hymenopteran parasitoids.

Our results suggest that interspecific variation in adult metabolic rate may explain these differences: *A. pleuralis* had a very low metabolic rate, while *A. japonica* had a very high metabolic rate. A higher metabolic rate results in a shorter adult lifespan, because lipid reserves are used at a higher rate. Another potential explanation for a shorter adult lifespan is provided by Harman's (1956) theory of the role of reactive oxygen species (ROS) in ageing. As the result of respiration, free radicals are generated in the mitochondria of organisms. The ROS react with macromolecules and so contribute to aging. As the production of ROS increases with metabolic rate, a higher metabolic rate can cause earlier death in organisms. Some recent studies have shown increased longevity in mutant organisms that resist to oxidative stress by reducing ROS production For example, Neretti

(2009) showed significant reduction in ROS and protein damage in long lived mutant *Drosophila* in comparison with control flies. Although a recent study suggests that natural selection may favour long lived species to reduce mtDNA mutation rates caused by ROS accumulation (Galtier et al. 2009), metabolic rate generally plays a crucial role in aging. Consistent with these studies our results showed a negative relationship between longevity and metabolic rate in *Asobara*.

Providing adult wasps with a carbohydrate food source significantly improved their survival. However, the strength of the effect differed between species. Two species *A. persimilis* and *A. tabida* were more dependent on supplementary carbohydrate food than others and showed a sharp decline in longevity when deprived from food whereas the reduction in longevity of *A. pleuralis* and *A. citri* in absence of food was substantially lower. *A. japonica* lived very short in both food and no food treatments. Extended longevity in time-limited parasitoids is likely to be important as it allows them to exhaust their egg supply during their lifetime (Rosenheim 1996). The effect of supplementary food sources and the ability to digest different polysaccharides by adult parasitoids has been documented (Jones and Jackson 1990; Jervis et al. 1996;Williams and Roane 2007). Longevity of a hymenopteran egg parasitoid, *Anaphes jole*, which was limited in the absence of food to a maximum of 3 days, exceeded 10 days when honey was provided as food (Jones and Jackson 1990). Williams and Roane (2007), in a comparison of the effect of different carbohydrates on survivorship of *A. jole*, demonstrated that the largest effect on longevity was found for three main sugars in nectar: sucrose, glucose and fructose. Our finding that adult feeding enhanced longevity in some species was thus consistent with previous studies. Lipid acquired during development can be used to either survive longer or to increase egg production (Ellers 1996). Differences in allocation of lipids to survival and reproduction may explain why species differ in their dependency on supplementary food as adults.

We expected to find a trade-off between longevity and initial allocation to reproduction. However, our results showed no relation between initial egg load and longevity in experiments either with or without food. Initial egg load in synovigenic species, therefore, should not be used to estimate lifetime fecundity and total allocation of resources to reproduction. A better estimate may be the ovigeny index (OI), which is the ratio of the number of mature egg at eclosion to potential lifetime egg production (Jervis et al.2001). It is a measure for the timing of reproduction from early life to later in life and may provide an indication of how much lipid will be used in future reproduction. In chapter 4, we establish that the different species of *Asobara* differ in ovigeny index. We showed relatively low OI for both *A. tabida* (0.64 ± 0.01) and *A. pleuralis* (0.53 ± 0.02) in comparison with species with high OI like *A.persimilis* (0.80 ± 0.02).

The reason why the two species, *A. pleuralis* and *A. tabida* with higher numbers of immature eggs behaved differently in absence of food, could be their different strategies in using the lipids for the maturation of eggs. Egg maturation comes at the cost of a decline in adult life span in the absence of food. Ellers (1996) showed a rapid decline in lipid reserves of *A. tabida* a few days after eclosion in both food and no food treatments, which shows that this species uses lipids to mature eggs early during adult life. Converting more lipids into eggs in early life could result in early death due to the exhaustion of resources. This may explain the short life span of *A. tabida* in the no food treatment, despite its high level of stored fat at emergence. In contrast to *A. tabida*, *A. pleuralis* had a long lifespan in the no food treatment and low lipid reserves. This species most likely postpones egg maturation until later in life, and would thus be able to use its lipid reserves to live longer when food is not available. In addition, the lower metabolic rate of *A. pleuralis* enables this species to live relatively long on low fat reserves.

Few studies on the fitness effects of developmental time are available for parasitoids. Godfray (1994) suggested that female wasps emerging early in spring may have easier access to unexploited hosts than later emerging conspecifics. Fast development may also be selected for in growing populations, where shorter generation times increase fitness. Fast development may further benefit parasitoids when they are suffering high pre-adult mortality due to natural enemies (Harvey and Strands 2002). The short developmental time we observed for *A. pleuralis* may partially be explained by high mortality risk during development in the tropical rain forest, due to vertebrate scavengers eating fermenting fruits from the forest floor, resulting in the death of all host larvae. The short generation time in this species would also allow its populations to recover, after crashes of the *Drosophila* populations during the dry season. However, studies of the population dynamics of these parasitoids and their natural enemies in the field are needed to assess larval mortality rates in different species and to assess the relative importance of these sources of selection.

Parasitoids are well known to manipulate their hosts to increase their own fitness (Stamp 1981; Fritz 1982, Brodeur and McNeil 1992, Grosman et al. 2008, Harvey et al. 2008). Our study on pupation site selection of the same five species of *Asobara* disclosed host manipulation by two species with relatively long development times, *A. tabida* and *A. citri*, but not by the other three species (chapter 2). It is likely that these parasitoids are able to reduce pupal mortality rate by manipulating the host's pupation site instead of reducing developmental time. By considering the fact that changing pupation site may result in less predation and mortality rate of parasitoids during developmental stages it is expected that these two species are less subjected to a high selection pressure for

shorter developmental time which is consistent with our findings.

Although resource uptake efficiency is a fitness related trait for all parasitoid species, this trait seems to be traded off with other traits and also varies in fitness value according to habitat. The five species of hymenopteran parasitoid used in this study differed in their resource uptake efficiency during development. For example, the fastest developing species - *A. pleuralis* - had relatively high lipid reserves compared to the two species with intermediate development times, *A. persimilis* and *A. japonica*. Fast resource uptake has been documented in other endoparasitoids (Slansky 1986; Sequeira and Mackauer 1993; Harvey 2005).

To conclude we suggest that lifespan and developmental time in parasitoids are genetically and physiologically uncoupled.

Chapter 4

Adult size and early investment in reproduction in five species of Asobara parasitoid wasps

Abstract

Adult parasitoid wasps are unable to synthesize lipids and are thus dependent on lipids obtained from their host during larval development. These insects therefore face a trade-off between the investment of lipids in eggs (reproduction) or in the maintenance of soma (survival). A theoretical study by Ellers & Jervis, 2003 predicted that resource allocation should depend on body size in parasitoids and reflect environmental selection pressures. We asked how body size should affect the timing of egg production in parasitoids. We measured the body size, lipid reserves, and reproductive investment (number of eggs, ovigeny index (OI) and egg size) at eclosion in five closely related species of *Asobara,* parasitoids of *Drosophila* larvae, originating from different geographic and climatic environments. Our results show significant interspecific variation in all these traits. A diagnostic test for phylogenetic independence revealed that closely related species did not resemble each other more closely than expected by chance for all traits measured. Lipid reserves scaled positively with body size both between and within species. In agreement with theoretical studies OI correlated negatively with body size both between and within species. Egg mass correlated negatively with lipid reserves both between and within species. This indicates the presence of a trade-off between allocation of lipids to reproduction and survival. With the exception of the most extreme pro-ovigenic species, *A. persimilis* we found that this strategy is compensated by small egg size. We discuss the potential role of habitat characteristics in shaping the interspecific variation in resource allocation strategies.

Introduction

Insect parasitoids seem unable to synthesize lipids as adults and thus depend on the limited lipid resources they obtain from their host

during larval development (Slansky 1986; Ellers, 1996; Rivero & Casas, 1999 ; Olson *et al.*, 2000; Rivero & West, 2002; Casas *et al*., 2003; Giron & Casas, 2003; Lee *et al.*, 2004 ; Visser & Ellers 2008). Lipids are needed both for the maintenance of the soma and for egg production, creating a trade-off between reproduction and survival (Roff 1992). Optimization of lipid allocation to these two components of fitness is thus a vital life-history decision (Ellers and Jervis 2003; Ellers et al. 2000b; Harvey et al. 2001; Jervis et al. 2008; Jervis et al. 2003; Jervis et al. 2001; Jervis and Ferns 2004; Thorne et al. 2006). Given that different reproductive rates are optimal in different environments, we expect resource allocation strategies to differ between environments.

Based on the timing of egg maturation, two modes of reproduction in parasitoids were recognized by early students of parasitoid life history, namely pro-ovigeny and synovigeny (Flander 1950). Pro-ovigenic parasitoids mature all of their eggs during pre-adult life and eclose with all eggs mature, while synovigenic parasitoids mature part or all of their eggs during adult lifetime. However, a wide range of variation in the number of mature or immature egg at emergence has been documented in synovigenic parasitoids (Quicke 1997). This variation can be described by the ovigeny index (OI), which is defined as the ratio of mature eggs at female eclosion to potential lifetime egg production (Jervis et al. 2001). Jervis et al. (2001) used the OI to describe inter- and intraspecific variation in the timing of reproduction. Jervis and Ferns (2004) argued that OI is a more useful and informative criterion for such comparisons than initial egg load, as the latter does not provide information on lifetime fecundity.

Various features of the habitat exert strong selection on fecundity and OI in parasitoids. These include host abundance and variance in the spatial distribution of hosts among patches. In a theoretical model Ellers et al. (2000b) showed that at low host densities, increasing variance in the numbers of hosts per patch and in the inter-patch distances selects for increased investment in early reproduction and hence in higher OI values. In rich habitats with low variance in the numbers of hosts per patch, lower OI values are favoured. This predicts that species occupying different habitats should show differences in OI that correspond to habitat quality and predictability.

A number of key life-history traits covary with body size (West et al. 1999). Body size should also affect the timing of egg production in insects. One reason for this is that smaller females have shorter expected life times and should therefore concentrate reproductive effort early in adult life (Ellers and Jervis 2003). If so, OI is predicted to covary negatively with body size in hymenopteran parasitoids. Later on Jervis and Ferns (2004) provided comparative empirical evidence for this prediction at the interspecific level. Another study also showed a negative correlation

between OI and body size within species (Thorne et al. 2006). However, fecundity is expected to increase with body size, simply because larger females should be able to produce more and/or bigger eggs. This would result in a positive correlation between body size and egg size (Berrigan 1991; Jervis et al 2001). In koinobiont parasitoids with hydropic eggs (*i.e.* eggs that absorb nutrients from the host hemolymph for embryonic growth) there is no clear relation between egg size and offspring survival. However, low OI has been documented for species with bigger and yolk richer, anhydropic egg (Jervis et al 2001). Variation in egg size may also cause substantial variation in initial egg load (IEL) in parasitoids with similar OI and body size. For example, Jervis and Ferns (2004) showed high variation in IEL of two Braconidae species, *Biosteres arisanus* (IEL = 180) and *Cotesia plutella* (IEL= 50) which have almost the same OI (0.3) and body size, but differ in egg size.

Here, we compare the resource allocation strategies of five closely related species of *Asobara*, synovigenic parasitoids of *Drosophila.* We investigate lipid reserves, egg size, egg load, OI and their relationships at both inter- and intraspecific levels. By reconstructing the phylogeny of these five species we were able to test for phylogenetic independence of the different life history traits measured.

Material and methods

Parasitoid and host

We used five closely related species of *Drosophila* parasitoids of the genus *Asobara* (Table 1). All species were cultured on *Drosophila melanogaster* from a laboratory stock that has been maintained in our lab since 1966 at 20 ± 1ºC and 16:8 h light:dark regime. These species originated from different climatic regions of the world (Table 1). Three species, *A. pleuralis, A. tabida*, and *A. japonica*, originated from wet forests (tropical for *A. pleuralis* and temperate for the other two). These represent habitats in which hosts are available throughout the season of adult activity of the parasitoids. The parasitoids produce successive generations throughout the season. For the other two species, *A. persimilis* and *A. citri*, host availability is periodically halted due to hot and dry conditions, during which *Drosophila* populations crash.

Table 1. Collection details of the *Asobara* species used in this study. All of the parasitoid species have been reared on *D. melanogaster* since collection in our lab at 20±1ºC&16hL: 8hD.

Parasitoid	Origin	Climate at origin	Collected
A. citri	Africa, sub-Saharan Africa	Dry & hot season alternated with a hot but wet season	Lamto, Côte, d'Ivoire, 1995
A. persimilis	Australia	Mediterranean hot & dry summer	Sydney, 1997
A. tabida	Holarctic, Europe& Asia and North America	Temperate & wet during the season when adults are active	Leiden, 2006
A. pleuralis	South-east Asia, Oriental tropic	Tropical wet forest	Manado, Sulawesi Indonesia, 2005
A. japonica	Japan Tokyo	Temperate & wet During the season that adults are active	Tokyo, 1995

DNA isolation, PCR amplification and sequences alignment

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, U.S.A.) from the whole body of female wasps. Three mitochondrial markers, COI, ND1 and 16S were used. PCR amplification was done using specific primers. The primers used in this study are listed in table 2. The PCR was performed using Thermocycler Perkin Elmer 240 under different thermal conditions for each primer combination as follows: 16S: 3 min at 94 ºC, then 37 cycles of 1 min at 94 ºC, 1 min at 52 ºC and 1 min at 72 ºC, ending with 5 min at 72 ºC, ND1; same thermal condition with 51 ºC annealing temperature and COI; two change in thermal condition, denaturing time from 1min at 94ºC to 15 sec and annealing temperature 45 ºC. Reactions were performed in a total volume of 25µl containing 2.5µl of 10X Buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 U of *Taq* polymerase and 0.3 mM MgCl2; all Qiagen's products. All sequences obtained were submitted to Genbank and their accession numbers are listed in table 3. Sequences were aligned to the outgroup sequence (*Aphaereta* sp.) using pairwise-alignment with MacClade 4.08 and then cleaned manually.

Phylogenetic analyses

PAUP* 4.0-test version 4.0d63 (Swofford 1998) was used to obtain the most parsimonious tree(s) (MP). We employed a heuristic parsimony search (Hillis et al. 1996) with 100 replicates of random addition sequences, and including the TBR (tree bisection reconnection) option for branch swapping. A bootstrap analysis with 100 replications was used to estimate statistical support (Felsenstein 1985). For the Bayesian analysis (BI) (Yang & Rannala 1997; Huelsenbeck et al. 2001), two Markov Chain Monte Carlo (MCMC) runs were conducted simultaneously with MRBAYES 3.0B4 (Huelsenbeck & Ronquist 2001). Each run was carried out for 10,000 generations with a sample frequency of 10 generations. The first 2,500 generations (250 trees) were discarded as burn-in. The best-fit model GTR+G was selected by Alike Information Center (AIC) criteria using MrModeltest version 2.2 (Posada & Crandall 1998). MP was analyzed on individual as well as the combined datasets, while BI was only conducted on the combined dataset. The Incongruence Length Different test (ILD) was performed before combining the datasets (Farris et al. 1994; Cunningham 1997; Posada and Buckley 2004).

Egg load, egg size, fat reserves and dry weight

Egg load, egg size, fat content and dry weight were measured on virgin female wasps harvested within five hours of eclosion. For each species 35 individuals were randomly chosen from 15 rearing jars, isolated and stored at -80ºC until further analysis. All four measurements were taken for each individual, as follows. Wasps were dissected on a cover glass, which had been weighed prior to dissection. The ovarioles were transferred to a small drop of demineralised water, opened and the eggs separated until they did not overlap (Ellers 1996). Two digital photographs were taken from each dissection, one containing all eggs to count the number of mature eggs (egg load) and one from a randomly chosen group of 20 eggs together with a small scale (to measure egg size). Egg size was calculated as the average surface area of 20 eggs per individual, measured relative to the scale, using Image-J image analysis software (Rasband, 1997-2005). Thecover glass containing the whole body including ovarioles and eggs was then dried at 80ºC for three days. The cover glass was then weighed again to determine the dry weight. After that, fat was extracted by submerging the cover glass in 3ml ether in a sealed glass tube for 24 hours (David et al. 1975, Ellers 1996). The ether containing the fat was then discarded and the cover glass washed again with fresh ether and dried again at 80ºC for three days. The fat contents of each individual were calculated by subtracting the dry weight before and after extraction of the fat reserves. We used egg mass (egg load × egg size) as our measure of the initial reproductive effort.

Table 2. List of the primers and their sequences used in this study.

Gene	Sequence of the primers (5´-3´)	References
COI		
Forward	Ron-5´GGA TCA CCT CAT ATA GCA TTC CC 3´	Monteiro and
Reverse	Nancy-5´CCC GGT AAA AAT TAA AAT ATA AAC TTC 3´	Pierce 2001
ND1		
Forward	ND1-5´ACT AAT TCAG ATT CTC CTT CT 3´	Smith and
Reverse	ND1-5´CAA CCT TTT AGT GAT GC 3´	Kambhampati 1999
16S		
Forward	16SWb-5´CACCTGTTTATCAAAAACAT 3´	Dowton and Austin 1994
Reverse	16S outer 5´CTTATTCAAATCGAGGTC 3´	Whitfield 1997

Table 3. List of species used for phylogenetic analyses with genbank accession numbers.

Code/ RMNH Ins. No.	Genus	Species	Source	16S	ND1	COI
100058	*Aphaereta*	sp.	Netherlands	SY157	SY157	SY157
MA501	*Asobara*	*citri*	Live stock	MA501	MA501	MA501
MA502	*Asobara*	*citri*	Live stock	MA502	MA502	MA502
MA401	*Asobara*	*japonica*	Live stock	MA401	MA401	MA401
MA402	*Asobara*	*japonica*	Live stock	MA402	MA402	MA402
MA201	*Asobara*	*persimilis*	Live stock	MA201	MA201	MA201
MA202	*Asobara*	*persimilis*	Live stock	MA202	MA202	MA202
MA301	*Asobara*	*pleuralis*	Live stock	-	MA301	MA301
100066	*Asobara*	*pleuralis*	East Malaysia	SY72	-	-
MA302	*Asobara*	*pleuralis*	Live stock	-	MA302	MA302
MA101	*Asobara*	*tabida*	Live stock	-	MA101	-
-	*Asobara*	*tabida*	No locality data	Z93715	-	-
RMNH-Jo643	*Asobara*	*tabida*	United Kingdom	-	-	AY935342

Ovigeny index

To measure the true ovigeny index we need to know the lifetime fecundity of an individual. Ellers (1998) showed that *A. tabida* uses its fat reserves for both survival and for producing more eggs during the

first week of its adult life. To measure both the fat reserve at emergence and the ovigeny index on the same individual females, we approximated ovigeny index as the ratio of mature eggs over total number of eggs at emergence of the wasp. Immature eggs, including the smallest visible oocytes, were counted on the same photographs used to count the number of mature eggs by increasing the magnification.

Test for phylogenetic independence

Closely related species may resemble each other in any trait because of their shared ancestry, rather than because of adaptation (Harvey & Pagel 1991). We assessed whether closely related species were more similar than distantly related species for the traits we measured using the test for serial independence (TFSI) as implemented in the software Phylogenetic Independence version 2.0 (Reeve and Abouheif 2003). TSFI is a parametric test to detect self-similarity among the adjacent observation and reveal phylogenetic autocorrelation (Abouheif 1999). The average value for each trait was calculated for each species and placed at the tips of the phylogenic tree. These values were then randomly reshuffled 1000 times. The observed topology of trait values was then compared to the generated distribution of random topologies. We report the P-value representing the chance that the observed topology could have arisen by chance, *i.e.* in the absence of phylogenetic autocorrelation.

Statistical analysis

We first examined between-species variation in dry weight using analysis of variance (ANOVA). Fat reserve, ovigeny index, egg size and egg load were compared between species using analysis of covariance (ANCOVA) in which dry weight was added as a covariate to represent body size. In each case we searched for the minimum adequate model by starting with the maximal model, then dropping the interaction term and then main factors from the model if non-significant. The simpler model was compared to the more complex model using an F-test. Traits were compared between species using post-hoc t-tests. In the cases where the correlation between body size and the trait of interest differed in sign between species, we dropped dry weight from the model and analysed between-species variation in the trait of interest using ANOVA.

Within-species correlations between traits were examined using Pearson correlations. We calculated both classical (r_c) and robust (r_r) correlations using the R package Mvoutlier (Gschwandtner and Filzmoser 2007). Robust correlation ignores outliers. For visualizing the results that included body size as the covariate we removed the effect of body size by regressing each trait on dry weight and plotting the residuals. All statistical analyses were conducted in the free statistical software R 2.5.1 (Ihaka & Gentleman, 1996).

Results

Phylogeny

Each marker showed a moderately resolved phylogeny of the five *Asobara* species. Phylogenies resulting from individual datasets were similar, but with low support. The ILD test showed significantly congruence (p = 0.03) of the markers. A total of 1319 bp were obtained after alignment of the combined markers (16S, COI and ND1). This included 180 (74%) phylogenetically informative and 63 (26%) uninformative characters. Cladograms resulted from the MP and BI analyses resulted in a fully resolved phylogeny among the five species of *Asobara*. Topologies obtained using both analyses were qualitatively identical (bootstrap values and posterior probabilities) (Fig. 1).

Phylogenetic independence

None of the traits under investigation showed significant phylogenetic autocorrelation (tests for serial independence: dry weight P = 0.09; fat content P = 0.19; ovigeny index P = 0.07; egg load P = 0.09, egg size P = 0.2; egg mass P = 0.17). We therefore did not correct for phylogenetic effects.

Body size

Significant differences in dry weight were observed among the species (ANOVA $F_{4,174} = 17.94$, $P < 0.001$, Fig. 2). *A. japonica*, *A. pleuralis* and *A. tabida* were the largest species, *A. citri* was intermediate, while *A. persimilis* was the smallest species (Fig. 2).

Fat reserve

ANCOVA revealed that fat content differed significantly between species ($F_{4,173} = 39.25$, $P < 0.001$, Fig. 3) and scaled with body size ($F_{1,173} = 351.65$, $P < 0.001$). However, the slopes of the relation between fat content and body size did not differ significantly between species (interaction effect $F_{1,173} = 1.11$, $P = 0.35$). Therefore, larger wasps contain more fat than smaller wasps in all five species. Post-hoc t-tests showed that the fat content of *A. japonica* was significantly lower than that of all other species (Fig. 3). *A. citri* and *A. tabida* had the highest fat contents, while *A. persimilis* and *A. pleuralis* had intermediate fat contents (Fig. 3).

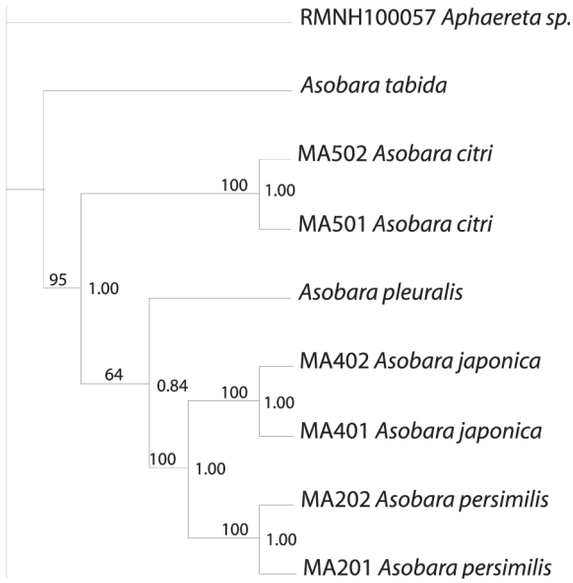

Fig.1: Phylogeny of five *Asobara* species. Cladogram resulting from analyses of Maximum Parsimony (MP) (numbers above nodes are the bootstrap values) and Bayesian analysis (BI) (number at the interior nodes are the marginal posterior probability of the clade being correct) of the combined markers (COI, ND1 and 16S). MP with 100 bootstrap replicates. 1 MP tree, tree length=330, CI=0.8152, HI=0.1848 and RI=0.8076.

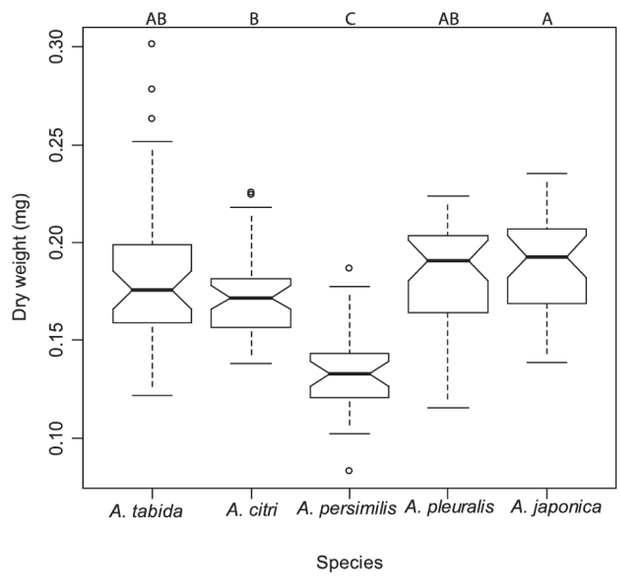

Fig.2: Variation in dry weight among the five species of *Asobara*

Ovigeny index

The ovigeny indices differed significantly between species (Fig 4. ANCOVA; $F_{4,173}$ = 46.69, P < 0.001). Ovigeny indices also covaried significantly with dry weight, indicating that larger species have lower ovigeny indices ($F_{1,173}$ = 25.43, P < 0.001). However, the pattern of covariance between ovigeny index and dry weight did not differ between species (interaction term, $F_{4,173}$ = 1, P = 0.41). *A. persimilis* displayed the highest ovigeny index, while *A. pleuralis* and *A. japonica* had the lowest ovigeny indices. Values for *A. tabida* and *A. citri* were intermediate (Fig. 4, Appendix 3.).

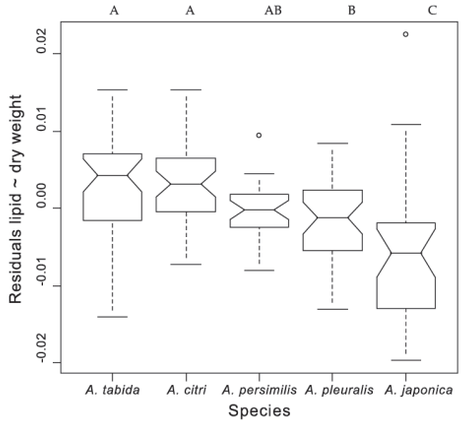

Fig.3: Variation in fat reserve among the five species of *Asobara*.

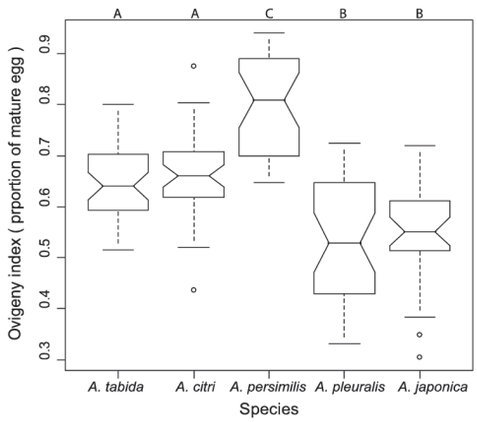

Fig.4: Ovigeny index in the five different species of *Asobara*

Intraspecific correlation between body size and ovigeny index

Interspecific comparisons showed that *A. persimilis,* which was the smallest species, had the highest median value for OI, (dry weight = 0.133mg, O.I = 0.81, Fig. 2 & 4). Two other species that were significantly bigger than *A. persimilis* showed the lowest median O.I values (*A. pleuralis*; D.W = 0.191mg, O.I = 0.55, *A. japonica*; D.W. = 0.193mg, O.I = 0.55, Fig. 2 & 4). The remaining two species, *A. citri* and *A. tabida*, with intermediate dry weights showed intermediate O.I. median values, (*A. citri*; D.W. = 0.172, O.I = 0.65, A. *tabida*; D.W. = 0.176, O.I. = 0.64, Fig. 2 & 4, Appendix 3).

Intraspecific comparisons revealed the same pattern for individuals within a species: a significant negative correlation between body size and ovigeny index was observed for all species except *A. persimilis* (*A. tabida*; r^2 = 0.15, P = 0.01, *A. citri*; ; r^2 = 0.24, P = 0.001, *A. japonica*; ; r^2 =

0.19, P = 0.01, *A. pleuralis*; ; r^2 = 0.12, P = 0.04, *A. persimilis*; r^2 = 0.06, P = 0.18). We found very similar robust and significant correlations within three species (Fig 5, *A. tabida*; $r_{classical}$ = -0.38, r_{robust} = -0.34, *A. citri*; r_c = -0.49, r_r = -0.45, *A. pleuralis*; r_c = 0.35, r_r = 0.37). Robust correlation was stronger than classical correlation in *A. japonica* r_c = -0.43, r_r = -0.73). In *A. persimilis* no significant correlations were observed (*A. persimilis*; r_c = -0.25, r_r = -0.08, Fig. 5).

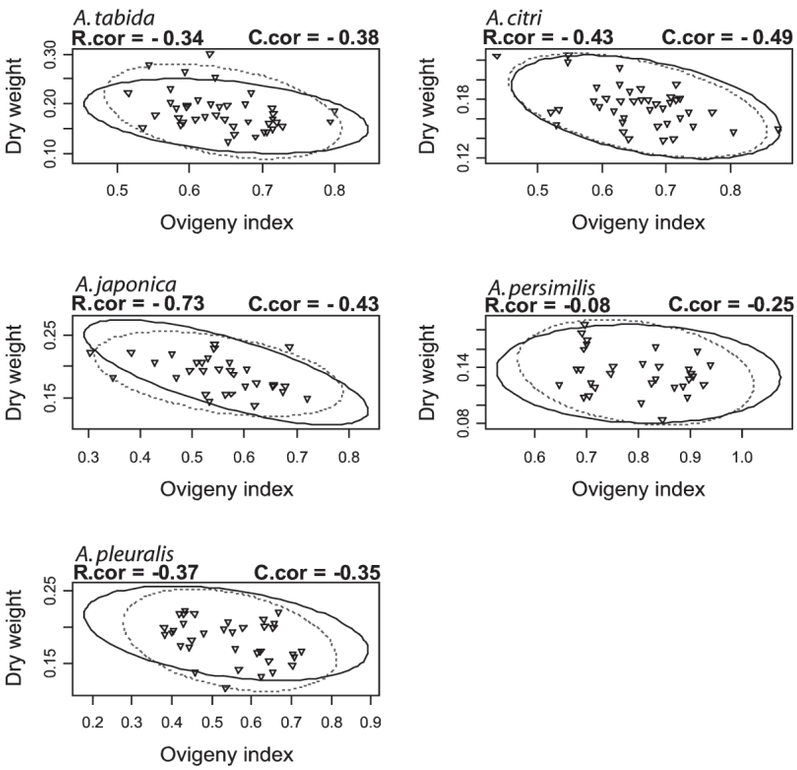

Fig.5: Within-species correlations between dry weight and ovigeny index in five species of *Asobara.* Solid lines: robust correlation (R.cor). Dashed lines: classical correlation (C.cor).

Egg size

ANCOVA revealed a significant interaction between the effects of dry weight and species on egg size (ANCOVA; $F_{4,173}$ = 12.27, P = 0.01). This indicates that the slopes of the relation between egg size and dry weight varied between species. We therefore examined the correlation between dry weight and egg size in the different species separately (Fig. 6). In two out of five species we found no significant correlation between egg size and body size (*A. tabida*; r^2 = 0.05, P = 0.14, *A. pleuralis*; r^2

= 0.012, P = 0.52), while in other two species we observed significant positive correlations (*A. citri*; r^2 = 0.14, P=0.014, *A. persimilis*; r^2 =0.16, P = 0.02). By contrast, egg size was negatively correlated to dry weight in *A. japonica* (r^2 = 0.37, P<0.001).

As this trait was not explained in similar directions by body size among the species, we dropped dry weight as the covariate factor from the model. We used ANOVA to assess the variation of egg size among the different species. The results showed significant variation in egg size among species (ANOVA; $F_{4,174}$ =118.47, P < 0.001). Two species from humid habitats, *A. pleuralis* and *A. japonica* showed considerably biger egg size in comparison with the other species (Fig.6). *A. tabida* was the only species from humid climate which showed a very small egg size. Among the species from dry habitats, *A. persimilis* showed a larger egg size than *A. citri* (Fig.6, Appendix 3).

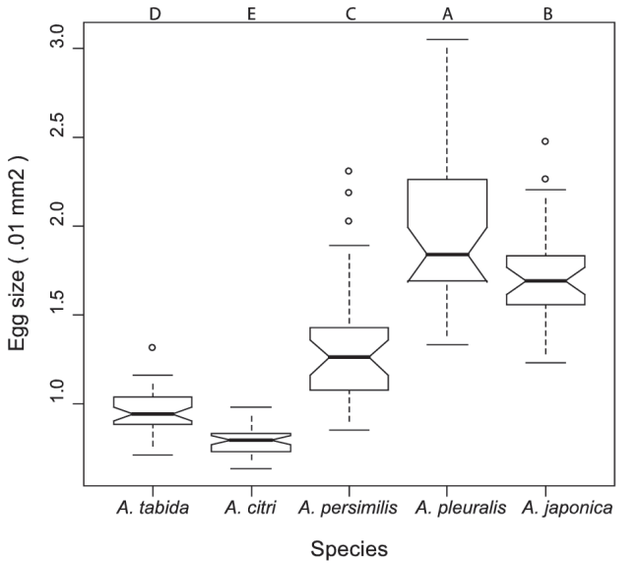

Fig.6: Variation in egg size among the five species of *Asobara.* Two species from humid habitats showed biger egg size (*A. pleuralis*; t = 36.78, P < 0.001, *A. japonica*; t = 32.68, P < 0.001). *A. persimilis* showed intermediate and other two species very small egg size (*A. persimilis*; t = 26.7,*A. tabida*; t=21.24, P<0.001, *A. citri*; t=18.399, P=0.004).

Egg mass

Egg mass, like egg size, did not vary in same direction in relation to body size for all species (ANCOVA; $F_{4,173}$ = 6.93, P < 0.001). An ANOVA on egg mass variation among the species showed significant interspecific variation in this trait (Fig.9, ANOVA; $F_{4,174}$ = 45.98, P< 0.001). Post hoc t-tests showed high egg mass for *A. persimilis* and *A. pleuralis*. No significant

differences in egg mass were observed between *A. tabida*, *A. citri* and *A. japonica*. (Fig.7, Appenix 3).

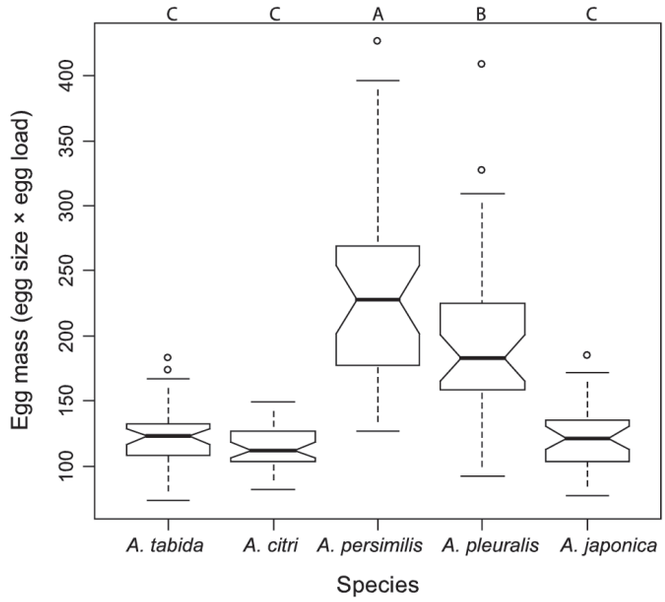

Fig.7: Variation in egg mass among the five species of *Asobara*. A biger egg mass was observed for two species, *A. persimilis* and *A. pleuralis* (t = 27.75, P < 0.001, t = 24.55, P < 0.001). No significant differences in egg mass were observed between *A. tabida*, *A. citri* and *A. japonica*. (t = 17.65, t = 16.78, t = 18.01).

Intraspecific correlations between egg size, egg load and egg mass

By using bivariate correlation plots in which each trait was corrected for body size, we found that *A. persimilis* was the only species with a strong positive correlation between egg size and egg mass (Fig.8, r_c = 0.82, r_r = 0.84). Weaker positive correlations were observed in two species (Fig.8, *A. tabida*; r_c = 0.54, r_r = 0.48, *A. pleuralis*; r_c = 0.66, r_r = 0.46). In two other species we found either a negative or no correlation (Fig.8, *A.japonica*; r_c = 0.25, r_r = -0.45, *A. citri*; r_c = -0.05, r_r = -0.21). All species except *A. persimilis* showed a strong correlation between egg mass and egg number (Fig.9, *A. tabida*; r_c = 0.77, r_r = 0.84, *A. citri*; r_c = 0.9, r_r = 0.9, *A. japonica*; r_c = 0.84, r_r = 0.95, *A. persimilis*; r_c = 0.31, r_r = 0.45, *A. pleuralis*; r_c = 0.66, r_r = 0.82). Thus egg number is the main determinant of total investment in eggs, except in *A. persimilis*, in which egg size is more important.

Trade-off between fat reserve and egg mass

Most of the species with high egg mass showed relatively low fat reserves which suggests a trade-off between fat reserves and egg mass. *A. japonica* was the only species which showed both low fat reserves and a low egg mass. *A. persimilis* and *A. pleuralis* which emerged with high egg mass had significantly lower fat reserves compared to *A. tabida* and *A. citri*. Similar indications for a trade-off between egg mass and fat reserves were also observed within species by plotting bivariate correlations. In these correlations we corrected all traits for body size. The results showed negative correlations between egg mass and fat reserve in all species except *A. tabida*. (Fig.10, *A. tabida*; r_c = 0.4, r_r = 0.37, *A. citri*; r_c = -0.54, r_r = -0.65, *A. japonica*; r_c = -0.52, r_r = -0.55, *A.persimilis*; r_c = -0.38, r_r = -0.42, *A. pleuralis*; r_c = -0.07, r_r = -0.75).

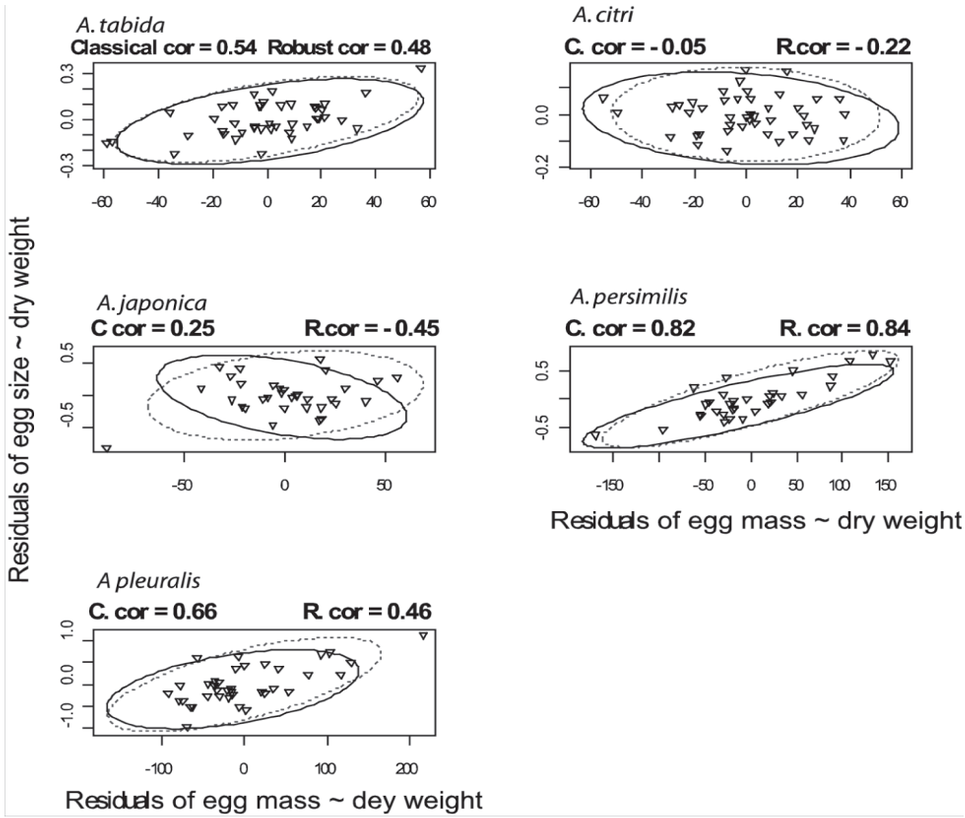

Fig.8: Within-species correlations between egg size and total investment in egg (egg mass) in five species of *Asobara.* Solid lines: robust correlation (R.cor). Dashed lines: classical correlation (C.cor).

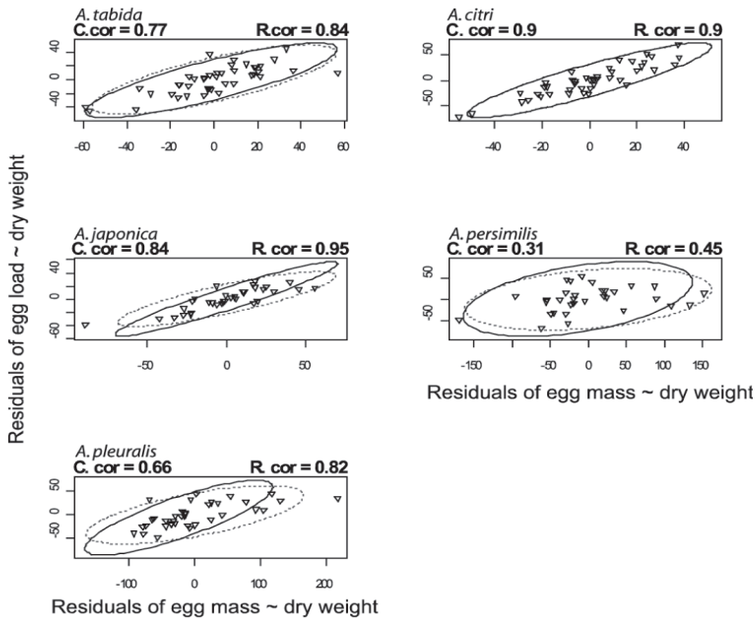

Fig.9: Within-species correlations between egg load and total investment in egg (egg mass) in five species of *Asobara*. Solid lines: robust correlation (R.cor). Dashed lines: classical correlation (C.cor).

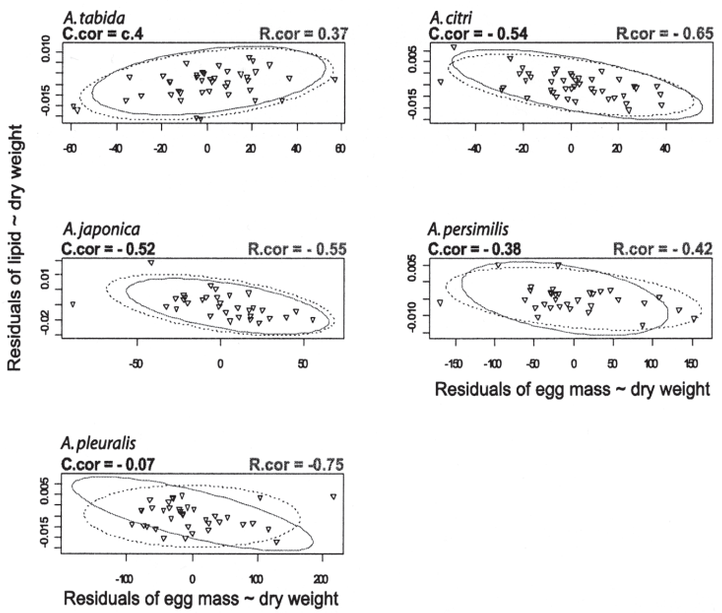

Fig.10: Within-species correlations between fat reserve and total investment in egg (egg mass) in five species of *Asobara.* Solid lines: robust correlation (R.cor). Dashed lines: classical correlation (C.cor).

Discussion

Our results demonstrate significant variation in resource allocation strategies among five closely related species of *Asobara*. All traits we measured (body size, fat reserves, ovigeny index (OI), egg size and initial reproductive effort (egg mass) showed considerable variation, both within and between species. We showed that each the life history traits under investigation was independent from its phylogenetic history. This indicates rapid evolution of reproductive traits and thus potentially rapid adaptation of each species to its own environment.

The number of mature eggs at eclosion and the total number of eggs which can be produced during adult lifetime varies widely in synovigenic parasitoids (Quick 1997) sugesting that allocation of resources to reproduction or survival is under strong selective pressure (Ellers at al 2000b). Jervis et al (2001) in a comparative study of 368 species of both pro-ovigenic and synovigenic hymenopteran parasitoids showed a wide range of variation in OI. Our finding of variation in OI among the five *Asobara* species provides the possibility to compare this variation both inter- and intraspecifically. Egg limitation is unlikely to play an important role in *Asobara* species (Ellers et al. 2000b, Jervis and Ferns 2004) as only a small fraction of the females in the populations of these parasitoids will be able to deplete all their eggs before the end of their adult life. Ellers et al (1998) showed, by comparing wild-caught and laboratory raised *A. tabida* that only 7% of the wild females were able to lay all of their eggs before dying.

OI measures how the energy budget is divided between reproduction and soma in synovegic parasitoids. Ellers and Jervis (2003) predicted a negative correlation between body size and OI in parasitoids. In a review, Jervis and Frens (2004) provided comparative evidence for these predictions. Our results are in agreement with these studies, as we found negative correlations between body size and OI both inter- and intraspecifically. *A. persimilis*, a species with a small body size and little variation in body size, was the only species in which we could not find this relationship. Within species, small females are unlikely to survive for long and should thus concentrate on early reproduction. Between species, the negative correlation between body size and OI indicates that certain habitats select for investment in early reproduction instead of survival and large body size. Habitat quality, defined as the host abundance and variance in the spatial distribution of the host can drive selection on resource allocation in parasitoid insects. In a theoretical study Ellers et al (2000b) showed that parasitoids in rich habitats with low variance in host distribution over patches are selected to carry fewer mature egg at the start of adult life and mature a larger proportion of their eggs during adult life (low OI) in comparison with parasitoids from poor habitats with

high variance in the spatial distribution of hosts. These predictions are supported by our study: *A. pleuralis* and *A. japonica* from humid habitats rich in fruits, showed low OI. By contrast, *A. citri* and *A. persimilis* from dry habitats where host densities are low during at least part of the season, had a high OI. Dry environments are apparently unfavorable for survival of adult parasitoids and select instead for investment in early reproduction. *A.tabida*, a species from temperate humid habitats had a high OI, and we think this exception could be explained by the spatial distribution of hosts in western Europe which is relatively unpredictable and patchily distributed (Ellers et al. 2001).

Egg size has rarely been studied in resource allocation studies of parasitoids. Jervis et al (2001) in a comparative study, showed a link between yolk-deficient hydropic eggs and pro-ovigeny. Thus, smaller eggs are expected for pro-ovigenic species and larger eggs for synovigenic species. Strong variation in initial reproductive effort of species with same OI also has been explained by variation in investment per egg (Jervis and Ferns 2004). Jervis and Kidd (1986) mention that hydropic eggs of koinobiont endoparasitoids are able to absorb and utilize protein from the host hemolymph for embryogenesis. If parasitoids were able to rely on host resources during the egg stage, the adults would be able to invest less in egg size and produce more and smaller eggs. Thus, it is unlikely that egg size will affect adult size in endoparasitoids with hydropic eggs and we expect no direct relation between egg size and fitness as has been found in other insects, e.g. butterflies (Boggs 1986). We found considerable variation in egg size for *Asobara* species which suggests different selection on egg size in different habitats. Species from humid and supposedly rich habitats (*A. pleuralis* and *A. japonica*) had larger eggs than the other species. Why larger eggs are advantageous in such habitats remains to be studied, but a possible explanation is that intraspecific competition by superparasitism is more frequent in such habitats, and that large eggs provide a competitive edge over smaller eggs. Of the species from habitats with a distinct dry season, *A. persimilis* showed relatively large variance in egg size compared to *A. citri*, which has very small eggs. The variation in egg size in *A. persimilis* was strongly correlated to variation in total egg mass. The observation that *A.persimilis* increases egg size with total investment in reproduction suggests that having large eggs is adaptive for this species. As the population density of *A. persimilis* becomes quite high in orchards during the autumn flush of *Drosophila,* it is possible that a higher incidence of competition in superparasitized hosts occurs during this part of the season (Price, 1976) and that thus may large egg size reward in *A. persimilis*.

The other species each showed a significant correlation between egg mass and egg load suggesting that they do not trade-off egg size against numbers of eggs, but egg number against lipid reserves. Fat reserves

in parasitoids may contribute to both egg production and survival. The role of lipid in somatic maintenance has been demonstrated by showing a positive correlation between lipid quantity and lifespan within several parasitoid species (Ellers 1996, Rivero and West 2005). Evidence for the role of lipids in egg production has also been demonstrated by Ellers at al (1998) for *A. tabida*, who showed that replenishment of the egg load drastically reduced fat reserves. Our study suggests that this trade-off holds both between and within species. Interspecific comparisons showed high fat reserves and low egg masses for *A. tabida* and *A. citri*, while the inverse was found for *A. persimilis* or/and *A. pleuralis*. *A. japonica* showed both low egg mass and low fat reserves. The very short life span for *A. japonica* both in the presence and in the absence of food (female *A. japonica* had an average life span < 4.5 days, while the other species varied from 10 to 20 days, see chapter 3) could be attributed to their low fat reserves, but possibly also to a higher basic metabolic rate and/or higher activity level (*A. japonica* showed significant higher metabolic rate than the other species see chapter 3).

In intraspecific comparisons, we found evidence for this trade-off in negative correlations between egg mass and fat reserves for all species except for *A. tabida.* The latter species showed a positive correlation in our data. However, Ellers and van Alphen (1997) demonstrated the presence of the trade-off in *A.tabida* using experimental manipulations. Our results are phenotypic correlations, and positive correlations can be caused by other variables and are not proof for the absence of a trade-off. Possibly the large variation in body size which is correlated with variation in OI (Ellers and Jervis 2003) may have caused the positive correlation found in our study for *A. tabida*.

In summary, our results showed enormous variation in life history traits in relation to body size directly linked to reproductive success in parasitoids. Functional models show that at least some of this variation is likely to be adaptive. Jervis et al (2008) in a review of resource allocation and reproductive strategies in parasitoids showed that additive genetic variation exist in traits related to reproductive success. This variation provides the potential to respond quickly to natural selection. Our finding that all of the measured traits are independent of the phylogeny of the species also suggests that the differences between them are due to selection and adaptation. We suggest that differences in the spatial and temporal distribution of the hosts, in addition to climatic differences are the selective forces driving the divergence in life history traits in these five species of *Asobara*.

Chapter 5

Genetic structure of Leptopilina boulardi populations from different climatic zones of Iran

Abstract

The genetic structure of populations can be influenced by geographical isolation (including physical distance) and ecology. We examined these effects in *Leptopilina boulardi*, a cosmopolitan parasitoid of *Drosophila* of African origin and widely distributed over temperate and (sub) tropical climates. We sampled 11 populations of *L. boulardi* from five climatic zones in Iran. The nuclear genetic variation among these populations was compared using amplified fragment length polymorphism (AFLP). To assess whether these populations had also diverged in their mtDNA, we sequenced part of the cytochrome oxidase (COI) gene. Genetic distances were calculated using Nei and Li's index (Nei & Li, 1979) and analysed using UPGMA cluster analysis and Principal Coordinates analysis (PCO). The AFLP results demonstrated clear-cut genetic differentiation between populations collected from the central part of Iran and those from north, which are separated by a desert. Both UPGMA and PCO analysis further separated two populations from the very humid western Caspian Sea coast (zone 3) from other northern populations from the temperate Caspian Sea coastal plain (zone 2) which are connected by rain forest. One population from the Caspian coast, Astaneh, was found to be genetically highly diverged from all other populations. An intermediate genetic structure between zone 2 and 3 was found for Nour from zone 2 which indicates some gene flow between these two populations. In all analyses a mountain population, Sorkhabad, was found to be genetically identical to those from the coastal plain (zone 2), which indicates high gene flow between these populations due to short geographical distance. A Mantel test showed a highly significant positive correlation between genetic and geographic distances ($r = 0.47$, $P < 0.001$). The COI gene was found highly conserved among all populations. Our results suggest that both geographic distances and physical barriers contribute to the formation of

genetically distinct populations of *L. boulardi*. Transfer of fruits containing *Drosophila* larvae parasitized by *L. boulardi* may have caused unexpected gene flow and changed the genetic pattern of populations, particularly in urban areas.

Introduction

Insect parasitoids have been a favorite model in ecology and evolutionary biology (Godfray and Shimada, 1999). *Drosophila* parasitoids have played a major role in these studies, because of their ease of maintenance in the laboratory, the enormous biological and genetic information on their *Drosophila* host which has been used as model organism for almost a century (Irving et al. 2004) and because of their diversity which allows comparative studies (Chapters 2 &3). One way to study adaptation in changing environments is to compare populations of a widely distributed organism across contrasting climates. Understanding the genetic structure of the insect populations is an important first step in the study of local adaptation (Mopper and Strauss 1998). Understanding the genetic structure of populations can provide essentional insights to interpret the results of ecological studies. Studying genetic population structure of insects is thus essential for comparative studies of behaviour and ecology and evolution of populations (Roderick, 1996). DNA markers provide powerful and efficient tools to study genetic diversity at both inter- and intraspecific level in insects' populations (Avise, 1994). A wide range of molecular markers has been successfully used to measure genetic diversity of insects, including RFLPs, RAPDs, SSRs and AFLP's. Among these techniques, Amplified Fragment Length Polymorphism (AFLP) is a useful DNA fingerprinting technique to study genetic diversity within a species (McMichael and Prowel, 1999; Hawthorne, 2001; Wu et al. 2006 and Tao et al. 2009). AFLP is a powerful technique, which is able to detect genetic variation of organisms based on DNA from any source and complexity without prior knowledge of the gene structure or sequences (Vost et al. 1995). In comparison to other methods, AFLP can provide higher resolution whilst needing less DNA (Tao et al. 2009). AFLP has been successfully used to study genetic diversity of insect populations from different geographic regions and belonging to different orders and families (Reineke et al., 1999; Ravel et al., 2001; Lu et al., 2002; Pannebakker et al. 2004; Samara et al. 2008; Alamalakala et al. 2009; Tao et al. 2009).

One of the main constraints on local genetic differentiation and adaptation is extensive gene flow between populations. Reduced dispersal of populations can thus accelerate the adaptation process and may lead to genetic subdivision of populations (Slatkin 1987). Environmental or physical barriers may promote isolation of populations. These include geographic distance and physical barriers like mountains, rivers and stretches of unsuitable habitat (Peterson and Denno 1998). AFLP markers

have been used to infer the role of geographical distance and barriers to gene flow in shaping the genetic structure of population in a variety of organisms (*e.g.* Salvato et al. 2002; Irwin et al. 2005; Clark et al. 2007). In the present study we used AFLP to investigate the role of geographical distance in shaping genetic variation in Iranian *L. boulardi* populations. *Leptopilina boulardi* (Hymenoptera: Figitidae) is a larval parasitoid of *Drosophila* of African origin (Allemand et al, 2003) which is widely distributed over tropical and warm temperate regions.

Mitochondrial markers have also been used in population genetic studies of insects (*e.g.* Crozier et al.1997; Olav et al. 2003; Smith 2005). For example, in the ant *Leptothorax rugatulus* (Olav et al.2003) a mitochondrial marker was found to be more informative than microsatellites and discriminated significantly better between populations due to maternal divergence resulting from reduced dispersal of females. Sequencing part of the cytochrome oxidase I gene successfully discriminated between two species of *Homalodisca*, a cicadellid leafhopper and demonstrated genetic differences between *H. coagulate* populations based on haplotype (Smith 2005). Using mitochondrial markers may provide valuable information on the migration of females between populations, because mitochondria are maternally inherited. Being able to trace back migration of female populations could provide crucial information in some insect parasitoids that have established asexual populations after infection with parthenogenesis-inducing *Wolbachia* (Stouthamer et al. 1999).

In this study, we used both AFLP and COI gene sequencing to study the genetic structure among 11 populations of *Leptopilina boulardi* collected from five contrasting climate zones in Iran. The zones were chosen to represent five distinct climates varying in precipitation, length of season and minimum and maximum temperatures. Many studies have combined genetic studies with experimental studies to measure the adaptive value of traits (Roderick, 1996; Kaltz and Shykoff 1998; Mopper and Strauss 1998; Lively 1999; Anderson et al. 2004). The main goal of this study was to describe the genetic structure of these populations, to be used later in a study of local adaptation in life history traits of these populations (chapter 6).

Material and methods

Field sampling

Leptopilina boulardi populations were sampled along a climatic cline stretching from the northern to the central regions of the Islamic Republic of Iran in July 2006. The transect covered five climatic zones

as follows (Table 1): cool mountains in Damavand (zone 1), wet forests (zone 2) and very wet rain forests along the Caspian sea coast (zone 3), a dry and hot climate with cold winters in the Esfahan region (zone 4) and a Mediterranean climate with very cold winters in Shahre Kord (zone 5). The locations of the sampling sites and climatic zones are shown in Fig. 1. Sampling was conducted in mid-summer to increase the likelihood of collecting adult wasps from all the zones. At each sampling site, we placed 12 traps. The traps consisted of plastic containers (diameter 10 cm; height 7.5 cm) with a 3 cm diameter circular hole in the lid. The lid holes were covered with a plastic mesh with a diameter of 2 mm to prevent large insects, snails, slugs and small rodents from entering the traps. This mesh was wide enough for *Drosophila* flies and their parasitoids to easily enter the traps. Several layers of filter paper were placed in each trap to absorb water and provide pupation sites for *Drosophila* larvae. The traps were baited with a piece of banana cut lengthwise with a smear of live yeast on top that was added to speed up fermentation and to attract fruit flies and parasitoids. The traps were suspended from trees using fishing wire and tilted downward to prevent rain from filling them. Each trap was placed in the shade of a bush or tree to protect it from overheating and its position was recorded by GPS. After a week the traps were collected and all pupae in the traps were wrapped in filter paper and transferred to lab. *Leptopilina boulardi* was the only parasitoid collected from all locations. Partially inbred lines were set up from each sampling site and 20 female wasps per population from early generations were frozen at -80ºC for genetic analysis.

DNA extraction

A number of DNA extraction methods were tried using either the whole body of a single female or a group of frozen female wasps. The highest yields of high quality DNA were obtained using an adapted CTAB protocol for insects (Reineke et al. 1998). The DNA extracted by using this method also resulted in the most reproducible AFLP patterns when DNA from the same extractions was amplified multiple times. Five female wasps were pooled per strain for DNA extraction. An extra RNA digestion was carried out by adding RNase A to the final concentration of 20µg / ml and samples were incubated at 37 ºC for 30 min. RNase A was then removed from the samples by adding phenol:chloroform:isoamylalcohol (25:24:1). We then suspended the DNA in the upper phase of the solution and transferred the upper phase to a new tube before DNA precipitation. DNA was precipitated using isopropanol (similar to the precipitation step for DNA extraction in CTAB protocol). The quality of the extracted DNA was checked on a 0.8% agarose gel and its quantity measured by a spectrophotometer (ND-1000, www.nanodrop.com). In the case of low quantity or sheared DNA (smear on the gel) the DNA extraction was repeated using five new females from the same strain.

Table 1: Description of the trapping locations of the populations including climate, vegetation and elevation

Zone	Population ID	Climate	Description of the location	Vegetation	Elevation (m)
1	Sorkhabad	Mountain	In the northern slope of the Damavand mountain, toward Mazandaran province	Tree and bushes along the river	1975
2	Qaemshahr 1	Temperate wet forest	In Mazandaran province, coastal Caspian sea	Forest	76
2	Qaemshahr 2	Temperate wet forest	In Mazandaran province in coastal Caspian sea	Forest	75
2	Nour	Temperate wet forest	In Mazandaran province in coastal Caspian sea	Natural park	20
2	Chalus	Temperate wet forest	In Mazandaran province in coastal Caspian sea	Forest	76
3	Astaneh	Temperate very wet forest	In Gilan province in coastal Caspian sea	Windbreak of rice field, close to city	8.5
3	Seyahkal	Temperate very wet forest	In Gilan province in coastal Caspian sea	Very dense forest	378
3	Lunak	Temperate very wet forest	In Gilan province in coastal Caspian sea	Very dense forest	485
4	Dorcheh	Semi dry desert with cold winter	Close to Esfahan along the Zayandehrod river	Orchard along the river	1625
4	Khairabad	Semi dry desert with cold winter	Close to Esfahan along the Zayandehrod river	Orchard and field along the river	1605
5	Zamankhan	Mediterranean with very cold winter	Close to Shahrekord and along Zayandehrod river	Orchard and field along the river	1873

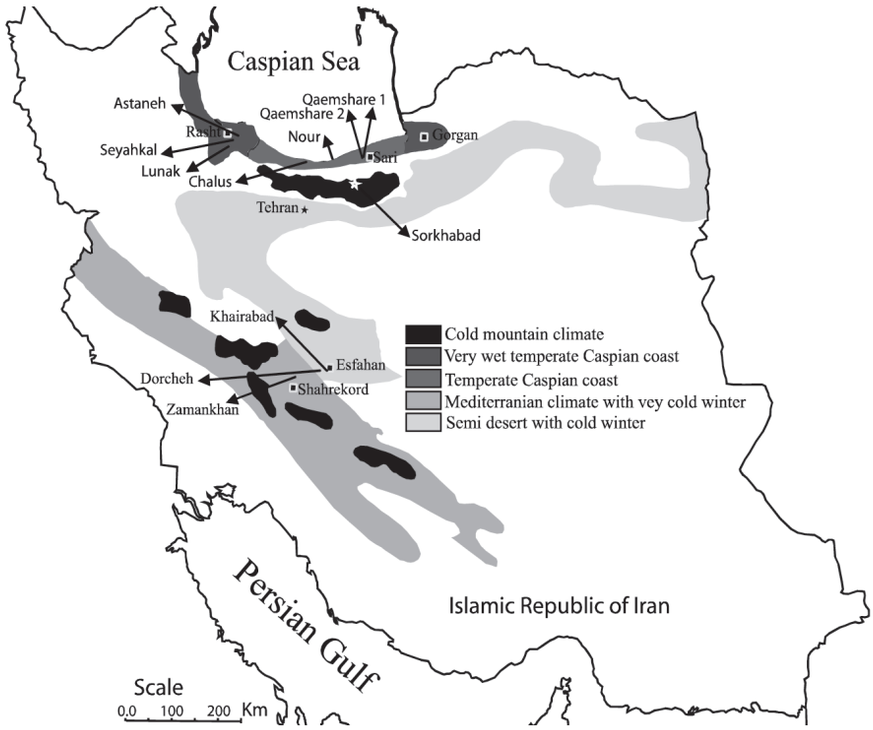

Fig 1: Map of the Islamic republic of Iran with climate zones indicated in shades of grey. Sampling points are indicated by the ID name of population, see table 1.

AFLP analysis

To asses the genetic diversity of the *L. boulardi* populations we employed the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) with a slight modification to the standard procedure. The AFLP procedure consisted of four steps. Restriction and ligation were carried out separately overnight. Step1 restriction digestion: 7 µl containing approximately 500ng of genomic DNA was incubated with EcoRI and MseI enzymes at 37 ºC overnight. Each reaction contained 5U EcoRI, 5U MseI , 4 µl of 10X restriction buffer (react 4) and 10 µg of BSA (bovine serum albumin), all from New England Biolabs (www.neb.com). The total volume was adjusted to 40 µl with autoclaved nanopure water. Step 2, restriction ligation: to each tube containing 40 µl of sample from step1, 10 µl of adaptor-ligation mix was added, which contained 5 µl EcoRI / MseI adaptor mix (one part 10µM Eco-adaptor and 9 parts 10µM Mse-adaptor; Table 1), 1 µl of 10 mM ATP, 1 µl 10X restriction buffer (react 4), 2U T4 ligase and 2.67 µl autoclaved nanopure water. The restriction-ligation reactions were incubated at 37 ºC overnight. Step 3, preamplification: this was the first

of two selection rounds of the fragments aimed at reducing background noise and nonspecific bands (Vos et al., 1995). Two primers, EcoRI and MseI with one base extension (Table 2) were used in the master mix of 16.7 µl per sample. Each reaction contained, 0.2 µl EcoRI+A primer (20 µM), 1.5 µl of Mse+C primer (20 µM), 15 µl AFLP Coremix (Applied bisystem, www.appliedbiosystems.com), and 4 µl of diluted restriction-ligation mix (concentration: 1 part restriction-ligation: 2.5 parts H2O) as DNA template. A touchdown profile amplification was used to increase the optimal primer selectivity in this step. The thermal cycling started with 2 min at 72 ºC to allow the polymerase to repair the nick caused by ligation of adaptors. This was followed by two cycles of 30 sec at 94 ºC, 30 sec at 65 ºC, 1 min 72 ºC, then 12 touchdown cycles during which the annealing temperature was dropped by 0.7 ºC in each cycle, then 23 cycles of 30 sec at 94 ºC, 30 sec at 56 ºC, 1 min 72 ºC and ended with 30 min at 60 ºC. This last step can promote addition of A (adenine) at the 3´ end of the strands by taq polymerase.

Table2: List of primers and adaptor used for the AFLP analysis

Type	Name	Sequence 5´-3´
Adaptor	EcoRI adaptor/F	CTCGTAGACTGCGTACC
	EcoRI adaptor/R	AATTGGTACGCAGTCTAC
	MseI adaptor/F	GACGATGAGTCCTGAG
	MseI adaptor/R	TACTCAGGACTCAT
Primer, pre select. amp.	EcoRI Preamp.	GAC TGCGTACCAATTC**A***
	MseI Preamp.	GATGAGTCCTGAGTAA**C***
Primer, select. amp.	EcoRI Select.	GACTGCGTACCAATTC**A**NN *
	MseI Select.	GATGAGTCCTGAGTAA**C**N *

*Nucleotides in bold are the fixed extended base in the 3´ end of the primers and N are the variable extension to provide different primer combination, see above.

The quality of amplified product was checked by loading 4 µl of PCR product on a 0.8% agarose gel prior to next step. Step 4, selective amplification: this last selection step was performed using one of three EcoRI primers (fluorescently labeled) and one of three MseI with three and two base extensions in each primer respectively. Different combinations of these primers resulted in 9 useful primer combinations (Table 3). Each PCR reaction contained 0.25 µl MseI C+X (20µM), 1.0 µl EcoRI A+XX (1 µM), 15 µl AFLP Coremix, 0.75 µl of autoclaved nanopure water, and 3 µl of PCR product from step 3 (diluted 1/10) in a total of 20 µl. Thermal cycling conditions were identical to those used in the preamplification, except that it started with 1 min at 94 ºC instead of 2 min at 72 ºC.

Table 3: List of selective amplification primers and fluorescent labels used in nine different combinations and the number of scorable and polymorphic bands generated by each combination.

Combination	Primers and labels	Scored band	polymorphic band
A	EcoRI+ACA (labeled Fam), MseI+CA	117	22 (18.8%)
B	EcoRI+AGG (labeled Joe), MseI+CA	63	20 (31.74%)
C	EcoRI+AAC (labeled Ned), MseI+CA	65	12 (18346%)
D	EcoRI+ACA (labeled Fam), MseI+CT	101	15 (14.85%)
E	EcoRI+AGG (labeled Joe), MseI+CT	65	25 (38.46%)
F	EcoRI+AAC (labeled Ned), MseI+CT	58	11 (18.96)
G	EcoRI+ACA (labeled Fam), MseI+CG	80	14 (17.5%)
H	EcoRI+AGG (labeled Joe), MseI+CG	69	16 (23.19%)
I	EcoRI+AGG (labeled Joe), MseI+CC	52	12 (23.08%)
Total band		670	147

For capillary electrophoresis, the PCR products from the selective amplification were diluted 10-fold. DNA was purified to remove the extra salt from the solution by following the MegaBace™ purification and precipitation protocol. DNA pellets were dissolved in 20 µl distilled water at 40 ºC for 30 min. Each sample contained 3.75 µl Megabace™ loading solution, 0.25 µl ET-500 R Size standard (both from Amersham Bioscience (www.amersham.com) and 2 µl of clean PCR product. Samples were denatured at 95 ºC for 3 min, immediately placed on ice and injected into LAP matrix (Amersham) capillaries at 4 kV for 54 sec and run on a DNA sequencer MegaBACE™ 1000 system (Amersham Pharmacia USA, www.amersham.com) at 10 kV for 75 min. The sequencer automatically captured and stored electrophoretic profiles for each sample. The fluorescent profiles were loaded into Fragment Profiler™ software ver1.2 (Amersham, Biosciences, www.amersham.com) using specific peak filters and were manually checked for correct alignment of the size standard. The positions of polymorphic markers between 80 and 500bp were scored and exported as a binary matrix in an excel sheet which '1' indicating presence and '0' absence of a band at a particular locus.

Amplification and sequence alignment for COI

A 638 bp portion of the cytochrome oxidase subunit I (COI) gene was amplified to evaluate the mitochondrial variation among the 11 *Leptopilina boulardi* populations from Iran. A *L. boulardi* population from Sospel (France) was used as out-group. PCR amplification was performed using a forward primer designed for a herbivorous hymenoptera from Torymidae family (Scheffer & Grissell 2003), COI-1775-F 5´-CGAATAAATAATATAAGATTTTG-

3´ and a reverse primer designed for *Leptopilina clavipes,* another species of the same genus (K. Kraaijeveld, unpublished), COI-2413-R, 5´-TCATCTAAAAATTTTAATCCCAGT-3´. The amplification was carried out on a Thermocycler PTC-2000 using the following thermal cycles: denaturing for 3 min at 92ºC followed by a touchdown with one degree drop in annealing temperature per cycle from 53-40 ºC (10 sec at 92 ºC ,10 sec at 53-40 ºC and 2 min at 72 ºC), then 25 cycles of 10 sec at 92 ºC,10 sec at 40 ºC and 2 min at 72 ºC, and ended with 5 min extension at 72 ºC. Each PCR reaction contained 1.2µl of 2.5mM DNTPs, 0.5µl of 10 µM of each primer, 0.15µl of Taq DNA polymerase(5 U), 1.5µl of 10X Buffer and 0.45µl of 15mM MgCl2 µl; all Qiagen products and was adjusted to 15µl with autoclaved nanopure water. The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, www.promega.com) following the manufacturer's protocol. The amplified bands were sequenced both forward and reverse on MegaBACE™ 1000 sequencer. The Sequencer software Version 4.2 (Gene Codes Corp.) was used to assemble the contigs and obtain consensus sequences. The sequences were aligned using pairwise-alignment in MacClade 4.08 (Maddison and Maddison 2005) and edited manually.

Data analysis

Pair-wise genetic distances between populations were calculated from the AFLP data using Nei and Li's index (Nei & Li, 1979) in Treecon version 1.3b for windows (van de Peer and de Wachter, 1994). UPGMA cluster analysis (Sokal and Michener, 1958) with 1000 replications of bootstraps was performed in Treecon and genetic distances were visualized in a dendrogram format. The geographic distances between collecting sites were calculated from their GPS locations and combined with the genetic distance values into a pair-wise genetic and geographic distance matrix. Isolation by distance was investigated with a Mantel test (Mantel, 1967) using the web-based program Isolation by distance web service (IBDWS) version 3.16 (Jensen et al. 2005). The combined binary AFLP data matrix was used to perform Principal Coordinate analysis (PCO) with GenAlex 6 (Peakall and Smouse, 2006) using Nei and Li's coefficient (Nei & Li, 1979) for calculating similarities, three first coordinates were used to graphically depict genetic variation among populations.

Results

AFLP analysis

The 9 primer combinations yielded 670 scorable bands of which 147 (21.94%) were polymorphic. UPGMA tree and bootstrap analysis indicated that the three populations from the central part of Iran (Dorcheh, Khairabad and Zamankhan) clustered together, but were differentiated from the northern populations sampled along the Caspian

Sea coast (bootstrap value = 88%, Fig. 2). One population from the very wet Caspian Sea coast (Astaneh) was considerably different t all other populations. UPGMA tree separated this population strongly from all other collected populations with high bootstrap value (bootstrap = 100). The Sorkhabad population, collected from a mountainous region on the slope of the Damavand mountain near Mazandaran clustered together with the wet Caspian Sea coastal populations.

Principal coordinates analysis revealed informative separation of populations. The first three coordinates explained 69.32% of AFLP variation. By plotting the first two coordinates (which together explained 52.73 % of the AFLP variation) the three populations from the dry central region of Iran clustered separately from the northern populations. These two principal coordinates also separated the populations from the two northern zones (zone 2 and 3), except Nour - a population from zone 2 clustered as zone 3 and Astaneh - the most divergent population which stood apart from all others. The other two PCO plots showed further evidence for divergence among the populations along the Caspian Sea coast. By plotting first and third coordinates and second and third coordinates (which in total explained 46.67% and 39.26% of AFLP variation respectively) two clusters were evident among the populations from the Caspian Sea coast, while Astaneh, the most divergent population, again stood apart from all other groups. Furthermore, the Zamankhan population from a Mediterranean climate (zone 5) appeared separated from the two other central populations from zone 4 (Khairabad and Dorcheh). Consistent with the UPGMA analysis, the Sorkhabad population from the mountain region clustered with populations from the geographically close, but ecologically different wet Caspian Sea coast.

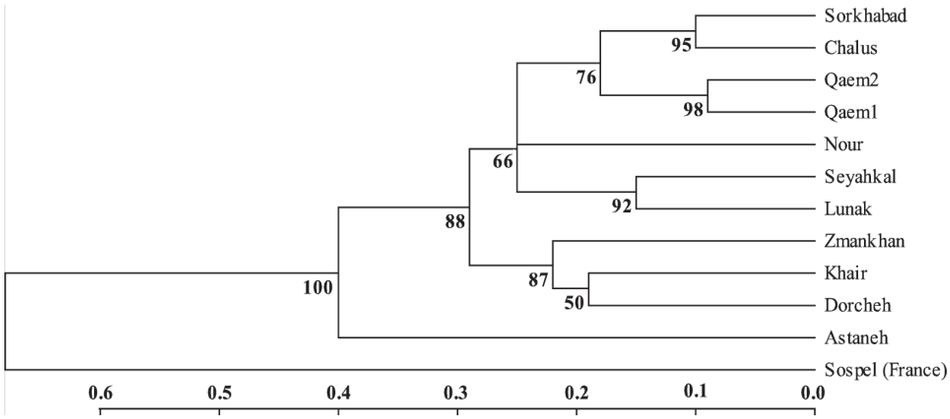

Fig. 2: Dendogram derived from the UPGMA (unweighted pair group methods of arithmetic averages) analysis of 147 polymorphic AFLP bands. Shown are the genetic distances of 11 population of Iranian *Leptopilina boulardi* and their outgroup (a population of *L. boulardi* from France). Scales indicate genetic distances (Nei & Li, 1979) and the numbers at nodes represent bootstrap value (1000 replication).

Isolation of populations by distance

A significant positive correlation between genetic and geographic distance was observed among the *L. boulardi* populations (Mantel test; r = 0.47, P < 0.001, Fig. 4a). The result of this test showed that a considerable part of the genetic variation was explained by geographic distance and supported the UPGMA and Principal Coordinate analyses since all geographically-close populations resembled each other. The only exception was the population from Astaneh, which was highly distinct from all others populations, even from those collected from a distance of less than 15 km. By excluding the Astaneh population from the Mantel test, the correlation coefficient increased substantially (mantel test; r = 0.73, P < 0.001, Fig. 4b).

Variation in cytochrome oxidase I (COI)

COI sequence was found to be highly conserved among the Iranian *L. boulardi* populations. Only two bases were found to be different in two Caspian Sea coast populations and four insertions and one replacement were found in the out-group compared to the Iranian populations (Table 4).

Table 4: Polymorphic site of the COI gene in 638 bp sequenced for 11 Iranian *Leptopilina boulardi* aligned with their out-group, Sospel - a population of *L. boulardi* from France.

Population	Position						
	10	60	131	321	338	440	459
Sospel	-	G	C	G	A	A	C
Zamankhan	-	-	C	-	-	G	-
Qaemshahr1	-	-	C	-	-	G	-
Qaemshahr2	-	-	C	-	-	G	-
Khairabad	-	-	C	-	-	G	-
Dorche	-	-	A	-	-	G	-
Nour	-	-	C	-	-	G	-
Astaneh	-	-	C	-	-	G	-
Seyahkal	-	-	C	-	-	G	-
Lunak	-	-	C	-	-	G	-
Sorkhabad	-	-	C	-	-	G	-
Chalus	C	-	C	-	-	G	-

Discussion

The AFLP analysis provided 147 polymorphic loci out of 670 reproducible and scorable bands in nine primer combinations. This provided enough information to allow a clear distinction among *L. boulardi* populations originating from different climatic zones in Iran. One of the main challenges in AFLP analysis for insects is generating enough reproducible and scorable bands and this is highly dependent on the quality of DNA (Reineke et al. 1998). In population genetic studies, pooling a number of individuals instead of extracting DNA from a single organism is a possibility to get enough DNA from small organisms and this solution has been suggested in previous studies (Pannebakker et al. 2004). A CTAB protocol modified for insects resulted in high quality DNA with highly reproducible bands in AFLP analyses in comparison with other extractions methods (Reineke et al. 1998). Pooling a number of female wasps and using the CTAB protocol modified for insects in the present study resulted in highly reproducible and scorable bands. The number of polymorphic bands among the populations depends on the primer combination and the genetic divergence between populations and can vary from very high to relatively low (*e.g.* high; Samara et al. 2008; Tao et al. 2009 and low; Najimi et al.2002; Pannebakker et al. 2004). Similar to the results of a previous study on genetic diversity in a different *Leptopilina* species, *L. clavipes* (Pannebakker et al. 2004) we found a relatively low number of polymorphic bands per primer combination (21.94% in total). By increasing the number of primer combinations (nine combinations) we obtained a high number of polymorphic bands (147 scorable polymorphic bands), providing highly informative data on the genetic structure of these populations. Similar to other AFLP studies, the consistency of scorable bands over all populations, the reproducibility of results and the presence of polymorphisms were the three criteria to choose the proper primer combination (Samara et al. 2008; Clark et al. 2007). As AFLP markers are dominant, the main assumption in the analysis is presence or absence of identical fragments (which are considered as homologous). To calculate the genetic distance between populations with Nei and Li's index (Nei & Li, 1979) only shared-presence bands were used. AFLP results showed the lowest distance values for two populations (Qaemshar1 and Qaemshar 2) collected at short range from each other (1.5 km) which was expected because of the high probability of gene flow and in agreement with a population genetic study using AFLP in a hymenopteran parasitoid (Samara et al. 2008). Excluding the Astaneh population, which was the most divergent population in both the UPGMA cluster analysis and the Principal coordinate Analysis (PCO), other northern populations clustered close together in two clades, with relatively high bootstrap value (76%). Nour, a population from zone 2 remained as an unresolved branch in UPGMA cluster-analysis between two northern populations (zone 2 and 3). PCO analysis provided a similar

pattern and supports the results of the UPGMA cluster-analysis. Based on PCO analysis, Nour grouped with populations from zone 3, based on the first two coordinates plot. By plotting the first and third coordinates in PCO, this population separated from all other northern populations and in plotting second and third coordinates of PCO, Nour grouped with populations from zone 2, the zone it originated from. The different results obtained with different plotting options in PCO analysis supported the unresolved position of this population found in the UPGMA cluster analysis. Genetically, it is intermediate between populations from zone 2 and 3. A clear distinction between northern and central populations resulted from both analyses (UPGMA; bootstrap value = 88%). A distinct difference between Zamankhan, a Mediterranean population, and the other two dry region populations, Khairabad and Dorche (zone 4) was also observed in UPGMA (bootstrap = 85%) and partially supported by PCO. Sorkhabad, the mountain population was almost identical to the populations from the Caspian coastal plain (zone 2) in both analyses. Both analyses resulted in strong separation of the Astaneh population, which was divergent from all other populations (UPGMA; bootstrap = 100 %).

Isolation of insect populations by distance or physical barriers has been shown in several studies (Salvato et al. 2002; Kerdelhue et al. 2006; Clark et al. 2007). Spatial distribution of organisms may prevent genetic differentiation of populations *e.g*. by massive gene flow between populations (Slatkin 1987). When suitable habitat connects distant sites, *Drosophila* parasitoids may be able to disperse over long distances. Barriers of unsuitable habitat or limited dispersal capabilities of parasitoids may hamper gene flow and cause the formation of divergent populations. Parasitized fruit flies may be transported with fruits and give rise to populations dissimilar from the neighbouring ones, in particular in urban areas. We found unexpected genetic divergence in two northern populations Astaneh (which was highly divergent from the neighbouring) and Nour (which showed intermediate structure between tow Caspian Coast zones). Both populations originated from urban areas and could have arrived with fruit transports.

Apart from these exceptions, our results demonstrate a highly positive correlation between genetic and geographic distance, which explained a large part of the genetic variation among populations in this study. To our knowledge studies on the genetic structure of *Drosophila* parasitoid populations are rare. A comparison of *L. clavipes* populations in western Europe found distinct genetic differentiation between sexual and asexual populations, but no correlation between geographic and genetic distances (Pannebakker et al. 2004). The positive correlation between genetic and geographic distances in our study is consistent with several other genetic structure studies on insect populations (Salvato et al. 2002; Kerdelhue et al. 2006; Clark et al. 2007).

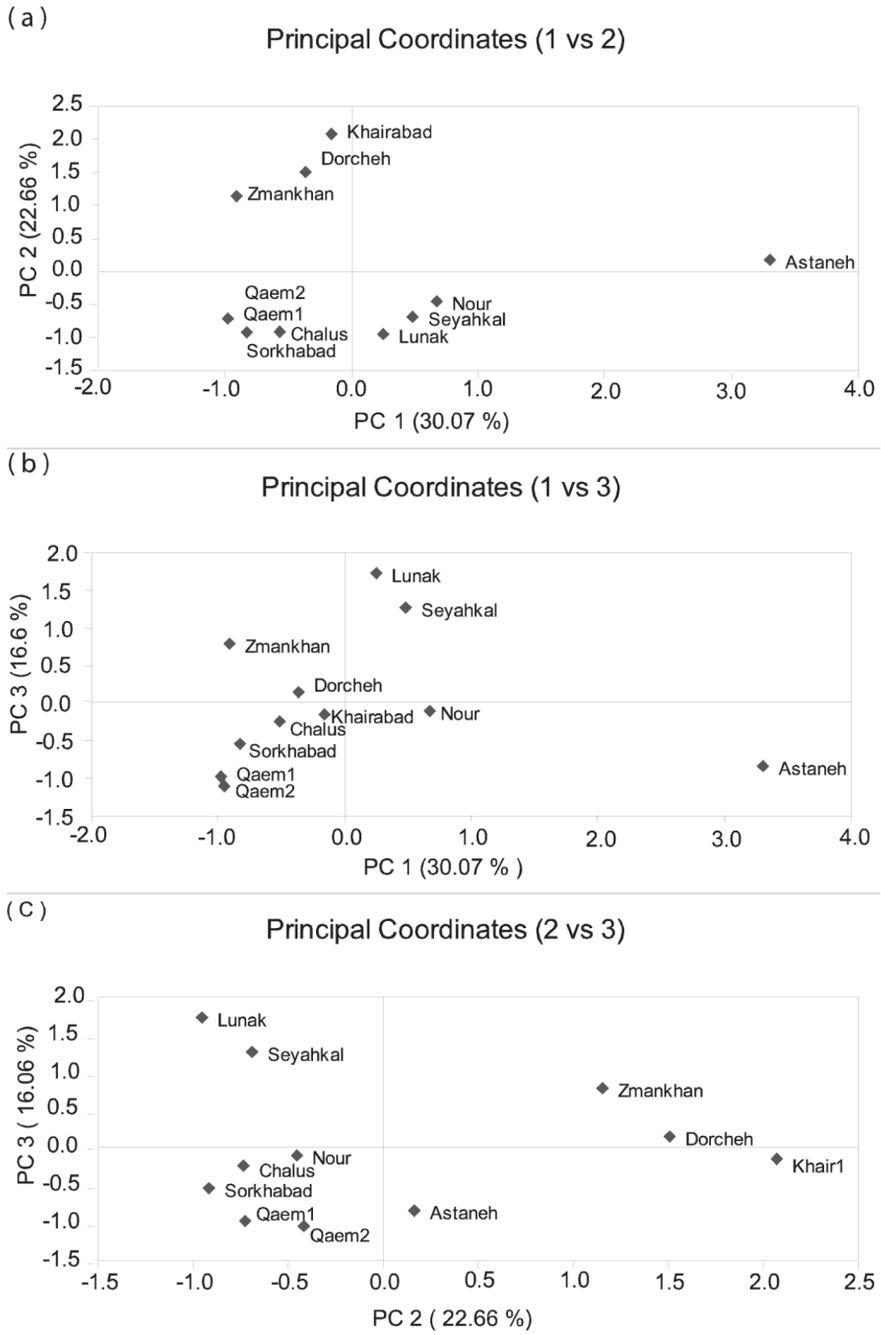

Fig. 3: Separation of Iranian *L. boulardi* populations by the first three coordinates in Principal Coordinates analysis. The first three coordinates explained 69.23 % of AFLP variations among Iranian *L. boulardi* populations. For the origin zone of populations see table 1.

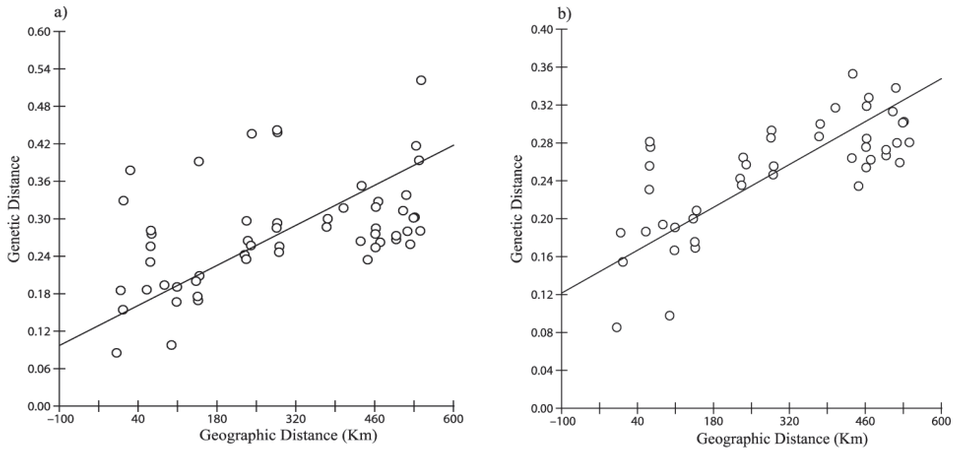

Fig. 4: Correlation between genetic and geographic distances, (a) including all populations and (b) excluding Astaneh, the most divergent population in all analyses.

The high genetic divergence over relatively short geographic distance between central populations than northern populations implies the role of a geographic barrier formed by unsuitable habitat (dry desert). Populations in the central part of Iran are from isolated locations surrounded by dry desert.

Sequence data on the cytochrome oxidase I gene were less informative in our study than ALFPs. Mitochondrial markers are potentially informative markers in genetic studies of insect populations (Crozier et al.1997; Olav et al. 2003; Smith 2005), sensitive to differences in the rates of male and female migration (Olav et al. 2003). However, most studies using CO I and II as molecular markers in insects have used them in phylogenetic and taxonomic contexts to discriminate between higher taxa than populations (Lunt et al. 1996; Caterino and Sperling 1999; Zaldivar-Riverón et al. 2006). Our results demonstrate a highly conserved pattern for COI among *L. boulardi* populations, as expected for sexually reproducing populations.

Our study adds to the evidence that AFLPs are convenient markers to study genetic diversity of populations within species (Yan et al., 1999; Sharbel et al., 2000; Schneider at al., 2002; Alamalakala et al., 2009) and can be used successfully for population genetic studies on hymenopteran parasitoids (*e.g*. Schneider et al. 2002; Pannebakker et al. 2004; Samara et al. 2008).

Chapter 6

Local adaptation in life history traits of *Leptopilina boulardi* populations from different climate zones of Iran

Abstract

Populations of parasitoids may vary in key life history traits due to differences in selection regimes that have resulted in local adaptation. Climate is one source of selection that may affect life history traits of parasitoids. We compared eleven populations of *Leptopilina boulardi* originating from different climatic zones in Iran to assess the effect of climate on several life history traits. We checked for *Wolbachia* infection and for phylogenetic autocorrelation of the life history traits, which might interfere with climate-induced patterns. None of the populations was infected with *Wolbachia*. Only egg-size showed evidence for phylogenetic non-independence. Measures for energy reserves (lipid, glycogen and sugar contents), as well as initial reproductive effort (egg load) showed significant divergence between climatic zones. Of the various factors that define climatic zones, two were especially powerful in explaining life history variation. These were the number of frost days per year and the number of rainy days per year. Lipid reserves were positively correlated with the number of rainy days per year, with the highest lipid contents found in very wet and mild climate, intermediate ones in dry regions with cold winters and low lipid reserves in temperate areas. Both glycogen and sugar reserves showed negative correlations with the number of frost days per year. The lowest glycogen and sugar reserves were found in regions that were extremely cold during winter and the highest glycogen and sugar were found in humid temperate regions with mild winters. Glycogen, thus, also showed a positive correlation with precipitation. The highest egg loads were observed in regions with only cold winters (and hot dry summers), while the lowest egg loads were observed in temperate areas with mild winters. Egg size did not correlate with any climatic factor. A trade-off between reproductive effort (egg load) and glycogen reserves is suggested by the negative correlation between the two. A positive phenotypic

correlation was found between egg load and lipid contents. We conclude that life history traits of parasitoid insects evolve quickly in response to climate, with the apparent exception of egg size.

Introduction

Local adaption in life history traits can occur through genetic change in response to local geographical selection pressures. Natural selection results in genetic change if phenotypic variation in a fitness-related trait is heritable (Endler, 1986). Different biotic and abiotic factors, such as the spatial and temporal distribution of resources, interactions with other species, or climate and geographical barriers may contribute to selection and divergence of geographically isolated populations (Schluter, 2001). Parasitoids are insect that lay their egg on or inside the body of pre-imaginal stages of other insects (eggs, larvae or pupae) and complete their development by consuming the host tissue and killing their host (Godfray 1994, Quicke 1997). The strong dependence of immature parasitoids on their hosts, and the arms race between parasitoids and hosts make parasitoids an interesting model system to study different aspects of ecology and evolution (Godfray and Shimada 1999).

Several recent studies consider the effects of climate on life history traits (*e.g.*, Stenseth and Mysterud, 2002, Stenseth et al. 2002; Winkler et al. 2002; Stenseth and Mysterud, 2005). Apart from the fact that parasitoid life histories may be under direct selection by climate factors, host abundance and the spatial and temporal distribution of hosts are also important sources of selection on key life history traits in parasitoids (Ellers et al. 2000b). Host abundance and distribution are highly dependent on temperature and precipitation, and thus depend on climate (Ellers et al. 2000b; Ellers and Jervis 2003). Hence, climate may affect life history traits of parasitoids directly or indirectly (*i.e.*, by changing host distribution pattern). Variation in host distribution patterns has been linked to intraspecific variation in foraging traits of several parasitoids, including *Cotesia glomerata* (Vos and Vet, 2004), *Asobara tabida* (Kraaijeveld & van der Wel, 1994; Kraaijeveld et al., 1995), and *Leptopilina boulardi* (Dubuffet et al., 2006). Hoffman et al. (2007) showed antagonistic selection between thorax and wing size in *Drosophila melanogaster* which resulted from resource distribution patterns. Populations that need to travel long distances between resource patches were selected for longer wings and shorter thorax than sedentary populations.

Differences in selection regimes due to variation in climate, have also been shown to play an important role in shaping life history traits of many other organisms. The effect of climate on life history traits is dictated by relative strength of natural selection and gene flow (Endler 1986). A wide range of climatic factors including temperature, precipitation, light

regime and seasonal length may all shape life history traits. Of these, temperature is generally regarded as the most influential factor in insect life history traits (Johnston and Benneth, 1996; Dahlgaard et al. 2001; Clarke, 2006). Insect life history traits may respond to temperature variation by phenotypic plasticity. Examples include the metabolic rate of a cricket, *Cratomelus armatus* (Nespolo et al. 2007), the number of eggs in the butterfly *Bicyclus anynana* (Steigenga and Fischer 2007) and fat and protein content of the butterfly *Lycaena tityrus* (Karl and Fischer, 2008). Inverse relationships between body size (Bazzocchi at al. 2003; Karl and Fishcher, 2008), longevity (Nilssen 1997; Dhileepan at al., 2005; Colinet at al. 2007) and egg size (Fischer et al. 2003) with temperature have also been documented for insects. In addition to phenotypic plasticity, variation in temperature may also result in local adaptation of insect populations. This may contribute to population differentiation and speciation. Ohtsu et al. (1999) studied life history trait adaptation of six different *Drosophila* species from three different climate zones, sampling two species from within each zone. The species varied in cold resistance and showed differences in the ratio of saturated to unsaturated fatty acids, and also differences in the composition and quality of unsaturated fatty acids in the cold tolerant species (Ohtsu e al. 1999). While we are still a long way from understanding the biochemical mechanism of cold tolerance in insects, some studies have shown that cold-tolerant populations show different energy reserve strategies compared to less tolerant populations. Often, these populations accumulate cryoprotectants such as glycerol, sorbitol, inositol or trehalose which provide colligative resistance against freezing (Lee et al. 1987; Masahiko, 2001). A comparative study of different populations of *D. buzzatii* collected along an altitudinal gradient with large climate variation revealed thermal adaptation in heat resistance, desiccation resistance and Hsp70 gene expression (Sørensen et al. 2005).

Other climate factors have also been shown to affect life history traits of insects. In an experiment investigating the effect of photoperiod on the phenotypic plasticity of life history traits of *Drosophila melanogaster*, a prolonged day/night cycle resulted in prolongation of developmental time on pupae and longer lifespan in male flies (Vaiserman et al 2008). Furthermore, humidity and precipitation have been reported to affect life history patterns of insects. In the hymenopteran parasitoid *Anisopteromalus calandrae* increased humidity resulted in significantly decreased developmental time and increased population growth rate (Smith, 1993). In *Drosophila melanogaster*, increased humidity resulted in increased survival of both eggs and pupae (Al-Saffar, et al. 1995).

The effect of single climate factors, as measured in the laboratory, cannot easily be used to predict changes in life history patterns of organisms in nature because of interactions between different climate factors.

Comparing life history traits of populations of organisms originating from different climatic regions, (*e.g*. clinal variation studies; Sørensen et al. 2005), can provide more information how climate affects life history, but does not easily allow one to disentangle the effects of the different climatic factors on particular life-history traits. Local adaptation in life history in response to climate has been demonstrated in several insect species. For example, individuals of the antlion, *Myrmeleon hyalinus* originating from a Mediterranean climate showed significant differences in body size and life span compared to those originating from a desert population (Schraf et al. 2008). *D. buzzatii* populations from highlands showed heritable differences in activity pattern from those of populations from lowland sites (Dahlgaard et al. 2001). Allele frequencies of genes that are potentially important for adaptation to environmental stress were shown to exhibit clinal variation (Bettencourt et al., 2002; Frydenberg et al., 2003). Most clinal variation studies revealed variation between populations over broad geographical scales. Latitudinal clines have less often been studied but may show similar patterns: Griffiths et al. (2005) showed an increase in starvation resistance with increasing latitude along a narrow geographical cline for *Drosophila birchii* populations from rainforests in Queensland, Australia (Griffiths et al. 2005). Variation in trade-offs between different life history traits (e.g., between reproductive and survival related traits; Roff, 1992) may also show clinal patterns. An example of clinal variation of two traits resulting in a trade-off relationship was observed for *D. buzzatii* where an increase in early reproduction with altitude was accompanied by shorter life span (Norry et al. 2006).

Here we tested the prediction that climate variation can result in adaptive differences in resource allocation of parasitoid populations. We measured life history traits of 11 populations of *Leptopilina boulardi* originating from five contrasting climate zones in the Islamic republic of Iran. Field sampling was conducted along a cline from very wet rain forest in the north to dry and hot desert in the centre of the country. In an earlier study we described the genetic structure of these populations (Chapter 4). We showed genetic differences between some of the local populations, indicating the potential for local adaptation to climate.

Material and methods

Parasitoids populations

We used 11 population of *Leptopilina boulardi* from five climatic zones of Iran (Table 1). The five different climate zones can be described briefly as follow: Zone 1: mountains with low vegetation along the valleys. A relatively dry climate with a long and cold winter. Most precipitation occurs as snow in winter. Zone 2: forest along the temperate Caspian coastal plain. A mild climate with high precipitation which is uniformly

distributed over the year. Winters are mild, with few freezing days, and periods of drought seldom occur. Zone 3: a very wet and cold region on the Caspian coast. It has a climate with the highest precipitation and cold winters with substantial snow and many freezing days. Most part of this region is covered with high dense deciduous forest and some rice fields. Zone 4: a dry region where vegetation is mostly limited to cultivated areas and orchards. It has dry and hot summers while the winters are cold and long with a high number of freezing days. This climate has the lowest precipitation of Iran. The variation in temperature between day and night is large in this region. Zone 5: a Mediterranean type climate in which most of the vegetation is limited to orchards and with some natural vegetation along the river. Summers are relatively dry, but there is substantial snow in winter. The amplitude in temperature (differences in day and night temperature) is also high in this climate.

The genetic structure of these populations has been characterized in a previous study (Chapter 4). The populations from the Caspian Sea coast were sampled from rain forests, except the ones from Astaneh and Nour. The Sorkhabad population originated from a mountain region and was collected on the northern slope of Damavand along a river which leads to forest in Mzandaran province near the Caspian Sea coast. Populations from the semi dry and Mediterranean region of central Iran were all collected along the Zayandehrod river between Esfahan and Shahrekord. All samples were collected in July 2006 and subsequently cultured on *Drosophila melanogaster* in our lab at 25 ± 1ºC and 16/8 h day/night regime.

Test for Wolbachia infection

All Iranian *Leptopilina boulardi* populations were checked for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* gene using forward primer *wsp*81F, 5´-TGG TCC AAT AAG TGA TGA AGA AAC-3´ and reverse primer *wsp*691R, 5´-AAA AAT TAA ACG CTA CTC CA-3´ (Braig et al. 1998, Zhou et al. 1998). DNA was extracted from a single female wasp randomly chosen from the each strain using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, U.S.A.). A DNA sample of *Wolbachia*-infected *Asobara japonica* was included as a positive control in all series of amplification. The PCR amplification was performed in a final volume of 20µl containing 1.0 µl DNA template, 2.0 µl 10x PCR-buffer , 0.25 mM of each dNTP, 0.25 µM each primer, 1.0 U of *Taq* polymerase; all Qiagen's products. The amplification was carried out on an MJ DNA Engine PTC-200 (BioRad, Mississaugua) with the following thermal cycles: 3 min at 94 ºC, then 35 cycles of 1 min at 94 ºC, 1 min at 55 ºC and 1 min at 72 ºC, ending with 5 min at 72 ºC. To visualize any possible amplified band a volume of 4µl of the PCR product were loaded and run on a 1 % agarose gel and stained with ethidum bromide.

Table 1: The origin and climate zone description of 11 population of Iranian *Leptopilina boulardi*.

Zone	Description	Location	Elevation (m)	Precip. mm & days/year	Ave. RH	Daily temp.	Max. Temp	Min. Temp	Freezing day / year
1	Cold mountain	Sorkhabad (Mountain)	1975	282 mm 70 days	51	8.8	16.4	1.2	160
2	Temperate coastal Caspian sea	Qaemshahr 1 forest	76	898 mm 106 days	82	17	20.8	13.1	7.5
2	Temperate coastal Caspian sea	Qaemshahr 2 forest	75	"	"	"	"	"	"
2	Temperate coastal Caspian sea	Nour recreation park	20	"	"	"	"	"	"
2	Temperate coastal Caspian sea	Chalus forest	76	"	"	"	"	"	"
3	Very wet coastal Caspian sea	Astaneh rice field	8.5	1359 mm 138 days	82	15.9	20.6	11.3	24.9
3	Very wet coastal Caspian sea	Lunak mountain forest	485	"	"	"	"	"	"
3	Very wet coastal Caspian sea	Seyahkal mountain forest	378	"	"	"	"	"	"
4	Dry and hotwith cold winter	Khairabad (bushes and tree)	1605	122.8 mm 44.5 days	40	16.2	24.5	9.1	73
4	Dry and hotwith cold winter	Dorcheh (bushes and tree)	1625	"	"	"	"	"	"
5	Mediterranean with very cold winter	Zamankhan (bushes and tree)	1873	317 mm 54 days	46	11.8	20.2	3.5	124

Test for phylogenetic independence

Closely related populations may resemble each other in life history traits because of their shared ancestry, rather than because of local adaptation (Harvey & Pagel 1991). We assessed whether closely related populations were more similar than distantly related ones for the traits we measured using the test for serial independence (TFSI) as implemented in the software Phylogenetic Independence version 2.0 (Reeve and Abouheif 2003). TSFI is a parametric test to detect self-similarity among the adjacent observations and reveal phylogenetic autocorrelation (Abouheif 1999). To apply TFSI we used a phylogenetic tree based on AFLP genotypes for these populations (see chapter 4). The average value for each trait was calculated for each population and placed at the tips of the phylogenetic tree. These values were then randomly reshuffled 1000 times. The observed topology of trait values was then compared to the generated distribution of random topologies. We report the P-values representing the likelihood that the observed topology could have arisen by chance, *i.e*. in the absence of phylogenetic autocorrelation.

Life history trait measurements

Collecting individual samples

All life history traits were measured on virgin female wasps harvested from the lab cultures within five hours of eclosion. We used 25 individuals from each location, except for the two climatic zones 1 & 5 (Sorkhabad and Zamankhan) with only one sampling point. In the latter case we used 40 individuals. Wasps were chosen randomly from 10 rearing jars, isolated and stored at -80ºC to halt all metabolic processes until further analysis. All measurements were taken for each individual. A photometric method was used to measure the egg load and egg size and the same dissected bodies were used for energy reserve measurements.

Egg load and egg size

Wasps were dissected on a cover glass and the ovarioles transferred to a small drop of demineralised water. The ovarioles were then opened and the eggs separated until they did not overlap (Ellers 1996). Two digital photographs were taken from each dissection, one containing all eggs to count the number of eggs (egg load) and the other from a randomly chosen group of 20 eggs together with a scale (to measure egg size). In all populations hardly any immature eggs were found. We decided therefore to classify all eggs as being mature. Egg size was calculated by

measuring the average area of 20 eggs per individual relative to the scale using Image-J image analysis software (Rasband, 1997-2005). The cover glass containing the whole body including ovarioles and eggs was washed into an eppendorf tube for further analysis (see below).

Lipid, sugar and glycogen analysis

To measure the lipid, glycogen and sugar contents of each individual we adopted a colorimetric analysis protocol (van Handel 1985a, b; van Handel and Day 1988). After dissection of the ovaries (see above) the wasps (including eggs) were individually washed in eppendorf tubes containing 80 µl of 2% sodium sulfate solution and grinded in the solution by a plastic pestle. 600 µl choloroform-methanol (1:2) was then added to tubes after which they were stored overnight at 4ºC. The next day, the tubes were vortexed for one minute and centrifuged for 15 minute at 1400 rpm. The supernatant was divided into two parts of 300 µl each in separated tubes. These were used for lipid and sugar assays respectively and the precipitant was used for the glycogen assay.

Lipid assay

The first series of tubes with 300 µl of supernatant was put on an aluminum heat block at 90 ºC under a fume hood until the solution had evaporated completely. Then 40 µl of 98% sulphuric acid was added to the tubes which were then heated for 2 minutes at 90 ºC and subsequently cooled down on ice. A volume of 960 µl of Vanillin reagent (premixed: 300mg vanillin, 200ml phosphoric acid 85% and 50ml distilled water) was added to the tubes and they were left for 15 minute at ambient temperature. The absorbance was read at 525 nm by a spectrophotometer (Unicam UV 1, Unicam Ltd, Cambridge, UK).

Sugar

The second series of tubes with 300 µl of supernatant were evaporated at ambient temperature using a Speed Vac plus SC110A (Fisher Scientific Bioblock, Illkirch, France). The samples were watched to avoid complete evaporation. A volume of 1 ml of Anthorone reagent (premixed: 125mg Anthrone, 63 ml sulphuric acid 98% and 25ml water) was added to the tubes, which were then put at 90 ºC for 10 minutes. After cooling on ice, the absorbances of the sample were read at 960 nm in a spectrophotometer (Unicam UV 1).

Glycogen

To each tube containing precipitant 400 µl of 80% methanol was added. These were mixed by vortexing for one minute and centrifuged for 5 minutes at 1400 rpm. The supernatant was discarded to eliminate

the sugar trace from the samples. One ml Anthrone reagent was added to each tube, which were then incubated at 90 ºC for 15 minutes and cooled down on ice. To eliminate protein fragments from the solution they were filtered through a membrane (PVDF 0.45µm; Millipore). The absorbance of the samples was read at 625 nm in a spectrophotometer (Unicam UV 1). As a control a blank tube without wasp was added to each set of samples and in each trial the absorbance of the blank was set at zero to correct the possible change in the strength of reagent over time. Reagents were stored at 4 ºC and they were refreshed every three days during the course of the experiment. The amounts of lipid, sugar or glycogen were calculated using the calibration curve made for each assay by measuring a serial dilution soybean (for lipid) or glucose (for sugar and glycogen).

Statistic analysis

All measurements of energy reserves (lipid, sugar and glycogen) and investment in reproduction (egg load and egg size) were compared between populations using analysis of covariance (ANCOVA) in which tibia length was added as a covariate to represent body size. We calculated egg mass as the number of eggs at eclosion times the egg size and used this value as total reproductive effort and to calculate total energy reserves we added the weights of lipids glycogen and sugars. In each case we searched for the minimum adequate model by starting with the maximal model, then dropping the interaction term and then main factors from the model if non-significant. The simpler model was compared to the more complex model using an F-test. Traits were compared between populations using post-hoc t-tests. For visualizing the size-corrected results in graphs we plotted residuals of the linear model of each trait against tibia size. To investigate possible trade-off between reproductive effort and stored energy within populations first we corrected all traits for tibia length and then tested correlations between egg load and related traits in linear models. If correlations were found to be significant we then examined both classical (r_c) and robust (r_r) Pearson correlations using the R package Mvoutlier (Gschwandtner and Filzmoser 2007). Robust correlation ignores outliers. The effect of climatic factor on life history traits was studied using linear models. All statistical analyses were conducted in the free statistical software R 2.5.1 (Ihaka & Gentleman, 1996).

Results

Wolbachia infection

No bands were observed on the agarose gels containing the PCR products of the *wsp* gene except in positive control. Therefore all 11 populations of *Leptopilina boulardi* were free of *Wolbachia*.

Phylogenetic independence

Only egg size showed evidence of phylogenetic autocorrelation (tests for serial independence: egg size P = 0.01; egg load P = 0.15; lipid reserve P = 0.09; glycogen reserve P = 0.17, sugar reserve P = 0.31). We therefore did not correct these traits for phylogenetic effects.

Egg load

ANCOVA revealed that egg load differed significantly between populations ($F_{10,290}$ = 22.74, P < 0.001, Fig. 1) and covaried with body size ($F_{1,290}$ = 227.31, P < 0.001). However, the slopes of the relation between egg load and body size did not differ significantly between populations (interaction effect $F_{1,290}$ = 1.22, P = 0.28). Therefore, larger wasps carry more egg than smaller wasps in all eleven populations. Apart from the Nour population from zone 2, a humid mild climate, which showed the lowest egg load of all populations, post-hoc t-tests distinguished three groups: Sorkhabad from a mountain region together with three populations from temperate Caspian Sea coast (zone 2) showing the lowest egg loads; three populations from the very wet Caspian sea coast (zone 3) showing intermediate egg loads and two populations from semi dry climate (zone 4) together with Zamankhan from a Mediterranean climate (zone 5) showing the highest egg loads (Fig. 1).

Egg size

ANCOVA revealed significant differences in egg size between populations ($F_{10,290}$ = 3.05, P < 0.001, Fig. 2) and significant covariation with body size ($F_{1,290}$ = 4.37, P = 0.03). The slopes of the relation between egg size and body size did not differ significantly between populations (interaction effect $F_{1,290}$ = 1.05, P = 0.41). Post-hoc t-test revealed that egg size was significantly smaller in three northern populations from the temperate Caspian coast (zone 2; Qaemshar 1, Qaemshahr 2 and Chalus) than in all others except Sorkhabad from the mountains.

Lipid reserve

Lipid content differed significantly between populations (ANCOVA $F_{10,290}$ = 13.49, P < 0.001, Fig. 3) and covaried with body size ($F_{1,300}$ = 41.33, P < 0.001). The slopes of the relation between lipid content and body size did not differ significantly between populations (interaction effect $F_{1,290}$ = 1.78, P = 0.35). Therefore, larger wasps contain more lipid than smaller wasps in all eleven populations. Post-hoc t-tests showed that two populations from very wet Caspian Sea coast (zone 3; Lunak and Seyahkal) carried significantly higher lipid content than the other populations. Two populations from the semi dry region (zone 4; Dorcheh and Khairabad) showed relatively high lipid reserves but differed significantly only from

two temperate Caspian sea populations (Nour and Chalus), which had the lowest lipid reserves. All other populations statistically group together as intermediate (Fig. 3).

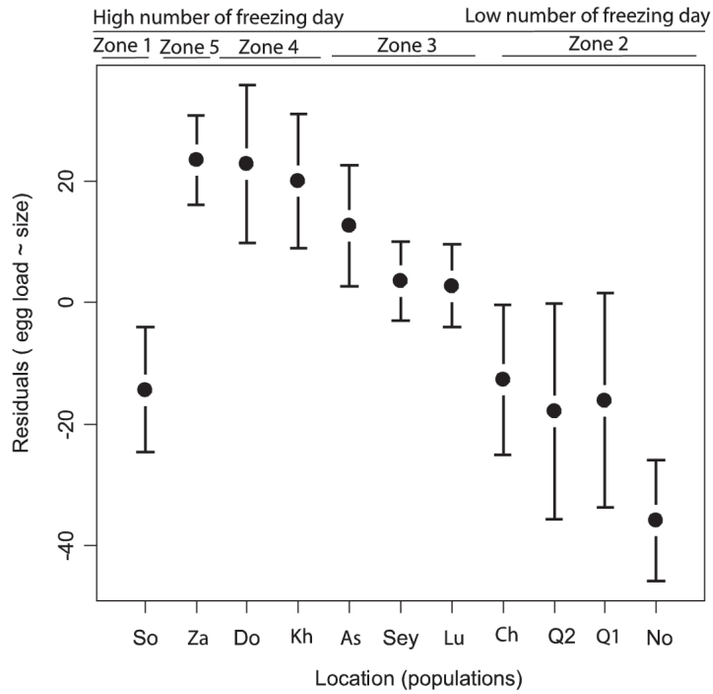

Fig.1: Egg load in 11 Iranian populations of *Leptopilina boulardi*.(values are residuals of the regression of egg load on tibia length to correct the effect of size; error bars represent Standard Error) Populations are ordered according to their ranked number of frost days per year (high to low). The correlation between egg load and freezing days is significant (t = 3.49, P < 0.001).

Glycogen reserve

ANCOVA revealed significant differences in glycogen reserve between populations ($F_{10,290}$ = 24.53, P < 0.001, Fig. 4) and covariation with body size ($F_{1,290}$ = 6.21, P < 0.001). The slopes of the relation between glycogen content and body size did not differ significantly between populations (interaction effect $F_{1,290}$ = 1.82, P = 0.056) and the only variation was in the steepness of the slopes, not in their direction. Thus larger wasps contained more glycogen than smaller wasps in all eleven populations. Post-hoc t-tests revealed that glycogen reserves of two populations from the dry region (Dorcheh and Khairabad), one from a Mediterranean climate (Zamanhan), one from a mountainous region (Sorkhabad) and one from temperate Caspian Sea coast (Nour) were significantly lower that those of all other populations. Overall, the populations from temperate and very

wet temperate Caspian sea areas showed significantly higher glycogen reserves than populations from mountainous and dry regions. Astaneh from zone 3 showed significantly higher glycogen reserve than all other populations (Fig. 4).

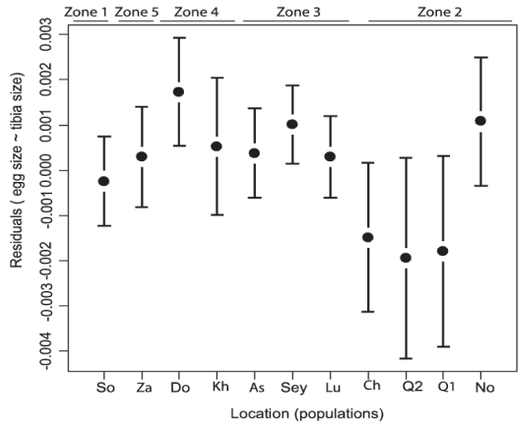

Fig.2: Egg size in 11 Iranian populations of *Leptopilina boulardi* (values are residuals of the regression of egg size on tibia length to correct the effect of size and error bars represent Standard Error). The climatic zones of each population are shown at the top of the graph.

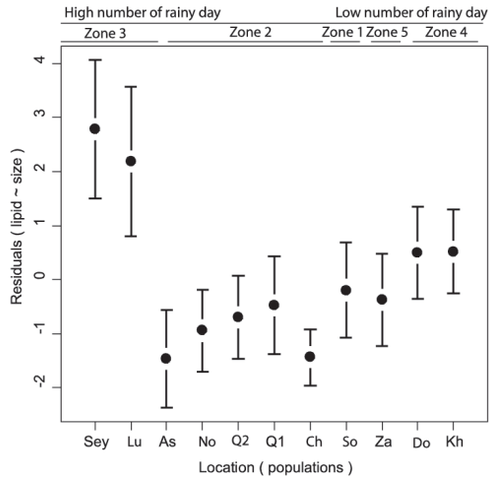

Fig.3: Lipid reserves in 11 Iranian populations of *Leptopilina boulardi* (values are residuals of the regression of lipid reserve on tibia length to correct the effect of size; error bar represent Standard Error). Populations are ordered according to their ranked number of rainy days per year (high to low). Correlation between lipid reserve and rainy days is significant after excluding first two populations ($t = -3.45$, $P < 0.001$).

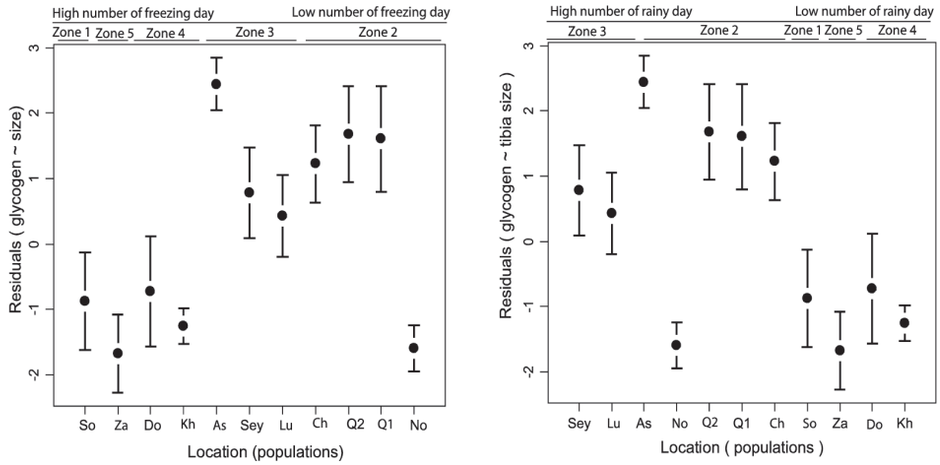

Fig.4: Glycogen reserves in 11 Iranian population of *Leptopilina boulardi* (values are residuals of the regression of glycogen reserve on tibia length to correct the effect of size and error bars represent Standard Error). Populations are ordered according to their ranked number of frost days per year (left panel: high to low) and their ranked number of rainy days per year (right panel: high to low). Both correlations between glycogen reserve and freezing days and rainy days per year are significant (Freezing days; $t = -8.17$, $P < 0.001$, rainy days; $t = 9.66$, $P < 0.001$).

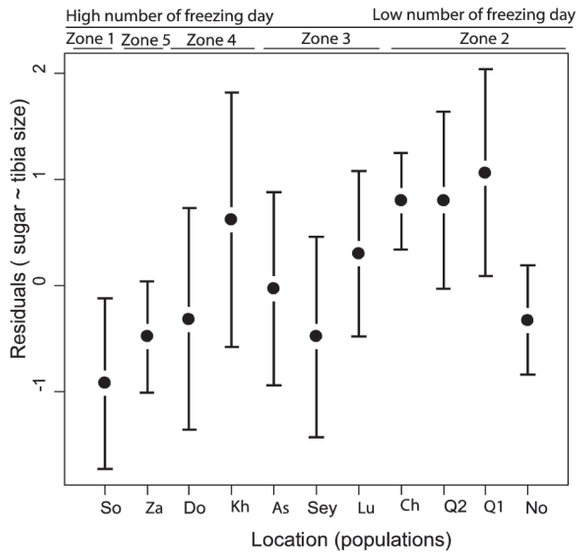

Fig.5: Sugar reserves in 11 Iranian populations of *Leptopilina boulardi* (values are residuals of the regression of sugar reserve on tibia length to correct the effect of size and error bars represent Standard Error). Populations are ordered according to their ranked number of frost days per year (high to low). The correlation between sugar reserve and freezing days is significant ($t = -3.77$, $P < 0.001$).

Sugar reserve

Sugar reserves differed significantly between populations ($F_{10,290}$ = 5.94, P < 0.001, Fig. 5) and covaried with body size ($F_{1,290}$ = 5.95, P = 0.015). The slopes of the relation between sugar content and body size did not differ significantly between populations (interaction effect $F_{1,290}$ = 1.08, P = 0.38). Therefore, larger wasps contained more sugar than smaller wasps in all populations. Post-hoc t-tests revealed higher sugar reserves for populations originating from two Caspian coastal zones in comparison with populations from drier regions including Sorkhabad from the mountains (Fig. 5). A slightly lower sugar reserve was also observed for two northern populations (Seyahkal and Lunak) from zone 3 in comparison with other northern populations, but these differences were only significant in one case (Fig. 5). One population from zone 2 (Nour) and one population from zone 3 (Astaneh) showed intermediate sugar reserves.

Interaction between climate and life history traits

We tested the effects of several climatic factors (average daily temperature, average maximum and minimum temperature, thermal amplitude, number of frost and rainy days per year) on life history traits. Two factors, the number of frost days per year and the number of rainy days per year showed significant correlations with certain life history traits. Egg load was correlated with the number of frost days (Fig. 1) and lipid reserves with the number of rainy days rainy days. For lipid reserve, two populations from the very wet Caspian coast showed a pattern different from the rest. Excluding these two populations yielded a significant negative correlation between the number of rainy day per year and lipid reserves (Fig. 3). Glycogen reserves were correlated with both the number of frost days and the number of rainy days per year (Fig. 4). Sugar reserves correlated with the number of frost days per year (Fig. 5).

Trade-off between energy reserves and reproductive effort

When all data from the 11 populations was pooled, lipid reserves correlated positively with egg loads (reproduction effort; r = 0.19, P < 0.001, Fig. 6). Glycogen was the only stored resource which showed a negative correlation with egg load (Glycogen; r = -0.16, P = 0.004, Fig. 6), suggesting a trade-off. A negative correlation between sugar reserves and egg load was not significant (r = -0.11, P = 0.06). These patterns were even stronger when analyzed using robust correlation (Fig 6, Glycogen; r_r = -0.35, Lipid; r_r = 0.3).

Egg mass and energy reserves

Both egg mass and energy reserves differed significantly among populations (ANCOVA egg mass; $F_{10,290} = 8.57$, $P < 0.001$, energy reserve; $F_{10,290} = 13.31$, $P < 0.001$ Fig. 7) and covaried with body size (egg mass; $F_{1,300} = 70.35$, $P < 0.001$; energy reserve; $F_{1,300} = 35.29$, $P < 0.001$). The slopes of the relation between either egg mass or energy reserve and body size did not differ significantly between populations (interaction effect, egg mass; $F_{1,290} = 0.68$, $P = 0.74$, energy reserve; $F_{1,290} = 1.47$, $P = 0.14$). Therefore, larger wasps contain more or larger eggs and more energy than smaller wasps in all eleven populations. Post-hoc t-tests showed that all populations from the temperate Caspian Sea coast (zone 2) together with the mountain population, Sorkhabad, had significantly lower egg masses than other populations. The wet coastal Caspian populations (zone 3) had intermediate egg masses and populations from dry zones (zones 4 and 5) had the highest egg masses but the values were not significantly higher than for those of the wet coastal Caspian populations. All populations from the Caspian coastal zone (zones 2 and 3) showed significantly higher energy reserves than those from central Iran and mountains. With the exception of the Sorkhabad population and one population from the temperate Caspian coastal plain, Nour, both of which had low energy reserves and relatively low egg masses, the occurrence of a higher egg mass comes at the cost of lower energy reserves (Fig. 7).

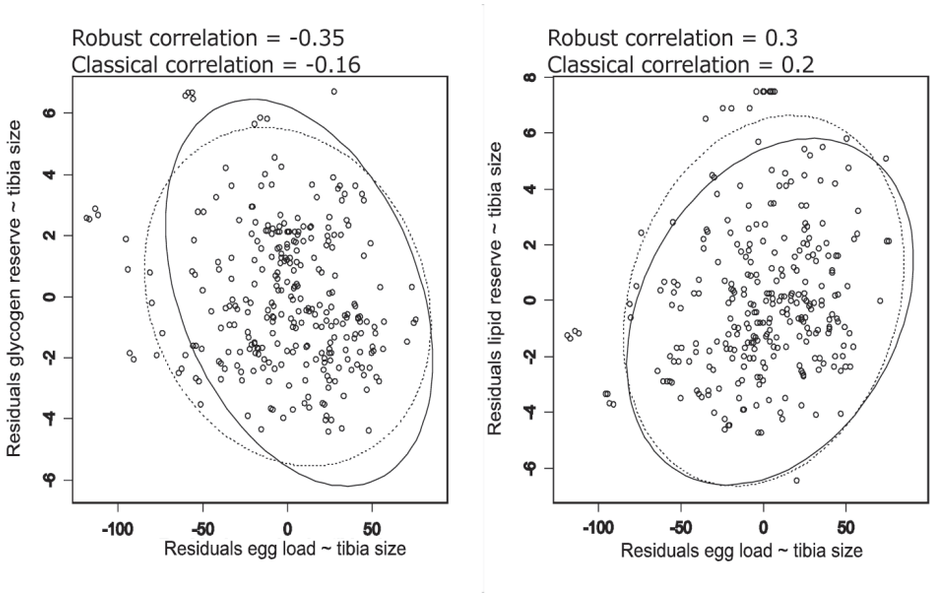

Fig.6: Classical and robust correlations between size-corrected egg load and size-corrected glycogen and fat reserves in Iranian *Leptopilina boulardi*. The data from the 11 populations have been pooled. Solid lines showed robust and dotted line classical correlation.

Discussion

Our results demonstrate substantial variation in most of the life history traits we studied. Four out of the five traits showed no phylogenetic autocorrelation which indicates the potential adaptive value of trait variation. Egg size, the only trait with significant phylogenetic autocorrelation, was also found to be the most conserved trait in all populations.

Direct effects of climate

Consistent with the idea that climate can influence life history traits, our results demonstrate a strong correlation between life history traits and two components of climate, namely the frequency of frost and rain per year. As *L. boulardi* spends the winter season in pre-pupal diapause inside the host's puparium, a direct effect of the number of frost days on life history traits of adult *L. boulardi* females seems unlikely. However, the number of frost days may itself be correlated with other important aspects of climate, *e.g.*, the length of the reproductive season for *L. boulardi*. Moreover, sudden and unexpected drought can make zone 4 and 5 unpredictable. In contrast to these zones, the climate data for zone 2 show a relatively stable pattern with almost no frost in spring and autumn, few freezing days during winter and almost no periods of drought during summer. Population crashes due to droughts, and the lack of such crashes in more stable climatic regions could be important as sources of selection on life history traits. Climate also affects life history traits indirectly, by shaping the biotic environment and with it the spatial and temporal patterns of host distribution.

Climate and host availability

Ellers *et al*. (2000b), in a theoretical study on optimal resource allocation strategy in parasitoid wasps, revealed a crucial role of abundance and variation in the spatial distribution of hosts on the evolution of initial egg load. This study predicted that parasitoids in habitats where host abundance is low and variance in the spatial distribution of hosts is high, should invest relatively more in early egg production than parasitoids in an environment with similar host abundance but low variance in the spatial distribution of hosts. In synovigenic parasitoids, which are able to mature egg during their adult lifespan, the optimal timing of egg maturation depends thus on the spatial variation in host distribution, but also on body size (Ellers and Jervis 2003). The *L. boulardi* populations that we studied seem to be pro-ovigenic and unable to mature eggs during adult life. According to the predictions of the Ellers *et al*. (2000b) model, they should also have higher egg loads at eclosion with increasing variance in the spatial distribution of hosts when average host abundance is low.

However, other theories concerning egg load predict that it should also increase with average host abundance and with increased mortality risk of immature parasitoids to balance mortality (Price, 1974). Lack of detailed data on the spatial distribution of hosts and on the population dynamics of the populations we studied, makes it hard to predict the evolution of egg loads and to explain differences observed between populations. However, for the populations collected along the Zayandehrod river between Esfahan and Shahrekord, the habitat consisted of a small strip of irrigated soils along the river, otherwise surrounded by arid desert country. In the irrigated zone along the river orchards producing fruits of a variety of species are scattered among vegetable fields. Females from these populations have to move between different fruit crops during successive generations, and will often need to disperse to find suitable reproductive habitat. However, once arrived in an orchard with ripe fruits they can easily find a large number of hosts. We predict therefore that these wasps have high egg loads and lower lipids and carbohydrate reserves in comparison with other populations, and this is indeed what we found. The coastal plain along the Caspian sea is mainly used as a rice growing area, and orchards and fruit trees are scarce. Although the long season and the high frequency of summer rains provides a stable climate and prevents that host populations crash due to droughts, parasitoids must travel long distances between sites with fruits. We thus expected egg loads to be lower in females from populations in this region and expected them to have higher energy reserves at the start of adult life. We predicted the populations collected on the mountain slopes facing the Caspian Coast (Lunak and Seyakhal) to have egg loads intermediate between those of the populations collected along the Zayandehrod river and those from the coastal plain, because there are more fruit trees in this region than on the coastal plain, but in densities much lower than along the Zayandehrod river. As this is confirmed by our data, we suggest that abundance of suitable host habitat (fruits) is an important factor in the egg load evolution, and that climate affects the evolution of egg load only indirectly by shaping the biotic aspects of the parasitoid's habitat.

Egg size adjustment in parasitoids

Egg size showed the least divergence between populations. Only the three populations from zone two (the wet Caspian coastal region), had smaller egg sizes compared to other populations. In other insects, egg size is positively related to offspring fitness. In koinobiont parasitoids with hydropic eggs there is no clear relation between egg size and offspring survival. In idiobiont parasitoids with anhydropic eggs, on the other hand, egg size may affect the success in larval competition. Collier et al. (2002) studying two *Encarsia* species, showed that *E. pergandiella* with smaller eggs performed better in interspecific competition than *E. formosa* which has larger eggs. In coleopteran parasitoids a positive correlation between

egg size and larval weight has been found, in which larger parasitoid larvae emerging from bigger eggs performed better by surviving longer and parasitizing their host more rapidly than smaller ones originating from small eggs (Boivin and Gauvin 2009). In *L. boulardi*, a koinobiont species with hydropic eggs, a relation between offspring fitness and egg size is not evident. We suggest that the small eggs of *L. boulardi* populations in climate zone 2 are the result of a trade-off between reproduction and energy reserves. Populations in this zone invest less in reproduction than other populations by having both fewer eggs and smaller eggs.

Lipid reserve and climate factors

The relationship between parasitoid lipid reserves and the number of rainy days per year showed a mixed pattern. The two populations from the very wet mountain slopes facing the Caspian coast (zone 3) showed high lipid reserves compared to other populations. However, the other nine remaining populations showed a steady increase in lipid reserves when we ranked them from very wet to dry zones. One of the most important roles of lipid in insect physiology is in maintenance of the soma, *e.g.* it plays an important role in starvation resistance and protection against desiccation and frost (Downer and Matthews 1976, Udonsi 1984, Djawdan et al. 1997, Minois and Le Bourg 1999, Colinet et al 2006). Lipids are also essential for egg production and hence play an important role in reproduction. As adult parasitoids seem unable to synthesize lipids *de novo* (Slansky 1986; Ellers, 1996; Rivero & Casas, 1999 ; Olson *et al.*, 2000; Rivero & West, 2002; Casas *et al*., 2003; Giron & Casas, 2003b ; Lee *et al.*, 2004 ; Visser & Ellers 2008), they are often a limiting resource in these insects causing a trade-off between investment in reproduction and longevity. This can result in between-population variation in lipid contents at the start of adult life as was shown by Ballard et al. (2008) who found clinal variation in starvation resistance in wild caught populations of *D. simulans* which correlated with lipid content. Our results revealed lower lipid reserves in less stressful environments.

We found the lowest lipid reserves in zone two, which is relatively humid, with little or no frost and relatively little desiccation stress. The negative correlation between the number of rainy days and lipid reserve in four out of five zones, could possibly be explained by selection for desiccation resistance in populations from dry regions. However, comparison of the data on egg mass and energy reserve suggests that populations from drier environments invest more in reproduction at the expense of the total energy reserves at the start of adult life, rather than just at the expense of lipids. We did not find a negative correlation either between energy reserves or lipid contents and egg mass (pooling the data of all populations).

This lack of a correlation may have been caused by two exceptional

populations, Nour and Sorkhabad, which showed relatively low egg masses

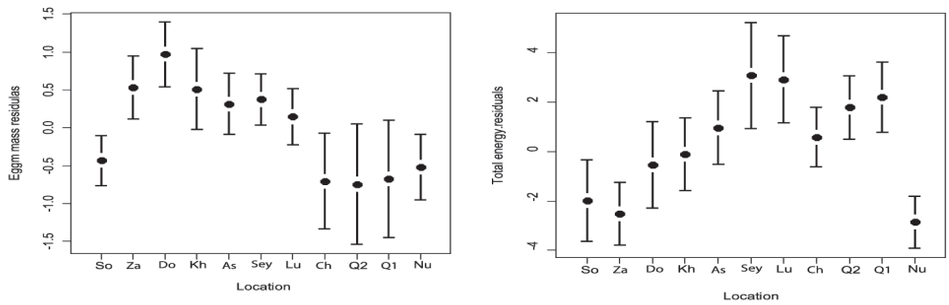

Fig.7: Egg mass and total energy reserve in 11 Iranian populations of *Leptopilina boulardi* (values are residuals of the regression of egg mass and total energy reserve on tibia length to correct the effect of size and error bars represent Standard Error).

and very low energy reserves. Another possibility is that reproduction in these populations is traded off against other investments of energy that we did not measure, e.g. in flight muscles. The climate in zone 3 contrasts with that of other areas sampled on the Caspian coast by its cool summer weather and long cold winters. This results in a relatively short season for *L. boulardi*, and the presence of extended natural forests and low human population density result in fruit being relatively scarce (not a good habitat for *D. melanogaster* and *D. simulans*, the hosts of *L. boulardi*). We think that large lipid reserves in the Seyahkal and Lunak populations may have been selected for by the long distance between resources, while we have argued earlier that the Astaneh population is an exception which may have been founded by flies transported via fruit from elsewhere (Chapter 4).

Carbohydrate reserves and climate factors

Beside lipids, carbohydrates play a key role as energy source in insect life. Carbohydrates can be mobilized more quickly than lipids and may thus display different environmental patterns (Schmidt and Mathur 1967). We found the highest glycogen and sugar levels in wasps from the wet and mild Caspian Coastal plain (zone two). Glycogen correlated negatively with the number of frost days per year and correlated positively with precipitation. Like lipids, glycogen has been suggested to provide stress resistance in insects (Djawdan et al. 1997, Graves et al. 1992, Marron et al. 2003, Zwaan et al. 1991, but see Sharmila Bharathi et al. 2003, Kostal et al. 2001). However, our results do not indicate a role for glycogen in desiccation and cold stress resistance in these populations. Instead, the pattern of glycogen storage may be explained by differences in dispersal behaviour, as the main flight fuel used by hymenopteran parasitoids are carbohydrates, in particularly glycogen (Vogt et al. 2000; Suarez et al. 2005). Only parasitoids that need to make very long

distance flights should preferentially store their energy reserves as lipids, as they can store more energy in the form of lipids in a limited space than as carbohydrates. Insects living in a more fine-grained habitat, where they can restore energy reserves more frequently, should store there energy reserves preferably as sugars and glycogen, and benefit from the fact that these can be mobilized quickly when needed. We have argued above that populations collected in zone 2 need long dispersal flights to find hosts, because the extended rice fields are unsuitable as habitat. Interestingly, Pelosse et al (2007) found a similar difference in glycogen allocation strategy in two populations of *Venturia canescens*, Arrhenotokous populations which live in natural habitats with scattered hosts and travel regularly between host patches stored more glycogen than thelytokous population which live in anthropogenic environments with aggregated hosts and no need to travel between host patches.

Trade-off between energy reserve and reproduction

Given that hymenopteran parasitoids lack the ability to synthesize lipids in adult life (Ellers 1996; Rivero and West 2002; Giron and Casas 2003; Visser and Ellers 2008), it has been suggested that they face a trade-off between allocating resources to reproduction or survival (Ellers 1996; Ellers et al. 2000a; Jervis et al. 2003; Jervis et al. 2008). In many cases this trade off may be masked by other factors, such as replenishing resources through host feeding (Rivero and West 2005; Ferracini, et al. 2006) or using resources to overcome habitat stress (Minois and Le Bourg, 1999; Colinet et al 2006; Ballard et al. 2008). We attempted to find support for this trade-off by pooling the data of all 11 populations and checking for a correlation between egg load and lipid and carbohydrate stores. Our results did not show a negative correlation between lipid reserve and egg load.

However, we did find a negative correlation between glycogen reserves and egg load. It should be noted however, that the only valid evidence of a trade-off is a negative causal relation between traits (*e.g.*, negative pleiotropy, or different allocation of a limiting resource to different functions). Our data are phenotypic correlations that can neither prove, nor disprove the existence of a trade-off. To demonstrate trade-offs, either experimental manipulation of phenotypic traits, or artificial selection experiments should be done. Our results demonstrate both high lipid reserves and egg-loads for parasitoids from the dry regions in zone 4 and 5 and also from two populations from cool wet Caspian mountain forests (climate zone 3). In these climate zones, we expect high variance in inter-patch travel times and thus selection for high initial fecundity (Ellers et al. 2000b).

To conclude, we have shown that large interpopulation variation exists in key life history traits between populations from different climatic

zones. We suggest that host distribution pattern, host abundance, length of the season and sources of a-biotic stress all have played a role in shaping the life history traits of these populations. We advocate the development of theories to predict the evolution of life-history trade-offs, which combine spatial and temporal patterns of host distributions and climate factors as sources of selection.

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Appendices

Appendix 1: Emergence, diapause and survival (emergence + survival) post-hoc t-test results from translocation experiment. BB = pupae transferred from bottom to bottom (control), WW = wall to wall (control), BW = bottom to wall,

		Emergence		
		BB	BW	WB
D. melanogaster	BW	-2.1 0.04		
	WB	4.2 < 0.01	5.9 < 0.01	
	WW	4.6 < 0.01	-6.2 < 0.01	0.6 0.5
A. citri	BW	-5.7 < 0.01		
	WB	1.0 0.32	6.6 < 0.01	
	WW	-4.9 < 0.01	0.9 0.34	-5.8 < 0.01
A. persimilis	BW	2.1 0.04		
	WB	-1.8 0.07	-3.6 < 0.01	
	WW	-1.1 0.27	-3.0 < 0.01	0.7 0.47
A. tabida	BW	6.9 < 0.01		
	WB	2.6 0.01	- 3.4 < 0.01	
	WW	5.4 < 0.01	0.57 0.57	3.1 < 0.01
A. japonica	BW	-3.6 < 0.01		
	WB	1.7 0.09	4.9 < 0.01	
	WW	-1.6 0.10	1.9 0.06	-3.2 < 0.01

WB = wall to bottom, NA = not applicable (no significant treatment effect observerd). *A. pleuralis* is not depicted, because no significant treatment effects were observed for all three traits. Upper number = t-value, lower number = P-value.

	Diapause			Survival		
	BB	BW	WB	BB	BW	WB
	5.2 < 0.01			3.0 < 0.01		
	0.6 0.6	-6.2 < 0.01		1.1 0.29	-2.0 0.04	
	5.2 < 0.01	-0.2 0.84	6.2 < 0.01	4.0 < 0.01	1.3 0.19	3.2 < 0.01
	NA			1.4 0.18		
	NA	NA		-2.5 0.01	-3.7 < 0.01	
	NA	NA	NA	-1.5 0.13	-2.7 < 0.01	1.1 0.30
	-7.2 < 0.01			4.0 < 0.01		
	-2.6 0.01	-4.5 < 0.01		2.3 0.03	-1.0 0.32	
	-7.2 < 0.01	0.0 1	-4.5 < 0.01	3.3 < 0.01	0.55 0.58	1.3 0.21
	3.6 < 0.01			NA		
	-2.0 0.05	-5.0 < 0.01		NA	NA	
	1.8 0.07	-1.7 0.10	3.6 < 0.01	NA	NA	NA

Appendix 2 : Comparisons of developmental time, survival (with and without food), metabolic rate, lipid reserve and egg load at eclosion in five species of

Trait	**Developmental time**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	NA				
citri	-15.0 <0.01	NA			
pleuralis	-75.5 <0.01	-60.7 <0.01	AN		
persimilis	-42.7 <0.01	-27.8 <0.01	32.8 <0.01	NA	
japonica	-41.5 <0.01	-26.9 <0.01	33.5 <0.01	0.74 0.45	NA
Trait	**Survival without food**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	Na				
citri	5.86 <0.01	NA			
pleuralis	8.46 <0.01	2.61 <0.01	NA		
persimilis	-6.25 <0.01	-12.05 <0.01	-14.55 <0.01	NA	
japonica	-14.69 <0.01	-20.5 <0.01	-23.13 <0.01	-8.23 <0.01	AN
Trait	**Lipid reserve at eclosion**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	AN				
citri	0.51 0.61	NA			
pleuralis	-2.70 <0.01	-3.18 <0.01	NA		
persimilis	-1.38 0.17	-1.87 0.06	0.915 0.36	NA	
japonica	-5.92 <0.01	-6.32 <0.01	-3.18 <0.01	-3.59 <0.01	NA

Asobara from a post-hoc z-test for first three traits and t-test results for last three traits. NA = not applicable. Upper number = z-value or t-value, lower number = P-value.

	Survival with food				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	-1.09 0.27	NA			
	-3.57 <0.01	-2.14 0.013	NA		
	-12.33 <0.01	-11.23 <0.01	-8.74 <0.01	NA	
	-24.23 <0.01	-23.11 <0.01	-20.65 <0.01	-11.9 <0.01	NA
	Metabolic rate				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	8.66 <0.01	NA			
	-2.07 0.04	-4.05 <0.01	NA		
	6.01 <0.01	2.7 <0.01	5.71 <0.01	NA	
	9.85 <0.01	9.53 <0.01	13.93 <0.01	7.91 <0.01	NA
	Egg load at eclosion				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	3.09 <0.01	NA			
	-4.32 <0.1	-7.29 <0.01	NA		
	8.28 <0.01	5.4 <0.01	12.02 <0.01	NA	
	-8.9 <0.01	-11.7 <0.01	-4.56 <0.01	-16.09 <0.1	NA

Appendix 3: Comparisons of ovigeny index, dry weight, egg size, lipid reserve and egg load at eclosion in five species of

Trait	**Ovigeny index**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	NA				
citri	0.55 0.61	NA			
pleuralis	-5.31 <0.01	-5.98 <0.01	NA		
persimilis	6.83 <0.01	6.54 <0.01	11.54 <0.01	NA	
japonica	-4.36 <0.01	-4.97 <0.01	0.75 0.46	-10.79 <0.01	NA
Trait	**Lipid reserve at eclosion**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	AN				
citri	0.51 0.61	NA			
pleuralis	-2.70 <0.01	-3.18 <0.01	NA		
persimilis	-1.38 0.17	-1.87 0.06	0.915 0.36	NA	
japonica	-5.92 <0.01	-6.32 <0.01	-3.18 <0.01	-3.59 <0.01	NA
Trait	**Egg size**				
Species	*tabida*	*citri*	*pleuralis*	*persimilis*	*japonica*
tabida	NA				
citri	-2.85 <0.01	NA			
pleuralis	15.65 <0.01	18.37 <0.01	NA		
persimilis	5.65 <0.01	8.29 <0.01	-9.147 <0.01	NA	
japonica	11.63 <0.01	14.28 <0.01	-3.37 <0.01	5.61 <0.01	NA

Asobara from a post-hoc t-test. NA = not applicable. Upper number = t-value, lower number = P-value.

	Dry weight				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	-1.68 0.09	NA			
	-0.35 0.73	1.26 0.21	NA		
	-7.15 <0.01	-5.59 <0.01	-6.57 <0.01	NA	
	0.62 0.54	2.18 0.03	0.93 0.35	7.28 <0.01	NA
	Egg load at eclosion				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	3.09 <0.01	NA			
	-4.32 <0.01	-7.29 <0.01	NA		
	8.28 <0.01	5.4 <0.01	12.02 <0.01	NA	
	-8.9 <0.01	-11.7 <0.01	-4.56 <0.01	-16.09 <0.01	NA
	Egg mass				
	tabida	*citri*	*pleuralis*	*persimilis*	*japonica*
	NA				
	-0.86 0.39	NA			
	6.91 <0.01	7.73 <0.01	NA		
	10.0 <0.01	10.79 <0.01	3.21 <0.01	NA	
	-0.11 0.92	0.69 0.49	-6.54 <0.01	-9.47 <0.01	NA

Curriculum Vitae

Majeed Askari Seyahooei was born on the 21st of January 1967 in Bandar Abbas, Iran. He studied natural science and finished his high school education at Ebne-Sina high school in Bandar Abbas in June 1984. He started his university education in September 1985 at Shiraz University and finished an ASc in agricultural crop science in Feb. 1988. He continued his study at Shahid Chamran University and finished his BSc in plant protection in July 1990 then worked in the entomology lab in agricultural research center of Hormozgan in south of Iran. He started his MSc at Tarbiat Modares University in Tehran and he finished his MSc in June 1997. He worked as a researcher in Agricultural Research Center of Hormozgan as an entomologist for 6 years and two years he served for two years as the head of Agricultural Research Center in Hormozgan province. Based on the virtue of his work he was awarded with a grant to do his PhD abroad in 2005 and he started his PhD in Animal Ecology group in the Institute of Biology in the Leiden University (IBL) in October 2005.