CHAPTER 6

Variation of *Leptopilina boulardi* Success in *Drosophila* Hosts: What is Inside the Black Box?

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Abstract Interactions between *Drosophila* hosts and parasitoid wasps are among the few examples in which occurrence of intraspecific variation of parasite success has been studied in natural populations. Such variations can originate from three categories of factors: environmental, host and parasitoid factors. Under controlled laboratory conditions, it is possible to focus on the two last categories, and, using specific reference lines, to analyze their respective importance. Parasitoid and host contributions to variations in parasite success have largely been studied in terms of evolutionary and mechanistic aspects in two *Drosophila* parasitoids, *Asobara tabida* and, in more details, in *Leptopilina boulardi*. This chapter focuses on the physiological and molecular aspects of *L. boulardi* interactions with two *Drosophila* host species, while most of the evolutionary hypotheses and models are presented in Chapter 11 of Dupas et al.

6.1. INTRODUCTION

As for many parasites, the success of parasitoids in the host they infect is not guaranteed. In the first place, the suitability of different host species can vary for a given parasitoid species (host species specificity; Brodeur and Vet, 1995; Mohamed et al., 2003). Additionally, the outcome of a parasitoid species–host species combination can also be quite variable. For instance, the host resists in some cases the infestation through an immune response that kills the parasitoid, while in others the parasitoid escapes this immune response, resulting then in the death of the host. Against endoparasitoids, which develop inside the body cavity of their hosts, the immune response of insects is generally the encapsulation response, which consists in the elaboration of a multicellular and melanized capsule around the parasitoid egg. This encapsulation response can affect the overall parasitoid success significantly, as shown for *Drosophila* hosts (Carton and Kitano, 1981). The physiological and molecular basis of encapsulation is reasonably well characterized, due to numerous studies in lepidopteran and *Drosophila* spp. (Carton et al., 2008; Kanost et al., 2004). The virulence strategies and tactics used by parasitoids (namely the means employed to escape encapsulation) have also been investigated in many models and some of the molecular factors used to achieve these strategies (virulence factors) have been characterized (Carton et al., 2008; Glatz et al., 2004; Moreau and Guillot, 2005; Pennacchio and Strand, 2006;

Poirié et al., 2009). By comparison, less is known about the mechanisms affecting the ultimate outcome of host–parasitoid interactions. In other words, why some interactions resolve in the encapsulation of the parasitoid while others lead to the parasitoid success?

It is generally recognized that the variation in the outcome of any host–parasite interaction can originate from three sources: the variation in host resistance, the variation in the parasite ability to escape host resistance (parasite virulence), which are both genetically determined and the environmental factors. Moreover, there is an increasing evidence for complex interactions between host and parasite genotypes (G_H x G_P interactions; Carius et al., 2001; Lambrechts et al., 2005), which themselves can interact with the environment (G_H x G_P x E interactions; Lazzaro and Little, 2009). In host–parasitoid interactions, the role of environmental factors on the overall variation of success has been studied in various biological models (Bensadia et al., 2006; Calatayud et al., 2002; Oliver et al., 2003). However, most studies on the contribution of host resistance and parasitoid virulence have been restricted to the interactions between *Drosophila* hosts and parasitoid wasps and concern variations in parasitoid encapsulation exclusively. In particular, the parasitoids *Asobara tabida* and *Leptopilina boulardi* have been thoroughly studied. Extensive variation in host resistance and parasitoid virulence in natural populations have been evidenced in these models, and the coevolutionary outcomes largely discussed (Dupas et al., 2003; Kraaijeveld et al., 1998). Recently, significant progress has been made in understanding the genetic and molecular mechanisms underlying variations in immune interactions between *L. boulardi* and *Drosophila* hosts. Here, we review these mechanisms, while the evolutionary hypotheses and models concerning *Drosophila*–parasitoid interactions are presented in Chapter 11 by Dupas et al. First, we show how to ''dissect'' the variation in parasitoid success in order to identify the factors that influence the outcome of the host–parasitoid interaction (presence or absence of encapsulation). We then review recent data obtained for host resistance and parasitoid virulence. Finally, we discuss the diversity of virulence mechanisms in *Drosophila*–parasitoid interactions, and highlight how the progress in molecular comprehension of host–parasite interactions may help to understand the evolution of pairwise host–parasitoid interactions as well as the evolution of a parasitoid's host range.

6.2. DISSECTION OF THE NATURAL VARIATION OF ENCAPSULATION

Various kinds of environmental factors are known to influence the outcome of host–parasitoid interactions. Abiotic factors, such as temperature (Blumberg and Van Driesche, 2001), presence of insecticides (Delpuech et al., 1996) or host diet (Karimzadeh and Wright, 2008; Ojala et al., 2005),

can considerably influence the presence and efficiency of the encapsulation response. Moreover, the host immune response can be affected by biotic factors. The presence of another parasitoid in the host, either from the same species (superparasitism) or from another species (kleptoparasitism) can impair the immune response, eventually increasing the success of a given parasitoid (Kraaijeveld, 1999; Sagarra et al., 2000). More recently, symbionts were shown to influence the success of some parasitoids considerably, impairing or increasing host resistance ability as well as parasitoid virulence (Fytrou et al., 2006; Haine, 2008). Working under controlled laboratory conditions, it is possible to reduce environmental variation and focus on the genetic contribution of hosts and parasitoids.

To assess the occurrence of genetic variation in host resistance or parasitoid virulence within populations, two methods can be used. The first consists of performing selection experiments. If genetic variation exists in the studied trait (resistance or virulence), then its frequency is expected to change as a response to selection. Using this method in *D. melanogaster*, increases in encapsulation rates from less than 5% to more than 40% were obtained for the parasitoids *L. boulardi* and *A. tabida* in less than 10 generations (Fellowes et al., 1998; Kraaijeveld and Godfray, 1997). The second method consists of comparing the resistance or virulence abilities of different host or parasitoid isofemale lines obtained from a population under the same conditions of parasitism. Isofemale lines are each derived from a female that has been inseminated once, and whose progeny inbred during several generations until most loci are homogeneous. Heritability can then be measured by analyzing resistance or virulence of these isofemale lines over two successive generations (Carton and Boulétreau, 1985; Carton et al., 1989). Advantage of founding a series of isofemale lines is that while variation within a line will be lost, a series of independent lines will maintain heritable variation from within the population of interest, and mixing the lines will reconstitute the majority of variation (David et al., 2005).

Between-population variations in resistance and virulence can be assessed either by comparing freshly collected populations or by using the isofemale line method. In this case, several isofemale lines are constituted, thus allowing a ''snapshot'' of the genetic diversity occurring in this population to be taken. Between-population variation in resistance is then tested by comparing the encapsulation rate of a ''reference parasitoid line'' in host populations coming from different geographical areas. Similarly, between-population variation in virulence is then tested by comparing the encapsulation rate of parasitoid populations coming from different geographical areas in a ''host reference line'' (Fig. 6.1A). This method has been largely used in *Drosophila*–parasitoid models (Table 6.1). Substantial variation for both resistance and virulence has been shown in

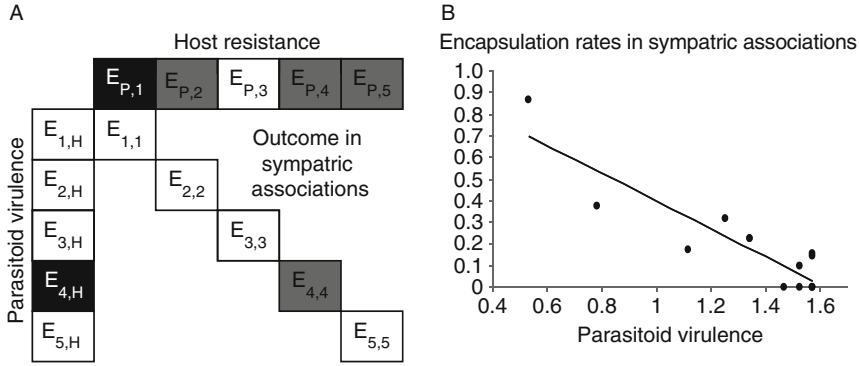

FIGURE 6.1 (A) Assessment of host resistance and parasitoid virulence in populations from different areas, and the outcome of the interaction in sympatric associations. $E_{i,j}$: encapsulation rate of parasitoid from locality *i* in host from locality *j*. Intensity of coloration of each square represents the level of encapsulation rates. Black: high, gray: medium, white: low. H: host reference strain, P: parasitoid reference strain. Resistance of each host population ($E_{P,j}$) is evaluated through the encapsulation rates of a reference parasitoid strain by the host population. Virulence of each parasitoid population ($1-E_{i,H}$) is evaluated through the encapsulation rates of this parasitoid population by a host reference strain. (B) Impact of parasitoid virulence on encapsulation rates in sympatric associations. Encapsulation rates measured in sympatric associations ($E_{i,j}$) are plotted against parasitoid virulence, which is tested using a host reference strain ($1-E_{i,H}$). Variables are arcsine transformed. *Note: Drawn from* Dupas et al. (2003).

populations from various geographical areas, and their ecological and evolutionary consequences are discussed elsewhere (Dubuffet et al., 2007; Dupas and Boscaro, 1999; Dupas et al., 2003; Kraaijeveld and Godfray, 1999; see also Chapter 10 by Kraaijeveld and Godfray and Chapter 11 by Dupas et al.).

Interestingly, the evidence for variation is strongly dependent on the line used for the experiment, since some lines fail to reveal variation. The strain ISm of *L. boulardi*, for example, is encapsulated by all the *D. yakuba* strains tested so far, but always escapes encapsulation in *D. melanogaster* (Carton, unpublished data; Dubuffet et al., 2007; Table 6.1). Similarly, the susceptible strain 1088 of *D. melanogaster* encapsulates none of the strains of *L. boulardi* we tested (Carton, unpublished data; Table 6.2). These strains are thus unsuitable to study variations in resistance and virulence in natural populations. Other laboratory lines can evidence genetic variations in the tested natural populations, but they might fail to reveal the whole range of responses. In theory, encapsulation rates obtained using a ''good'' reference line should range from 0% to 100%. For example, the encapsulation rates obtained using the parasitoid line ISy of *L. boulardi* range from less than 5% to more than 85% using laboratory lines of

TABLE 6.1 List of parasitoid and host species and strains used to demonstrate variations in resistance

Host species tested	Geographical origin of strains tested	Parasitoid species	Strain used for the test	Variation (YES/NO) range of encapsulation rates obtained (mean)	References
D. melanogaster	Europe	*A. tabida*	Sospel	YES 0–63.5 (26.7)	Kraaijeveld and van Alphen (1995)
D. melanogaster	Europe	*L. boulardi*	Tasagil	YES 0–24.2 (5.0)	Kraaijeveld and van Alphen (1995)
D. melanogaster	Worldwide	*L. boulardi*	ISy (G486)	YES 9.6–68.8 (55.2)	Dupas et al. (2003)
D. melanogaster	Worldwide	*L. boulardi*	ISm (G431)	NO (<5)	Carton and Frey (unpublished)
D. yakuba	Africa	*L. boulardi*	ISy (G486)	YES 6–97.9 (65.0)	Dubuffet et al. (2007)
D. yakuba	Africa	*L. boulardi*	ISm (G431)	NO (100)	Dubuffet et al. (2007)

Variation in resistance has been tested in host populations from various geographical origins (Worldwide, European or African distribution) using a single parasitoid strain. "Parasitoid reference strains" (shown in gray) are those which allow to evidence variation in resistance of host populations.

TABLE 6.2 List of parasitoid and host species and strains used to demonstrate variations in virulence

Host species	Strain used for the test	Parasitoid species tested	Geographical origin of strains tested	Variation (YES/NO) range of encapsulation rates (mean)	References
D. melanogaster	R (940)	*L. boulardi*	Worldwide	YES 0–74.2 (12.3)	Dupas and Boscaro (1999), Dupas et al. (2003)
D. melanogaster	S (1088)	*L. boulardi*	Worldwide	NO (<5)	Carton and Frey (unpublished)
D. yakuba	R_1 (1880-D)	*L. boulardi*	Worldwide	YES 10–100	Dupas and Boscaro (1999)
D. simulans	Ds1448	*L. boulardi*	Worldwide	Yes 10–40	Dupas and Boscaro (1999)
D. melanogaster	InHam	*A. tabida*	Europe	Yes <25–100	Kraaijeveld and van Alphen (1994)

Variation in virulence has been evidenced by comparing the encapsulation rate of parasitoid populations from various geographical origins (Worldwide, European or African distribution) on a single host strain. "Reference strains" (shown in gray) are host strains which allow to evidence variation of virulence.

D. melanogaster or *D. yakuba* (Carton et al., 1992; Dubuffet et al., 2007). In contrast, the parasitoid strain ''Tasagil'' of *L. boulardi* used by Kraaijeveld and van Alphen (1995) does not cover the whole range of encapsulation rates in *D. melanogaster* since the maximum encapsulation rate obtained using this strain is only 50% (Fellowes et al., 1999). The partial virulence of this strain might thus explain the low encapsulation rates measured by Kraaijeveld and van Alphen (1995) in European populations of *D. melanogaster* in comparison to the ones measured by Dupas et al. (2003) using the ISy line, and it might also hide part of the genetic variation of resistance. The choice of the ''reference line'' is thus critical for those wishing to reveal the genetic variations in resistance and virulence and investigate rationally these genetic interactions. Many well-characterized laboratory lines have been called ''reference lines,'' but we suggest, at least for the present chapter, that the term ''reference line'' should be restricted to the lines that allow the detection of genetic variation in natural populations of the antagonistic species. We will as well use the term ''resistance'' as the encapsulation rate of a parasitoid reference line measured in a host population or line, and the term ''virulence'' as one minus the encapsulation rate of a parasitoid population or line by a host reference strain (see Box 6.1).

Since the amount of genetic variation observed is strongly dependent on the choice of laboratory lines, the use of the reference lines could be questionable for the study of natural variation. Can the outcome of a host–parasitoid interaction be predicted by separate estimation of resistance and virulence levels of each partner using these reference lines? Fortunately, measurements of resistance and virulence using these reference strains actually give good predictions of levels of encapsulation measured in sympatric conditions (hosts and parasitoids coming from the same area, but tested in controlled laboratory conditions; Dupas et al., 2003; Kraaijeveld and Godfray, 2001). Interestingly, in the *L. boulardi*–*D. melanogaster* model, in which host resistance, parasitoid virulence and sympatric outcome were all evaluated, it appears that most of the variation in sympatric host–parasitoid associations comes from the variation in parasitoid virulence (Dupas et al., 2003; Fig. 6.1A and B). It explains 81% of the variance in encapsulation (calculated from Dupas et al., 2003 using the method from Kraaijeveld and Godfray, 2001; $F_{1,11}=$ 47.4; $P < 0.001$). The addition of host resistance to the regression does not increase the variance explained ($F_{1,4} = 3.045$, $P = 0.156$), due to the fact that most parasitoid populations are highly virulent on *D. melanogaster* (Fig. 6.2). As a result, resistance variation in *D. melanogaster* only accounts for the overall host–parasitoid outcome in tropical Africa, where parasitoid virulence is low. By comparison, both virulence and resistance explain the overall variance in the *A. tabida*–*D. melanogaster* model, but with

BOX 6.1 Definition of terms used in this chapter

As stressed in this chapter, the presence or absence of encapsulation of parasitoids by their hosts can depend on the interaction between the host and parasitoid genotypes (Dubuffet *et al.*, 2007). This means that a host genotype that is resistant to one parasitoid genotype is not necessarily resistant to all parasitoid genotypes. Similarly, virulence is relative to the antagonistic partner. The terms ''resistance'' and ''virulence'' have then to be considered carefully, because they do not design the overall outcome of the host or parasitoid, but their genetic potential toward one particular genotype of the interacting partner. In order to clarify all the terms related to the topic of this chapter, we give their definitions below.

Host reference line: Host line that is used to evidence the genetic variation of virulence in parasitoid populations.

Parasitoid reference line: Parasitoid line that is used to evidence the genetic variation of resistance in host populations.

Resistance: Ability of a host to encapsulate a parasitoid reference line.

Variation in virulence strategy: Genetic variation in the means used by parasitoids to successfully overcome encapsulation by hosts.

Variation of resistance: Genetic variation in the ability of hosts to encapsulate a reference parasitoid line. Individuals from the ''**resistant**'' line encapsulate the parasitoid while the ''**susceptible**'' ones do not.

Variation of virulence: Genetic variation in the ability of parasitoids to overcome encapsulation by a reference host line. Individuals from the ''**virulent**'' line escape encapsulation while the ''**avirulent**'' ones do not.

Virulence: Ability of a parasitoid to overcome encapsulation by a host reference line

Virulence factors: Molecules employed by parasitoids to achieve their virulence tactic.

Virulence strategy: Set of means used by parasitoids to escape encapsulation. It includes the general effects on host encapsulation ability (local immunoevasion or overall immunosuppression), the underlying virulence tactics and the effects of each virulence factor on host targets.

Virulence tactic: Describes each of the mechanisms involved to achieve the virulence strategy, that is, the potential effects of the parasitoid on specific components of the encapsulation response, as cellular or humoral effectors.

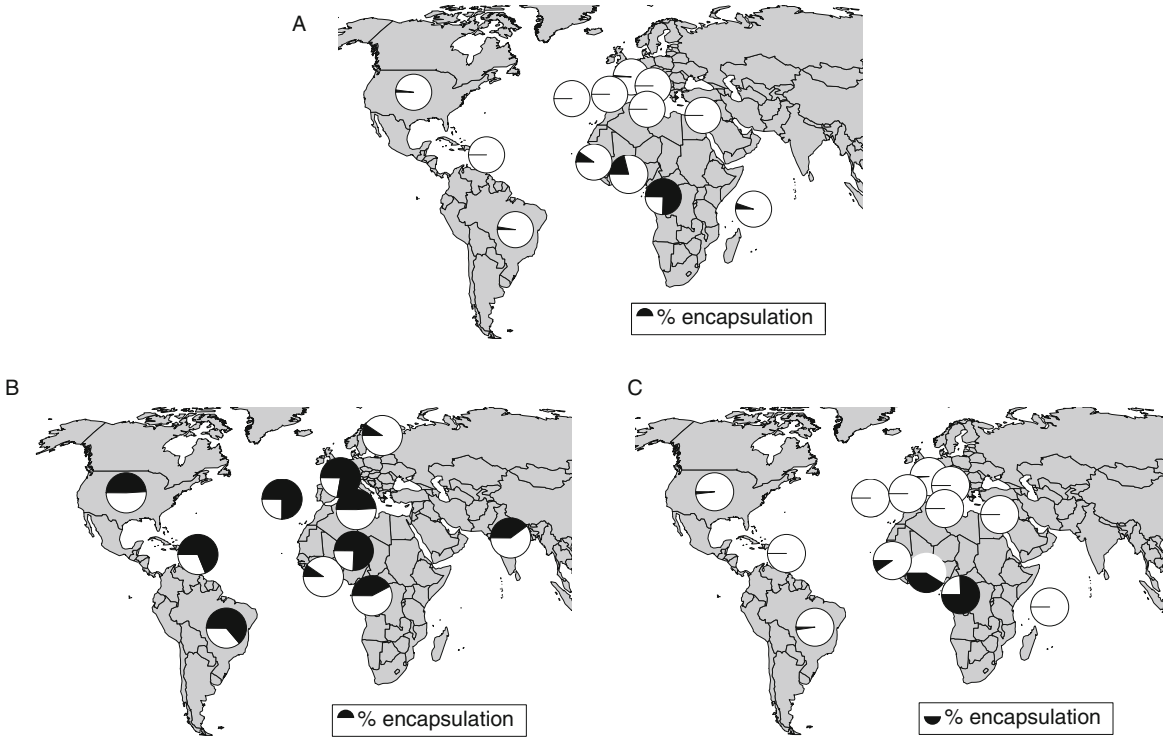

FIGURE 6.2 (A) Geographical distribution of the encapsulation rate of *L. boulardi* populations in sympatric *D. melanogaster* populations (% represented by the black portion of the pie chart). (B) Geographic distribution of resistance in *D. melanogaster* populations. The resistance level (represented by the black portion of the pie chart) is estimated from the rate of encapsulation of the reference ISy line of *L. boulardi* in *D. melanogaster* populations. (C) Geographical distribution of the virulence in *L. boulardi* populations. The level of virulence (represented by the white portion of the pie chart) is estimated from the rate of encapsulation of various natural populations of *L. boulardi* by the reference resistant strain of *D. melanogaster*. *Note:* From Dupas et al. (2003).

virulence again being the most important factor (Kraaijeveld and Godfray–2001).

In order to investigate the genetic, physiological and molecular basis of the variation of virulence and resistance observed in natural populations of *L. boulardi*, the isofemale lines of *L. boulardi*, *D. melanogaster* and *D. yakuba* showing the most contrasting virulence and resistance abilities have been chosen. Each host–parasitoid combination results either in a very low (<10%) or a very high (>85%) percentage of encapsulated parasitoid eggs (Fig. 6.3). This matrix of interactions reflects the situation observed in natural populations. The parasitoid ISm line, which originates from Tunisia, represents the pattern observed in most places: it is highly virulent in *D. melanogaster*, whichever the host strain, but is completely unable to escape encapsulation in any *D. yakuba* strain (Dupas and Boscaro, 1999). The success of this strain is thus host-species specific. By contrast, the parasitoid ISy, which originates from Congo, can infect both *D. melanogaster* and *D. yakuba* but is host-genotype specific, which means that its success depends on the genotype of the host (susceptible *vs.* resistant; Dubuffet et al., 2007; Dupas et al., 2003). When observing the whole matrix of interactions, it appears that *L. boulardi* has specific interactions with its hosts, since the parasitoid success depends both on the host and parasitoid lines considered (Dubuffet et al., 2007).

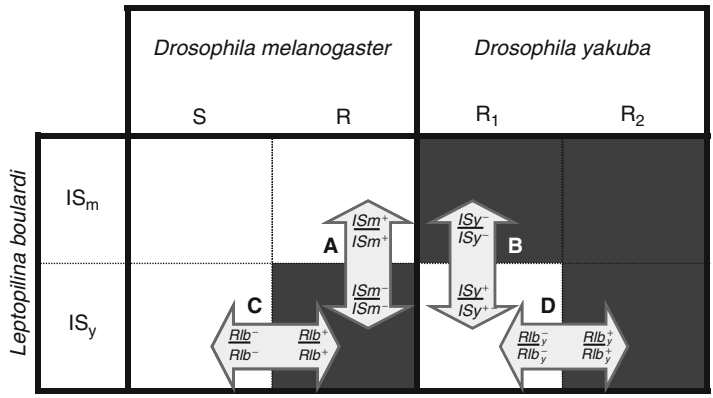

FIGURE 6.3 Genetic interactions between *L. boulardi* and its *Drosophila* hosts. Dark boxes: parasitoid failure (encapsulation); white boxes: parasitoid success. Variation in parasitoid virulence in *D. melanogaster* (A) and *D. yakuba* (B) is encoded by two distinct major biallelic loci (*ISm, ISy*). Variation in host resistance both in *D. melanogaster* (C) and *D. yakuba* (D) is encoded in each case by a single major biallelic locus (*Rlb, Rlb$_y$*). Success of the ISm line of *L. boulardi* is species-specific while the success of the ISy line is host-genotype specific. *Note:* Redrawn from Dubuffet et al. (2007).

6.3. HOST RESISTANCE: ORIGIN OF VARIATION

6.3.1. The actors of physiological resistance

An exhaustive description of the current knowledge regarding the molecular bases of immune defenses in *Drosophila* would largely exceed the purpose of this chapter (for a review, see Lemaitre and Hoffmann, 2007). However, data on the encapsulation process will be required to understand the next parts of this chapter fully. It has now been well described that large eukaryotic parasites, such as parasitoid eggs, that invade the hemocoel of insects generally provoke a series of immune responses mediated in large part by circulating blood cells (hemocytes) that form multilayer capsules around the foreign organism (Carton et al., 2008). In addition to some lepidopteran species, the model organism for studies on parasitoid encapsulation has been *D. melanogaster*. However, we have to keep in mind that if other species of the *melanogaster* subgroup such as *D. yakuba* and *D. simulans* use apparently rather similar immune components to those described in *D. melanogaster*, the death of the parasitoid is not associated with encapsulation in other species like *D. paramelanica* (Nappi, 1970; see Chapter 4 by Nappi et al.). Moreover, the specific hemocytes devoted to the formation of the capsule in the *melanogaster* group are not found in all *Drosophila* spp. (Eslin and Doury, 2006; see Chapter 7 by Eslin et al.). The mechanisms responsible for a parasitoid success or failure in *D. melanogaster* might thus strongly differ from those involved in the outcome of its interactions with other *Drosophila* spp., leading to a diversity in virulence strategies as well as resistance systems.

One of the first detectable events following parasitism in *D. melanogaster* larvae is the proliferation, release and/or differentiation of host hemocytes (Carton et al., 2008; Markus et al., 2009). In *Drosophila*, plasmatocytes and lamellocytes are the principal cells involved in cellular encapsulation. The proportion of lamellocytes, which are rarely observed in nonparasitized flies, is greatly enhanced in parasitized larvae (Lanot et al., 2001; Rizki and Rizki, 1992; Russo et al., 2001; Sorrentino et al., 2002). Six h following infection, a thin layer of melanin is observed on the surface of the parasitoid (Russo et al., 2001), which suggests that biochemical reactions associated with the production of melanin, for example, activation of the phenol oxidase (PO) cascade, are triggered very early following infection (Williams et al., 2005). They are associated with the production of cytotoxic radicals that are thought to be responsible for the parasitoid death. By 24 h after infection, the wasp egg is completely surrounded by plasmatocytes. By 40 h, lamellocytes are found attached around the egg and at 48 h after infection a fully formed melanotic capsule is visible in the host hemocoel (Williams et al., 2005). Lamellocytes also appear as sources for PO-mediated melanogenesis (Irving et al., 2005;

Nam et al., 2008), as is a third type of hemocyte, the crystal cell (Rizki and Rizki, 1985; Rizki et al., 1980). Besides physical damage such as rupture of the basal membrane, parasitism but also injection of female parasitoid venom can induce the proliferation of hemocytes and specifically of lamellocytes (Labrosse et al., 2005a) but at the moment, no ''immune-inducing'' component has been identified yet and the mechanisms that lead to the ''recognition'' of the invader are also largely unknown.

Regulation of the hemocyte number is controlled by different pathways including genes from the Ras-mitogen-activated protein kinase pathway, while the Jak/Stat and Jun kinase pathways strongly affect lamellocyte formation (Zettervall et al., 2004; Fig. 6.4). Other signals, such as those mediated by aop(ACT), Toll(10b) or Rac1, cause a simultaneous increase in lamellocytes and total hemocyte number. Adhesion and cell-shape changes are also an essential part of the encapsulation process. One family of proteins central to the processes involved in cell shape is the Rac GTPases. Once activated, Racs are involved in many cellular processes including: cytoskeletal organization, regulation of cellular adhesion, cellular polarity and transcriptional activation. Both *Drosophila* Rac1 and Rac2 genes are required for proper encapsulation of *L. boulardi* eggs (Williams et al., 2005, 2006). Rac2 is necessary for hemocyte spreading and cell–cell contact formation and melanization is disrupted in capsules recovered from Rac2 mutants (Williams et al., 2005). Rac1 is involved in the increase in hemocyte number as well as induction of lamellocyte formation.

In insects, PO is present as inactive prophenoloxidase (PPO) and cleaved into active PO by a serine protease (prophenoloxidase-activating enzyme; PPAE), that itself becomes activated through a sequential process involving other serine proteases (Cerenius et al., 2008; Jiang et al., 2003; Satoh et al., 1999). In *D. melanogaster*, the expression of several serine

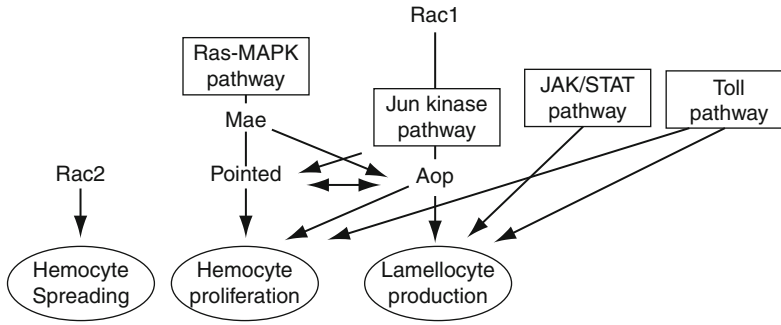

FIGURE 6.4 Part of the known pathways and genes involved in proliferation and spreading of hemocytes as well as production of lamellocytes in *D. melanogaster*. The mae gene is a candidate for the *Rlb* resistance gene to the ISy line of *L. boulardi*.

proteases is significantly increased within the few h following infection, which is concomitant with the melanin deposition (Schlenke et al., 2007; Wertheim et al., 2005). Serine proteases are themselves negatively regulated by serine protease inhibitors, the better described in *D. melanogaster* being Spn27A, which inhibits PPAE (De Gregorio et al., 2002). Also, introducing Spn27A into otherwise immune reactive *D. melanogaster* larvae reduces the frequency of melanotic encapsulation of eggs of *L. boulardi* (Nappi et al., 2005).

6.3.2. Variation and genetic determinism of *Drosophila* resistance to parasitoids

Naturally occurring resistance variation between populations, evidenced by the use of parasitoid reference lines, has been well described in *D. yakuba* against *L. boulardi* (Dubuffet et al., 2007) and in *D. melanogaster* against *L. boulardi* and *A. tabida* (Dupas et al., 2003; Kraaijeveld and van Alphen, 1995). Interestingly, *D. melanogaster* resistance levels toward the Congolese parasitoid line ISy of *L. boulardi*, are quite high while encapsulation rates toward sympatric parasitoid populations are in general very low (Dupas et al., 2003; Fig. 6.2A and B). This comes from the fact that virulence overcomes the effects of resistance in this model: encapsulation only occurs if the host is resistant and the parasitoid has a low virulence, a situation observed in Congo (Dupas et al., 2003; Fig. 6.2C). In this area, within-population variation of resistance has been described, encapsulation rates of the reference line ISy ranking between 12% and 90% (Carton and Boulétreau, 1985; Carton et al., 1992). In other areas, *L. boulardi* is highly virulent, which results in low encapsulation rates in sympatric associations. It is difficult to explain the high levels of resistance to ISy parasitoids found in these areas since it is totally inefficient toward *Leptopilina* parasitoids found in sympatry. They could be maintained as the result of a selection pressure coming from cooccurring parasitoid species. In the south of France, for instance, *L. boulardi* was shown to cooccur sometimes with *L. heterotoma* and other parasitoids (Fleury et al., 2004). Alternatively, resistance might be pleiotropic and its polymorphism maintained by completely different selection factors. By comparison, most populations of *D. melanogaster* encapsulate a significant proportion of *A. tabida* eggs in sympatric associations, due both to moderate virulence of the parasitoid as well as a significant proportion of resistant hosts (Kraaijeveld and Godfray, 2001). In *D. yakuba*, levels of encapsulation of *L. boulardi* in sympatric associations are not known, but are likely to be high, due to the low virulence of *L. boulardi* toward this host—the ISy parasitoid line is, to our knowledge, the only one described that can escape encapsulation in this species—and the high resistance levels to this line we found in populations (Dubuffet et al., 2007). So far,

parasitoid success has only been reported in Congo (Dupas and Boscaro, 1999). This might be correlated with the fact that *D. yakuba* is intrinsically a much better encapsulator than *D. melanogaster*, probably because unparasitized larvae have on average more hemocytes (Carton and Kitano, 1981; Eslin and Prévost, 1998).

To analyze the genetics of resistance to parasitoid wasps in *D. melanogaster*, selected inbred resistant (R) and susceptible (S) lines were obtained from the same population (Brazzaville, Congo) using the ISy parasitoid line (Carton et al., 1992). Resistance to *A. tabida* WOV was analyzed using the same R strain that proved resistant also to *A. tabida* and Canton S as a susceptible strain (Benassi et al., 1998). Resistance of *D. yakuba* was analyzed using the isofemale lines 1880-D (R1 line, susceptible) and 1907 (R2 line, resistant) chosen from two populations in Tanzania using the ISy line of *L. boulardi* (Dubuffet et al., 2007).

Considering the high number of genes potentially involved in insect immune response to parasitoids (Irving et al., 2005; Zettervall et al., 2004), variation in resistance was expected to be multigenic. However, in both *D. melanogaster* and *D. yakuba* host species, resistance to parasitoids is always explained by a single diallelic locus inherited autosomally, with the resistant phenotype showing complete dominance over the susceptible one. In *D. melanogaster*, the loci were named *Rlb* (resistance to *L. boulardi*) and *Rat* (resistance to *A. tabida*; Benassi et al., 1998; Carton et al., 1992), and in *D. yakuba* the locus was named Rlb_y (resistance to *L. boulardi*; Dubuffet et al., 2007; Fig. 6.3). The use of isofemale lines might have favored the recovery of simple genetic systems, but a study dealing with genetic variation of resistance to *A. tabida* in *D. melanogaster* from different localities in Europe also concluded on a simple genetic basis of resistance (Orr and Irving, 1997).

These results raised the question whether the same *D. melanogaster* locus was involved in resistance to *A. tabida* and *L. boulardi*. Using recombination experiments, we showed that *Rlb* and *Rat* are 35 cM apart (Poirié et al., 2000). Besides, there is no correlation between the field capacity to encapsulate these two parasitoid species (Kraaijeveld and van Alphen, 1995) and a strain susceptible to *L. boulardi* was resistant to *A. tabida* (Vass et al., 1993). This suggested that resistance has parasitoid-specific components and that at least two separate genetic systems explain resistance to parasitic wasps in the same host species.

6.3.3. Physiological and molecular bases of *Drosophila* resistance to parasitoids

Occurrence of different genes responsible for resistance to *L. boulardi* and *A. tabida* in *D. melanogaster* was in agreement with selection experiments showing that lines selected for resistance to *L. boulardi* also increased in

resistance to *A. tabida* while only a slight increase in resistance to *L. boulardi* was observed in lines selected against *A. tabida* (Fellowes et al., 1999). This led to consider that improved resistance had a nonspecific component more or less effective against both wasps and a specific component required for encapsulation of *L. boulardi*.

The nonspecific component might correspond to an increase in hemocyte number as observed in lines selected for increased resistance to *A. tabida* (Kraaijeveld et al., 2001). Accordingly, it has been shown that the hemocyte number can affect the resistance potential of *Drosophila* hosts against *A. tabida* (Eslin and Prévost, 1998). The *Rat* locus has not been cloned yet but it has been localized on chromosome 2R, near the centromere (Poirié et al., 2000) and may correspond to the major resistance locus characterized in a QTL mapping experiment (Orr and Irving, 1997).

The physiological basis of variation of resistance to *L. boulardi* in *D. melanogaster* and *D. yakuba* is still unknown. However, one difference between the R and S strains of *D. melanogaster* has been described by Russo et al. (2001): in larvae parasitized by ISy parasitoids, the number of hemocytes is about twofold higher in the R strain than in the S strain at 15 h postinfestation and higher in the S strain than in the R strain at 24 h postinfestation. It is then possible that the earlier ''proliferation response'' in the R strain plays a role in variation of resistance. Genetic experiments have been used to localize the locus *Rlb* on the right arm of chromosome 2, at a genetic location of ca. 2-86.7 (Poirié et al., 2000). Its localization was then restricted in a 300 kb region, in 55E2-E6; F3, using strains bearing deletions (Hita et al., 1999). Indeed, despite dominance of the Rlb^{+} allele, F1 larvae bearing a deletion in front of the Rlb^{+}-containing region show a decreased encapsulation rate, probably because of transvection effects. The *Rlb*-containing region was then restricted to 100 kb by controlling the molecular limits of the deletions using *in situ* hybridization and Southern-blotting experiments. Finally, male recombination experiments were performed to localize *Rlb* to the right or to the left of a P-element inserted in this region. Results showed that *Rlb* was close to the P-element leading to characterization of two candidates, the *mae/edl* gene and CG15086 of unknown function (Hita et al., 1999). *mae* (modulator of the activity of Ets)/*edl* (Ets-domain lacking) is the more likely candidate for *Rlb*. It encodes a protein with an ETS-specific pointed domain (SAM domain) and acts as a signaling intermediate that directly links the RTK/RAS/MAPK signaling pathway to its downstream transcription factor targets (Baker et al., 2001). *mae/edl* mediates MAPK phosphorylation of the Ets transcription factors yan/aop and pointed P2, yan/aop being involved in cell choice between cell proliferation and differentiation following RTK signaling (Rogge et al., 1995). The fact that ectopic expression of *yan/aopACT*, a *yan/aop* constitutively active allele, stimulates both proliferation of hemocytes and formation of lamellocytes in *Drosophila*

larvae (Zettervall et al., 2004) supports the possible involvement of *mae/edl* in resistance to *L. boulardi*. Differences between resistant and susceptible alleles, their expression or their regulation might explain differences in the timing of hemocyte proliferation in response to parasitism (Fig. 6.4).

It would be interesting now to determine whether the locus that determine the variation of resistance to *L. boulardi* in *D. yakuba* is homologous to the locus *Rlb*, or if completely different loci explain variation to the same parasitoid in the two different host species. Explaining the high level of resistance against *L. boulardi* in the field both *D. yakuba* and *D. melanogaster* will indeed require understanding the function of resistance genes as well as their degree of specificity.

6.4. PARASITOID VIRULENCE: ORIGIN OF VARIATION

Among the few well-studied *Drosophila* parasitoids, variation of virulence has largely been evidenced in some species, such as *L. boulardi* (Carton et al., 1989; Dupas and Boscaro, 1999) and *A. tabida* (Kraaijeveld and van Alphen, 1994) but not in other species like *A. citri* or *Ganaspis xanthopoda*. Occurrence and genetic analysis of such a variation in *L. heterotoma* was reported by Walker in 1959 but it has never been documented since then. Recent analyses of virulence of six *Leptopilina* spp. against three *Drosophila* host species (Dupas, unpublished data) also suggest that intraspecific variation in virulence can be easily observed in some but not all species of the same genus.

L. boulardi is undoubtedly the species whose variation of virulence has been best described, both for its occurrence in natural populations and its physiological and molecular mechanisms. The only description of intrapopulation variation in virulence concerns the host *D. simulans* (Carton et al., 1989), whereas interpopulation variation has been documented for *D. melanogaster*, *D. simulans* and *D. yakuba* (Dupas and Boscaro, 1999). The mechanisms underlying the interpopulation variations, which we present below, have been investigated in *D. melanogaster* and *D. yakuba* using the parasitoid lines ISm and ISy, which originate from different populations.

6.4.1. Genetic determinism of virulence variation

So far, *Leptopilina* spp. remain the only parasitoid genus for which the genetic determinism of virulence variation has been investigated. Both in *L. heterotoma* and *L. boulardi*, these analyses revealed that the success of parasitoids is more related to the genotype of their mothers than to their own genotype, since the success of hybrid eggs issued from crosses

between virulent and avirulent lines remained the same as that of the maternal line (Dupas and Carton, 1999; Dupas et al., 1998; Walker, 1959). This suggested that variations in maternal secretions, like venoms, may determine the intraspecific variations of success of *Leptopilina* spp.

In *L. boulardi*, genetic crosses have been performed during two generations between the lines ISm and ISy, which have opposite virulence abilities on the species *D. melanogaster* and *D. yakuba* (Fig. 6.3). These crosses revealed that variations of virulence on each of these host species have a simple determinism, with a diallelic locus explaining these variations (Dupas and Carton, 1999; Dupas et al., 1998). In *D. melanogaster*, virulence and avirulence alleles are semidominant while in *D. yakuba* there is dominance of the avirulence phenotype.

Dupas and Carton (1999) mixed the lines ISy and ISm for 16 generations and tested the females obtained from this experimental population for their virulence abilities on *D. melanogaster* and *D. yakuba*. They found no correlation between these virulence abilities, which led them to conclude that the locus responsible for variation of virulence on *D. melanogaster*, called *ISm*, is distinct from the locus responsible for the variation of virulence on *D. yakuba*, called *ISy*. The absence of correlation in parasitoid virulence on the three species *D. melanogaster*, *D. yakuba and D. simulans* in natural populations tallies with distinct virulence genetic systems against each *Drosophila* spp. (Dupas and Boscaro, 1999).

6.4.2. Physiological determinism of virulence variation

As described elsewhere, parasitoids use various strategies to escape encapsulation (Pennacchio and Strand, 2006; Poirié et al., 2009). Some evade encapsulation due to surface characteristics that make them inaccessible to the host immune system, or due to a local decrease in efficiency of the immune response, which does not impair the overall host encapsulation response (local immunoevasion). Others modulate or suppress the whole host encapsulation response (systemic immunosuppression). Among *Drosophila* parasitoids, the first mechanism has been described in *A. tabida* (Prévost et al., 2005; see Chapter 9 by Prévost et al.) while immunosuppression has been reported for *A. citri*, *G. xanthopoda*, *L. heterotoma* and *L. victoriae* (Chiu et al., 2000; Morales et al., 2005; Prévost et al., 2005; Rizki et al., 1990). In *L. boulardi*, we have combined description of the virulence strategy used by successful parasitoids and investigation of the causes of failure of avirulent parasitoids to understand the physiological causes of virulence variation. Variations of parasitoid virulence can roughly originate from two main mechanisms: either they differ in their ability to evade locally the host immune system, or they differ in their ability to suppress the whole encapsulation response.

One way to distinguish between local immunoevasion and systemic immunosuppression strategies is to determine whether parasitism of a host larva by a virulent parasitoid can protect or not from encapsulation another foreign body that would be normally encapsulated in the same host. This foreign body can be the egg of a nonvirulent parasitoid or a drop of paraffin oil injected into the host larva. According to this criterion, virulent lines of *L. boulardi* have a systemic immunosuppression strategy on *D. yakuba* and *D. melanogaster*. In *D. yakuba*, a drop of paraffin oil is protected from encapsulation for 24 h postparasitization by the ISy line. However, this protection is only transient since the drop is fully encapsulated 48 h postparasitization (Dubuffet et al., 2008), at a time a parasitoid egg has reached the larval stage. The parasitoid larva might then use a local immunoevasion strategy that follows the initial systemic immunosuppression. Interestingly, the line ISm, avirulent on *D. yakuba*, does not affect its capacities to encapsulate the oil drop, which suggests that the variation of success of *L. boulardi* on *D. yakuba* is linked to a variation of the immunosuppressive abilities between the parasitoid lines (Dubuffet et al., 2008). On *D. melanogaster*, multiparasitism experiments have been performed using the lines ISm and ISy, respectively, virulent and avirulent on *D. melanogaster* (Labrosse et al., 2003). About 48 h postparasitization, ISy parasitoids are normally found encapsulated. However, in multiparasitized host larvae, larvae of the two parasitoid lines, easily distinguishable, were found free in the host hemolymph. This indicates that the ISm line can protect ISy parasitoids from encapsulation in *D. melanogaster*. In that case, ISm immunosuppression in *D. melanogaster* might be more durable than ISy immunosuppression in *D. yakuba*, lasting at least 48 h, or it might protect ISy eggs only until they hatch, where a local immunoevasion mechanism would then again take over the protection.

As in *L. heterotoma* and *L. victoriae*, the venom injected during oviposition by *L. boulardi* females was shown to be responsible for the suppression of the encapsulation response (Dubuffet et al., 2008; Labrosse et al., 2003; Morales et al., 2005; Rizki and Rizki, 1990). Both in *D. melanogaster* and *D. yakuba*, injection of venom from the virulent line (ISm for *D. melanogaster*, ISy for *D. yakuba*) can protect from encapsulation a foreign body that is usually encapsulated. In contrast, injection of venom from the avirulent line (ISy for *D. melanogaster*, ISm for *D. yakuba*) does not confer any protection. These observations led us to conclude that qualitative and/or quantitative variations in the venoms of the two parasitoid lines were responsible for the observed variations of virulence (Dubuffet et al., 2008; Labrosse et al., 2003). This assumption is strengthened by results from genetic analyses that suggested that the maternal secretions were responsible for the success/failure of the parasitoid progeny (see Section 6.4.1).

To investigate the functional basis of the variation of immunosuppressive effects in *L. boulardi* further, a first approach was to describe and quantify the physiological modifications that parasitism induces in *Drosophila* hosts. As for most parasitoids, studies focused on effects on the host hemocytes and the PO cascade since changes in the levels of production/effects of cytotoxic radicals are particularly difficult to evidence (see Chapter 4 by Nappi et al.).

Hemocytes were observed and counted after parasitization by the ISm and ISy lines, both in *D. yakuba* and *D. melanogaster* (Dubuffet et al., 2008; Russo et al., 2001). In all cases, the total number of hemocytes and specifically of plasmatocytes and lamellocytes increased significantly after parasitization in comparison to unparasitized controls. This suggests that *L. boulardi* does not prevent the production/release of hemocytes following recognition of the intruder. However, larvae parasitized by virulent parasitoids show a much lower increase in the number of some categories of hemocytes compared to larvae attacked by avirulent parasitoids: this is the case for the lamellocyte number in the *D. melanogaster*/ISm interaction and for the plasmatocyte number in the *D. yakuba*/ISy interaction. These interactions might simply elicit a weaker immune cellular response. Alternatively, immunosuppressive factors injected by ISm and ISy females might be responsible for these effects. The parasitoid wasps *L. heterotoma*, *A. citri* and *G. xanthopoda* are known to induce the atrophy of the *D. melanogaster* hematopoietic organ (Chiu et al., 2000; Prévost et al., 2005). Parasitism by *L. heterotoma* also leads to apoptosis of circulating plasmatocytes and hematopoietic precursors in the lymph gland, as well as to destruction of circulating lamellocytes (Chiu et al., 2000; Rizki and Rizki, 1984, 1990). However, none of these modifications are observed following parasitism of *D. melanogaster* with the ISy avirulent strain of *L. boulardi* (Chiu et al., 2000). The potential effects of *L. boulardi* virulent wasps on hemocytes or hematopoietic organs remain to be elucidated.

In addition to a variation of effects of ISy and ISm parasitoids on lamellocytes number in *D. melanogaster*, we observed a variation on the lamellocytes morphology: a significant proportion of lamellocytes (up to 50%) became bipolar following parasitization by the virulent line ISm, but not in ISy-parasitized larvae (Russo et al., 2001). Such change in lamellocyte morphology has also been reported in *L. heterotoma* and *L. victoriae*, and was suggested to correlate with a decreased ability to adhere and to form capsules (Morales et al., 2005; Rizki and Rizki, 1984). In both species, the factor responsible for these effects, called lamellolysin in *L. heterotoma*, is localized in the venom gland (Morales et al., 2005; Rizki and Rizki, 1991). Accordingly, injection of venom from the ISm line of *L. boulardi* mimicked parasitism effects on lamellocytes number and shape, while injection of venom from the ISy line had no effect, which indicates that

variation in venoms is responsible for variations of effects on lamellocytes (Labrosse et al., 2005a). In *D. yakuba*, no effect on lamellocyte morphology or number has been observed, neither following parasitism by the ISm line nor in ISy-infected larvae (Dubuffet et al., 2008), which suggests that, considering these two host species only, effects on lamellocytes morphology are specific to *D. melanogaster*.

We recently started investigating the effects of *L. boulardi* venom on the humoral components of the encapsulation response. We showed that venom from the line ISy, virulent on *D. yakuba*, inhibits the activation of the proenzyme prophenoloxidase into PO in this species, in a dose-dependent manner (Colinet et al., 2009; Fig. 6.5A). In contrast, venom from the avirulent line ISm did not show such an inhibiting effect (Dubuffet, unpublished data). This suggests that variations of virulence in *D. yakuba* could be linked to a variation in the venom capability to inhibit the PO cascade of *D. yakuba*. The question remains if parasitoid success of ISm on *D. melanogaster* is also associated with an inhibition of the PO cascade.

Our physiological data, even incomplete, support the idea that some, if not all, mechanisms underlying the variation of virulence of *L. boulardi* on *D. melanogaster* and *D. yakuba* differ from each other: the variation of virulence in *D. melanogaster* is correlated to a variation of effects on the lamellocytes number and morphology while the variation of virulence in *D. yakuba* is correlated to variation of effects on plasmatocyte number and on the phenoloxidase cascade. The existence of mechanisms underlying the variation of virulence, different for each host species, is supported by the existence of the two distinct loci for virulence evidenced by Dupas and Carton (1999).

6.4.3. Parasitoid components at the origin of virulence variation

Both in *D. melanogaster* and *D. yakuba*, it appears that the variation of virulence observed between the ISy and ISm lines of *L. boulardi* is due to a variation of the immunosuppressive effects induced by the venom. Characterization of the virulence factors contained in these venoms, and particularly their quantitative and/or qualitative variations is thus crucial to determine the basis of virulence variation.

6.4.3.1. Variation in virus-like particles

In all figitid parasitoids studied to date, *L. heterotoma* (Rizki and Rizki, 1990, 1994), *L. victoriae* (Morales et al., 2005) and *L. boulardi* (Dupas et al., 1996; Labrosse et al., 2003), virus-like particles (VLPs) are observed in the venom of females. This characteristic is not unique to figitidae since other parasitoid families, including the well-studied braconidae and ichneumonidae, produce VLPs either in the venom apparatus or in the ovaries (Barratt et al., 1999; Reineke et al., 2006; Suzuki and Tanaka, 2006).

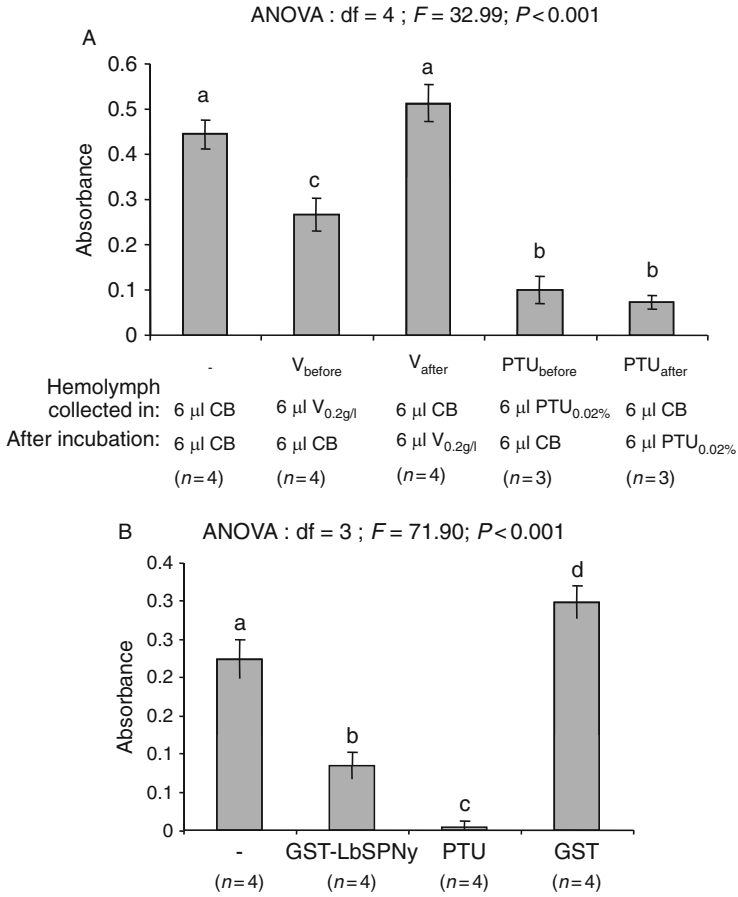

FIGURE 6.5 (A) Effect of ISy venom on PO activation in *D. yakuba*. PO activity was measured in hemolymph samples in which parasitoid venom (0.2 g/l) or PTU (a PO inhibitor) was present before or after a 45-min incubation at 37 °C. The proPO proenzyme is normally activated into active PO during this incubation period by the serine protease cascade. In samples collected in presence of ISy venom, PO activity is lower. This effect is not due to an inhibition of the PO enzyme itself, since it is not observed if the venom is added after the incubation period. In PTU controls, however, inhibition is observed whatever the moment where PTU is added. CB: cacodylate buffer; V 0.2 g/l: venom extract. PO activity is measured through the conversion of L-DOPA into dopachrome, which absorbance is measured at 490 nm. Mean values (standard error (SE)) are given for each category and numbers within brackets indicate numbers of replicate. (B) Effect of the recombinant serpin LbSPNy on PO activity. Hemolymph of *D. yakuba* larvae was collected in cacodylate buffer alone (–), in presence of the GST-LbSPNy fusion protein (0.3 g/l), in presence of a PO inhibitor (PTU) or in presence of the GST protein alone (0.3 g/l). Mean values (SE) are given for each category and numbers within brackets indicate numbers of replicates. *Note*: From Colinet et al. (2009).

The nature of these VLPs, which do not contain deoxyribonucleic acid (DNA) and can have very different aspects, remains to be elucidated. VLPs are injected into the host together with the eggs and have been described, for instance, to target hemocytes, inducing morphological changes and/or apoptosis (Rizki and Rizki, 1990; Suzuki and Tanaka, 2006). In *L. heterotoma*, VLPs have been shown to enter *Drosophila* host hemocytes (Chiu et al., 2006; Rizki and Rizki, 1994). They can be observed free in the cytoplasm of lamellocytes or in engulfed vesicles in plasmatocytes, suggesting that these last cells are able to phagocyte them (Rizki and Rizki, 1990, 1994). Besides, the so-called ''lamellolysin'' factor, injected by this parasitoid and responsible for changes in host lamellocyte morphology, has been demonstrated to be composed of VLPs (Rizki and Rizki, 1990, 1994). However, neither the nature of these VLPs nor the molecular nature of the factors responsible for these changes has been identified in this species.

In *L. boulardi*, VLPs have been detected in all lines studied including the ISm and ISy lines, but they strongly differ in the morphology and the number of the particles (Dupas et al., 1996; Labrosse et al., 2003; Fig. 6.6). The particles of the ISm line as well as of two other lines also virulent on *D. melanogaster*, are round shaped and contain several vesicles, while the particles in the ISy line, avirulent on *D. melanogaster*, are more elongated and contain fewer vesicles. F1 hybrid females resulting from the cross between the ISm and ISy lines produce VLPs of intermediate morphology with less elongated particles containing more vesicles than in the ISy line (Dupas et al., 1996). Interestingly, these hybrids exhibit half-immune suppressive ability toward *D. melanogaster*. These data suggest that the morphology of the VLPs might be somehow related with the parasitoid virulence level against *D. melanogaster*. However, the morphology of VLPs might not be related to virulence toward *D. yakuba* since these hybrids are completely avirulent on this host species (Dupas and Carton, 1999). The ISm type of *L. boulardi*, but not the ISy type, induces changes in the morphology of *D. melanogaster* lamellocytes. If ISm VLPs have the ability to enter lamellocytes as *L. heterotoma* VLPs, they might be responsible for these changes, either by themselves or by transporting the responsible factor(s) inside these hemocytes. Purification experiments and proteomic analysis will allow identifying the proteins that constitute and/or are transported by VLPs. This will give more insights into how the VLPs are formed what explain the observed intraspecific morphological differences, and what role they play on observed differences in virulence levels.

6.4.3.2. Variation in proteinic content of venoms

Biochemical approaches have provided recently valuable information on the nature and variation of the immune suppressive factors in *L. boulardi*. Native and sodium dodecyl sulfate polyacrylamide gel electrophoresis

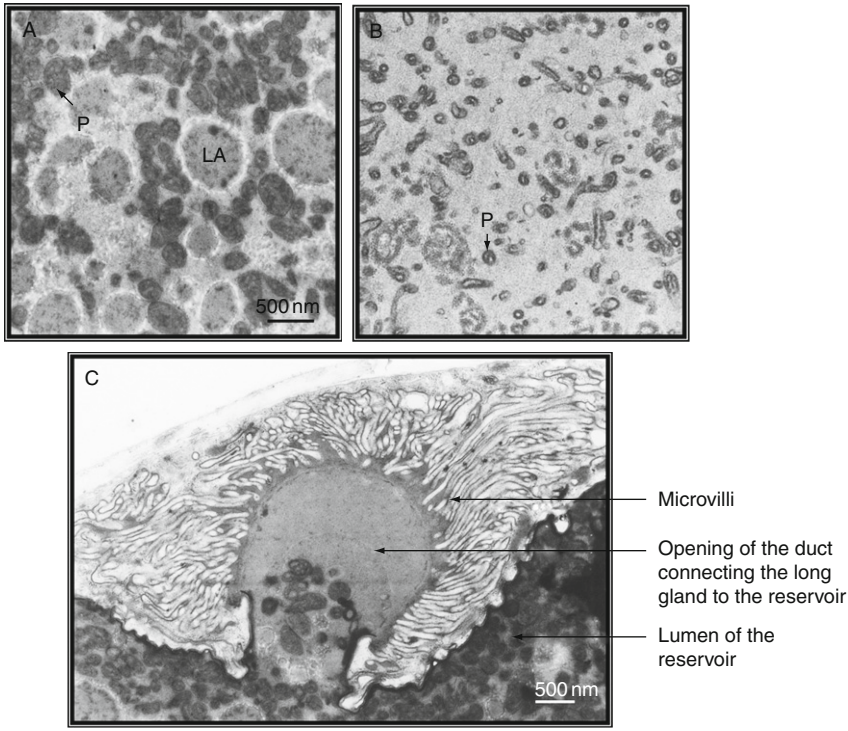

FIGURE 6.6 Results of transmission electron microscopy. (A) VLPs in the reservoir of a G431 virulent female. LA, large aggregates; P, particles. (B) VLPs in the reservoir of a G486 avirulent female. P, particles. (C) Transversal section of the apical part of the reservoir of a G431 virulent female. Numerous microvilli are observed. *Note:* From Labrosse et al. (2003).

(SDS-PAGE) of the protein content of the venom apparatus of the ISy and ISm *L. boulardi* revealed an impressive variation between these two lines (Colinet et al., 2009; Labrosse et al., 2005b; Fig. 6.7A and B). Nevertheless, all the lines virulent on *D. melanogaster* we tested harbored a proteinic profile more or less similar to the one observed for ISm parasitoids (Fig. 6.7C). This suggests that the intraspecific variation of virulence between the ISy line and the lines virulent on *D. melanogaster* is correlated with differences in venom gland protein profiles, resulting from qualitative and/or important quantitative differences in the protein content of these glands.

6.4.3.3. The LbGAP virulence factor and its variation

Among the major native proteinic bands in the venom of the line ISm and of all the tested lines virulent on *D. melanogaster*, the P1 and P4 bands have been the most studied. Each of these two bands, eluted from native PAGE,

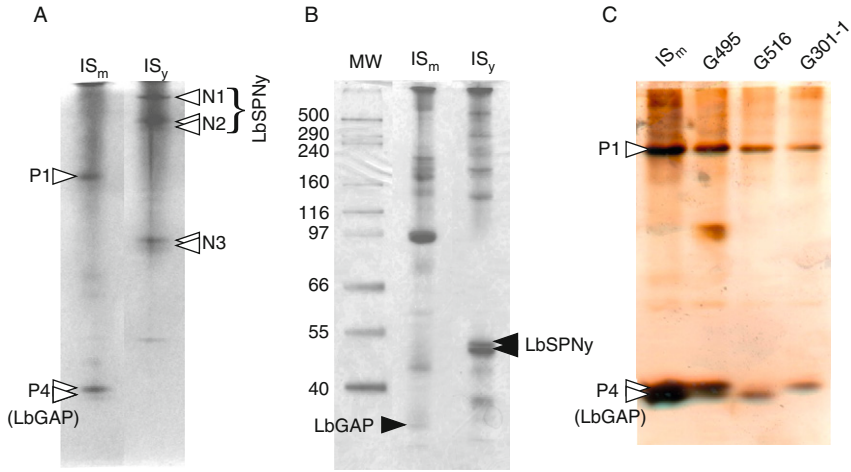

FIGURE 6.7 Comparison of the proteinic profiles of venom glands of *L. boulardi* from various localities. (A) Native-PAGE profiles of ISm and ISy strains (gel 8%). (B) SDS-PAGE profiles of ISm and ISy strains (gel 8%). (C) Native-PAGE profiles of *L. boulardi* strains virulent on *D. melanogaster* (gel 8%). Origin of strains: ISm: Nasr'allah, Tunisia; ISy: Brazzaville, Congo; G495: Lamto, Ivory Coast; G516:Toulouse, France; G301-1: Guadeloupe. White arrowheads: major native bands; black arrowheads: SDS bands containing identified virulence factors. MW: molecular weight in kDa. *Notes:* The profile of the ISy strain was published in Colinet et al. (2009); Fig. 6.7C is from Labrosse et al. (2005).

had a significant effect on the encapsulation rate of avirulent ISy eggs by *D. melanogaster* resistant larvae (Labrosse et al., 2005b). The strongest effect was nevertheless obtained with the band P4, for which injection had the same effect as that of whole venom gland extracts. Injection of this band also mimicked changes in the morphology of lamellocytes induced by parasitism (Labrosse et al., 2005a). These results led us to conclude that this band contains the major virulence factor of the line ISm, and that modification of lamellocytes is an essential part of the virulence strategy used by this line to escape encapsulation by *D. melanogaster*.

The protein band P4, eluted from native PAGE, was submitted to N-terminal sequencing allowing cloning the complete complementary DNA (cDNA). It encodes a RhoGAP (Rho GTPase-activating protein) domain-containing protein that was then renamed LbGAP (Labrosse et al., 2005b). Using Western blot experiments with a specific antibody against a recombinant LbGAP protein, it was confirmed that LbGAP is abundant in venom glands of ISm females, but it was not detected in the rest of the body (Labrosse et al., 2005b). Using immunofluorescence experiments, we showed that LbGAP enters plasmatocytes and lamellocytes and is directly involved in affecting the morphology of lamellocytes

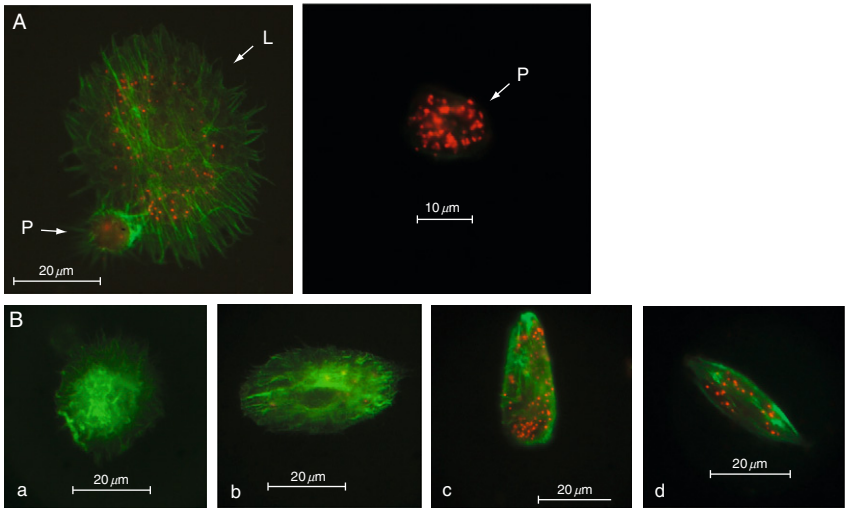

FIGURE 6.8 LbGAP enters *Drosophila* lamellocytes and plasmatocytes and affects lamellocyte morphology. (A) Example of LbGAP-containing hemocytes. L: Lamellocyte, P: plasmatocyte. (B) Classification of lamellocytes into four categories according to their morphological changes. a: Unmodified lamellocyte, b: slightly modified lamellocyte with 1–10 LbGAP spots, c: fairly modified lamellocyte with more than 30 LbGAP spots, d: strongly modified lamellocyte with 10–30 LbGAP spots. Hemocyte actin cytoskeleton was visualized using phalloidin (green). LbGAP was detected using a specific rabbit polyclonal antibody (red). *Note:* From Colinet et al. (2007).

(Fig. 6.8; Colinet et al., 2007). The quantity of LbGAP in a lamellocyte is indeed correlated with the degree of modification in the lamellocyte shape. Interestingly, LbGAP is observed as large spots in *Drosophila* hemocytes, which suggests that the protein is associated with larger structures.

The molecular bases of LbGAP effects have been further determined: using biochemical assays we showed that LbGAP has a RacGAP activity, and two-hybrid experiments allowed to characterize its targets in *D. melanogaster*. LbGAP specifically targets and inactivates the two Rac GTPases, Rac1 and Rac2 (Colinet et al., 2007). Rac GTPases are known to regulate cytoskeletal rearrangements necessary for cell-shape change and adhesion (Burridge and Wennerberg, 2004), which are an essential part of the insect cellular response against endoparasitoids. Moreover, both Rac1 and Rac2 were precisely reported to be required for successful encapsulation of *L. boulardi* eggs (Williams et al., 2005, 2006), thus explaining the physiological effects of LbGAP on host lamellocytes. These results were the first to describe the physiological effects of a parasitoid virulence factor together with its molecular function and its protein targets in the host.

By contrast to ISm, the ISy line of *L. boulardi* is encapsulated by resistant *D. melanogaster* flies and does not induce changes in lamellocyte morphology in *D. melanogaster* or in *D. yakuba* (Dubuffet et al., 2008; Russo et al., 2001). Further studies will be needed to determine whether this intraspecific variation of virulence results from qualitative differences in terms of functional activity of LbGAP or interaction with host targets or from a quantitative difference in its production. In agreement with the last hypothesis, LbGAP could not be detected in Western blots of ISy venom glands (Labrosse et al., 2005b). Besides, partial sequencing of the major bands in ISy venom did not reveal any peptide with similarities to Rho-GAP proteins (Colinet, unpublished data). This suggests that the LbGAP protein is not produced or is in a small amount in ISy venom but these data remain to be confirmed.

The characterization of LbGAP also allows us to address the question of host specificity of virulence. Indeed, the ISm line does not induce any modification of lamellocytes in *D. yakuba* (Dubuffet et al., 2008) and is totally avirulent on this host. Since there are no differences in Rac1 and Rac2 sequences between *D. melanogaster* and *D. yakuba*, the observed host specificity cannot be explained by a difference in the nature of the target of LbGAP. Another hypothesis would involve a difference in the capacity of LbGAP to enter lamellocytes between both *Drosophila* spp. The mode of entry of LbGAP in *D. melanogaster* lamellocytes is thus a central point to be elucidated. As reported above, VLPs can enter *Drosophila* hemocytes and we then suspect that LbGAP might be associated with VLPs thus facilitating its entry. This would explain why LbGAP is detected as ''large spots'' inside *D. melanogaster* hemocytes in immunofluorescence experiments. Moreover, we know that among the proteins characterized from samples of VLPs purified from the parasitoid *Venturia canescens* figures VLP2, a RhoGAP domain-containing protein such as LbGAP (Reineke et al., 2002). If VLPs act as ''transporters of virulence factors,'' then the difference in host specificity of ISm females might come from a difference in VLPs ability to target and enter lamellocytes of *D. melanogaster* and *D. yakuba*. A detailed comparison of these hemocytes in the two species should address this question.

6.4.3.4. The serpin SPNy virulence factor and its variation

Analysis of the protein content of the venom apparatus of ISy females led to the analysis of two major bands, named N1 and N2, which were not observed in the venom of ISm females (Fig. 6.7A). Mass spectrometry led to identification of similar peptides from the two bands, suggesting they contain the same protein. The corresponding cDNA encodes a serpin-domain-containing protein, LbSPNy (Colinet et al., 2009). Using real time polymerase chain reaction (qRT-PCR) experiments, this factor was shown to be specifically overexpressed in ISy venom glands compared to the rest

of the body (460-fold higher expression). Moreover, the recombinant LbSPNy protein reproduced the inhibition of the PO cascade observed with the venom (Fig. 6.5B; Colinet et al., 2009). LbSPNy is thus the first serpin demonstrated to be used as a virulence factor by a parasitoid wasp. Little is known on the *Drosophila* PO cascade as compared to Lepidopteran models. However, it seems that activation of the PO cascade, as well as melanization, occurs during the early parasitism period (Nappi and Christensen, 2005; Russo et al., 1996; Wertheim et al., 2005). These events seem to be important for the encapsulation to take place, since the injection of natural or synthetic serine protease inhibitors inhibits the encapsulation response (Ling and Yu, 2005; Nappi et al., 2005). Serpins act as suicide-substrate inhibitors, which means they become inactive once they inhibited their serine protease target (Law et al., 2006). The expression of many serine proteases is increased in the first 24 h following parasitization (Wertheim, 2005), which suggests that their production might at some point overcome the number of serpin molecules. The PO cascade could then be triggered, and the encapsulation could subsequently take place. Inhibition of the activation of the PO cascade by the serpin LbSPNy we described could thus be responsible for the transient immunosuppression observed in *D. yakuba* parasitized by ISy parasitoids. Our work opens the way to identification of the serine protease(s) targeted by LbSPNy, which will provide information on the regulatory pathways of *Drosophila* PO activation. An open area of research is now to determine how important is the use of serpins as virulence factors among parasitoids since they are known to be used by other parasites, such as nematodes, to evade the host immune responses (Knox, 2007; Zang and Maizels, 2001).

Interestingly, the venom of ISm *L. boulardi* females does not seem to contain any abundant protein potentially corresponding to LbSPNy (electrophoresis experiments and partial sequencing of major proteins, data not shown). Moreover, preliminary data suggest that ISm venom does not inhibit the PO cascade in *D. yakuba*. This supports the essential role of LbSPNy in targeting this cascade and might be one of the reasons why ISm females are not virulent on *D. yakuba* hosts. Further studies will be needed to explain the bases and evolutionary origin of these intraspecific differences.

6.5. DISCUSSION

6.5.1. On intra- and interspecific variability of virulence strategies in the *Leptopilina* genus

It remains a challenge to determine what makes a parasitoid ''virulent'' or ''avirulent'' against a given host, even when focusing on the immune aspects of the interaction. At first, the virulence strategies that allow a

successful parasitoid to escape the host immune defenses have to be known so that the aspect in which the avirulent parasitoid fails can be evidenced. In *Drosophila* parasitoids, the strategies that have been described are diverse and can differ even at the genus level. This corresponds to a difference in the virulence ''tactics'' used by these parasitoids. Most of our work aimed at elucidating *L. boulardi* virulence strategy, with the final objective of characterizing the processes underlying variations of virulence toward *D. melanogaster* and *D. yakuba*. However, it is also of interest to question the occurrence of variations in the means used to escape encapsulation within the *Leptopilina* genus.

One of our major results was to evidence the key role of LbGAP in the high virulence of the Tunisian ISm line of *L. boulardi*, and its ability to induce modification in lamellocyte shape (Colinet et al., 2007; Labrosse et al., 2005a,b). Such a strategy is probably the most common in *L. boulardi*. Indeed, the Guadeloupean line G301-1 also modifies the lamellocytes of *D. melanogaster* (Poirié, unpublished data). Besides, proteinic patterns of venoms of this line as well as lines from the south of France and Ivory Coast, all highly virulent on *D. melanogaster*, are roughly similar, and all include the bands that correspond to LbGAP (Labrosse et al., 2005b; Fig. 6.7C). *L. heterotoma* and *L. victoriae* have also been reported to suppress the ability of the host to encapsulate a foreign body (Morales et al., 2005; Rizki and Rizki, 1990; Schlenke et al., 2007) and to induce modifications in *D. melanogaster* lamellocytes. Considering these results, it would be tempting to conclude that the virulence strategy used toward the host *D. melanogaster* is largely conserved within the *Leptopilina* genus.

Data obtained with two *L. boulardi* lines, however, appear to question this conclusion: the Congolese line ISy and the Californian line Lb17 are consistently able to achieve successful parasitization of *D. melanogaster* larvae without inducing any modification of lamellocytes. Schlenke et al. (2007) performed microarrays to compare the transcriptional response of *D. melanogaster* larvae infected by Lb17 *L. boulardi* females and *L. heterotoma*. Based on the results that showed few changes in the transcription level of immune genes in hosts infested by *L. heterotoma* but upregulation or downregulation of several of these genes in Lb17-infected larvae, they concluded that *L. boulardi* and *L. heterotoma* have totally different virulence strategies. *L. heterotoma* would escape encapsulation by *D. melanogaster* through a ''near complete failure of attacked flies to mount an immune transcriptional response,'' while *L. boulardi* would escape encapsulation by attaching to the host tissues, a feature previously reported in other strains of *L. boulardi* (Rizki and Rizki, 1990). Whether this ''egg-sticking'' strategy explains the success of the line Lb17 remains to be determined. However, in the lines ISm, 301.1 and ISy, where we observed sometimes such egg attachment, we did not find any correlation

between attachment and parasitoid success, neither in *D. melanogaster* nor in *D. yakuba* (Dubuffet, unpublished data).

The Congolese line ISy of *L. boulardi* protects its eggs from encapsulation in the ''susceptible'' genotype of *D. melanogaster* but not in the ''resistant'' genotype (Dubuffet et al., 2007). Susceptible hosts are nevertheless immunocompetent, since they can encapsulate *A. tabida* (Poirié et al., 2000). The parasitoid success is not correlated with a modification of lamellocytes, which is consistent with the absence of immunodetection of the virulence factor LbGAP in the venom of the ISy line (Russo and Labrosse, 2005a). This result, together with a venom proteinic profile completely different from that of the other lines, suggests that the ISy line relies on an alternative virulence strategy to escape encapsulation in the susceptible larvae of *D. melanogaster*. Interestingly, this parasitoid line can also infest the ''susceptible genotype'' of *D. yakuba*. In this species, it inhibits the PO cascade activation due to the serpin LbSPNy and delays the proliferation of plasmatocytes (Colinet et al., 2009; Dubuffet et al., 2008). Future investigations will determine whether the virulence strategy used by ISy females on *D. melanogaster* is similar to the one described for *D. yakuba*.

The existence of lines such as ISy and Lb17 raises also an important question: is the modification of lamellocytes a conserved feature of the virulence strategy used by *Leptopilina* wasps (that was lost by both lines) or an example of convergence of effects? Such a convergence of effects is indeed commonly observed in host–parasitoid interactions. Roughly similar effects can be induced by various parasitoids on hosts that are as different as Lepidopteran caterpillars or *Drosophila* larvae, and due to completely different virulence factors. For example, disruption of actin cytoskeleton of hemocytes is induced by completely unrelated proteins such as those encoded by the polydnavirus gene CrV1 of *Cotesia rubecula* (braconid) or the polydnavirus gene VHv1.1 of *Campoletis sonorensis* (Ichneumonid), or by the factor LbGAP of *L. boulardi* (cynipid; Glatz et al., 2004; Labrosse et al., 2005b). Inhibition of PO activation is also caused by factors as various as a serine protease homolog in the braconid *C. rubecula*, a smapin in the braconid *Microplitis demolitor*, or a serpin in *L. boulardi* (Asgari et al., 2003; Beck and Strand, 2007; Colinet et al., 2009). The modification of the shape of lamellocytes observed after parasitism by *L. heterotoma*, *L. victoriae* or the lines ISm and G301-1 of *L. boulardi* could similarly result from totally different virulence factors that converge in their effects. Accordingly, preliminary data strongly suggest that RhoGAP proteins are not involved in *L. heterotoma* virulence against *D. melanogaster* (Colinet, unpublished data). To compare properly the virulence strategies used by parasitoids, we thus think that it is actually necessary to distinguish three levels within the term ''virulence strategies'': (1) the general strategy of the parasitoid, assessed through the

effects on host encapsulation ability (systemic immunosuppression or local immunoevasion); (2) the ''tactic(s)'' used to achieve this general strategy, that is, the immune components targeted by the parasitoid; and (3) the virulence factors used to achieve each of these tactics. Only the characterization of these virulence factors, and their resulting effects on specific components of the host immune system and on the whole encapsulation ability, will allow the full comprehension of the diversity of virulence strategies used by parasitoids.

Altogether, available data for different *L. boulardi* lines suggest that alternative virulence strategies exist in *L. boulardi*, the ISm/G301.1 strategy ''resembling'' more that of *L. heterotoma*. Surprisingly, within-species variability in the means to escape encapsulation is a question which has never been explored so far. Parasitoids species are usually considered as ''invariants,'' and comparisons between the virulence strategies used by different parasitoid species always rely on comparisons between single laboratory lines, as in the study performed by Schlenke (2007). In this case, comparison of the species *L. boulardi* and *L. heterotoma* could have resulted in quite different conclusions if other *L. boulardi* lines such as ISm were used in addition to the line Lb17.

6.5.2. On the variation of outcome in host–parasitoid interactions

The virulence strategy of parasitoids comprises multiple tactics, each achieved by one or many virulence factors. These tactics are used on diverse components of the host immune system, and in many models it appears that various tactics are employed at different periods of the parasitoid development (Dubuffet et al., 2008; Glatz et al., 2004; Schmidt et al., 2001). Similarly, encapsulation is a complex immune reaction that involves the coordination between recognition molecules, signaling pathways and immune effectors (Carton et al., 2008; Govind, 2008). Variations in the outcome of any host–parasitoid interactions can potentially originate from variations of any of the components of the parasitoid virulence strategy or host resistance.

Linking the molecular bases that underlie the variations of resistance and virulence in a host–parasitoid interaction is a thrilling objective in the field of evolutionary biology, since it aims to determine which genes in the host and in the parasitoid populations are potentially involved in coevolutionary processes. The achievement of this objective requires three important points: first, a genetic variation for resistance and/or virulence has to exist in the model. Second, it is necessary to have elucidated both the cellular and molecular processes leading to encapsulation (in unsuccessful infections) and the nature and function of effector virulence factors preventing encapsulation (in successful infections).

Third, tools have to be available to study these variations. When all these requirements are fulfilled, it is possible to determine what makes the difference between an avirulent and a virulent parasitoid, and/or between a resistant and a susceptible host and to assess whether these traits are under coevolution or not. Parasitoids of *Drosophila* are a model of choice to solve this puzzle, since extensive variations in the outcome of their interactions with *Drosophila* hosts are regularly reported in natural populations (Dubuffet et al., 2007; Dupas et al., 2003; Kraaijeveld and Godfray, 1999). The use of isofemale lines allows study of each factor that originate these variations, that is, the genetic variations of resistance and virulence. Moreover, *Drosophila* is the insect model for which the encapsulation response is the most studied, and the existence of genetic markers throughout the genome allows determination of which genes underlie the variation of resistance (Hita et al., 2006). We recently also characterized the virulence strategy used by the parasitoid *L. boulardi* on the hosts *D. yakuba* and *D. melanogaster*, and developed a method based on the comparison of physiological effects and virulence factors between avirulent and virulent lines to study the mechanisms underlying the variations of virulence (Colinet et al., 2009; Dubuffet et al., 2008; Labrosse et al., 2003, 2005a,b).

Studies performed on the parasitoids *L. boulardi* and *A. tabida* revealed that their success depends on both host and parasitoid genotypes (Dubuffet et al., 2007; Kraaijeveld and Godfray, 2001). However, the geographic variation in host–parasitoid outcomes is more explained by the variations in parasitoid virulence than by the variations in host resistance (see Section 6.2; Dupas et al. 2003; Kraaijeveld and Godfray, 2001). From other models, we know that environmental factors can also influence the host immune responses and the infective abilities of parasitoids (Blumberg, 1997; Calatayud et al., 2002; Delpuech et al., 1996; Fytrou et al., 2006; Karimzadeh and Wright, 2008). In order to investigate deeply the factors that influence the outcome of host–parasitoid interactions in the field, it is now necessary to determine whether the effects of environmental factors overcome those of host and parasitoid genetic factors, have on the contrary minor effects, or if all these factors interact altogether.

6.5.3. On the ways to reconcile the genetic and molecular data

Because host immune response and parasitoid virulence strategy are multifactorial, their variation was expected to be multigenic. However, genetic crosses or quantitative trait locus (QTL) analyses performed between resistant and susceptible host lines, or between virulent and avirulent parasitoid lines, always concluded in a simple genetic determinism (Benassi et al., 1998; Carton et al., 2005; Dubuffet et al., 2007; Orr and Irving, 1997). In other invertebrate–parasite systems, QTL analyses revealed that most of the genetic variation for resistance is generally

explained by few loci (2.47 on average), and in about 20% of the cases studied, resistance is explained by a single locus (Wilfert and Schmid-Hempel, 2008). However, when different parasites or host isolates were used, different QTLs were generally found (Wilfert and Schmid-Hempel, 2008). Whether this also applies in *Drosophila*–parasitoids systems has to be determined. For example, it would be interesting to assess whether the locus *Rlb* also explains genetic variation of resistance to the semivirulent strain Tasagil of *L. boulardi* (Kraaijeveld and van Alphen, 1995) or if it would be recovered from genetic analyses of resistance to the ISy line in other *D. melanogaster* strains. It would also be interesting to determine whether the gene corresponding to *Rlb* is also responsible for the genetic variation of resistance to the same ISy parasitoid line in *D. yakuba*.

Variation of virulence of *L. boulardi* toward *Drosophila* hosts was also found to be determined by single loci, the nature of which remains to be determined. Data from the field and from laboratory crosses both evidenced that the *ISm* and *ISy* loci, responsible for virulence against *D. melanogaster* and *D. yakuba* respectively, are distinct (Dupas and Boscaro, 1999; Dupas and Carton, 1999). This is in agreement with the variation of effects of ISm and ISy female venoms on the two host species, the ISm line containing the ISm^+ allele but not the ISy^+ and vice versa. A parasitoid which is ''strong'' on a host species is neither especially ''strong'' nor ''weak'' on the other, which would be the case if virulence alleles had positive pleiotropic effects on different hosts, or if virulence on each host species was allelic. Interactions between *L. boulardi* and these two host species (if not all *Drosophila* spp.) thus has to be considered independently (see Table 6.3).

There is an interesting challenge in linking each of the two loci *ISm* and *ISy* with variations in virulence factors contained in the venom of *L. boulardi* females. On *D. melanogaster*, we suspect that variation of virulence between the lines ISy and ISm might be linked to the presence or the quantity of the LbGAP protein in the venom (Labrosse et al., 2005b). It would be interesting to focus now on parasitoid lines that have intermediate levels of virulence, like the Turkish strain Tasagil, to see whether it is correlated with intermediate amounts of LbGAP. It also would be interesting to determine whether intrapopulation variations of virulence are linked with the variation of this factor. On *D. yakuba*, future investigations will determine whether qualitative or quantitative variations in serpins like LbSPNy could originate the variation of virulence between ISm and ISy lines (Colinet et al., 2009).

The loci *ISm* and *ISy* might thus encode for qualitative or quantitative variations of the factors LbGAP and LbSPNy, respectively, acting then as major loci for virulence. However, the presence/quantity of several venom proteins potentially involved in virulence, other than LbGAP and LbSPNy, differ between the lines ISm and ISy (Labrosse et al., 2005a, unpublished data). *ISm* and *ISy* loci might thus contain clusters of

TABLE 6.3 Genetic, immunologic and molecular determinants of virulence variation in *L. boulardi*

	Variation of virulence in *D. melanogaster*	Variation of virulence in *D. yakuba*
Parasitoid/host lines used to study the underlying mechanism	ISy (''avirulent'') and ISm (''virulent'')/ host reference line ''R''	ISy (''virulent'') and ISm (''avirulent'')/ host reference line ''R1''
Genetic determinism of virulence	Variation of encapsulation of the parasitoid eggs determined by the mother genotype One major biallelic locus, called *ISm*. ISm^{+} allele, associated with virulence, is semidominant over ISm^{-}	Variation of encapsulation of the parasitoid eggs determined by the mother genotype One major biallelic locus, called *ISy*. ISy^{+} allele, associated with virulence, is recessive to ISy^{-}
Variation of effects on the host encapsulation ability (virulence factor which variation might be responsible for the variation of virulence)	Variation in immunosuppressive effects of parasitoids (venom, LbGAP-containing proteinic band P4 in particular)	Variation in immunosuppressive effects (venom)
Variation of effects on the cellular immune response (virulence factor which variation might be responsible for the variation of	Less important proliferation of lamellocytes in ISm parasitized hosts Alteration of lamellocyte shape in hosts parasitized by	Less important plasmatocyte proliferation in ISy parasitized hosts No effect on lamellocyte shape

(*continued*)

TABLE 6.3 (*continued*)

	Variation of virulence in *D. melanogaster*	Variation of virulence in *D. yakuba*
parasitoid success)	virulent ISm parasitoids (venom, LbGAP contained in proteinic band P4, in particular)	
Variation of effects on the humoral immune response (virulence factor which variation might be responsible for the variation of parasitoid success)	?	Inhibition of the PO cascade activation and subsequent melanization by ISy parasitoids (venom, SPNy in particular). No effect of ISm parasitoid venom

virulence genes encoding these proteins, and/or correspond to a single gene with pleiotropic effects (e.g., it could encode a transcription factor responsible for increased expression of different genes in venom tissues). Alternatively, variations in the presence/quantity of proteins other than LbGAP and SPNy could originate from other virulence loci, each determining the virulence of *L. boulardi* on a specific host species. Differences in minor proteinic bands have been noticed between the venoms of lines having similar virulence properties on *D. yakuba* and *D. melanogaster* but not on *D. simulans* (Labrosse, unpublished data). Further investigation of the proteins contained in these bands, along with the characterization of the physiological mechanisms underlying variation of virulence of *L. boulardi* on *D. simulans* will generate interesting data for the comprehension of the diversity of virulence factors contained in parasitoid venoms.

6.5.4. On intraspecific variation of virulence and host specificity in parasitoids

Thompson hypothesized that host or parasitoid populations from various geographical areas should differ in their traits involved in the interaction with the interacting species, due to a geographical mosaic of selection

(Thompson, 2005). Such mosaic of selection is likely to occur in many parasitoid species, especially if the range of species available is highly variable depending on the localities, as for *L. boulardi* (Dupas et al. 1999; 2003; see Chapter 11 by Dupas et al.). The availability of each host species as well as their respective levels of resistance may then shape the evolution of the virulence strategies in each parasitoid population. They can eventually become quite different, with the involvement of different virulence factors. Such variation in the nature or quantity of virulence factors resulting from this geographical mosaic of selection could then lead to strong variations of virulence, as observed in *L. boulardi*.

Most parasitoids rely on factors contained in their venom glands and/or calyx fluids to escape encapsulation by their hosts. Many of these factors, injected during oviposition, are proteins produced by the wasps themselves (Moreau and Guillot, 2005), but others are viruses (polydnaviruses, PDVs) encoding for virulence factors which are expressed due to the host machinery during the parasitoid development (Bezier et al., 2009; Glatz et al., 2004; Renault et al., 2005). Future investigations will determine whether variations in parasitoid success are common, and whether they can be correlated with qualitative and/or quantitative variations in venom or calyx fluid secretions. To our knowledge, genetic variation of virulence has been documented in only three species in addition to parasitoids of *Drosophila*: *Cotesia sesamiae*, *Aphidius ervi* and *Lysiphlebus fabarum* (Henter, 1995; Ngi-Song et al., 1998; Vorburger et al., 2009). From these, only the braconid *C. sesamiae* was studied for the molecular basis underlying the variations of virulence. Injection of virulent wasp calyx fluid in hosts infected by the avirulent wasp allows development of the avirulent parasitoid (Mochiah et al., 2002). The virulent and avirulent lines differ in the presence of few proteinic bands in calyx fluid analyses (Gitau et al., 2006) and show qualitative and quantitative differences at the level of the CrV1 PDV gene, known to induce inactivation of host hemocytes (Gitau et al., 2007). CrV1 variants between virulent and avirulent parasitoids strains are also submitted to positive Darwinian selection (Dupas et al., 2008), which suggests that diversity is selected in this PDV gene, maybe in relation with changes in the host range.

The ability of parasitoids to parasitize a new host species successfully can rely either on the *de novo* production or overproduction of molecules that complete the repertoire of virulence factors already present or to subtle changes in the present virulence molecules. Such changes could allow the virulence factors to ''match'' with their targets in new host species, which might also present some subtle differences. These mechanism could explain the diversity of some gene families in PDVs as well as the positive selection pressures observed on some genes of these families, like CrV1 (Dupas et al., 2008; Espagne et al., 2004; Serbielle et al., 2008). Of course, the targets of these parasitoid virulence factors have to be identified, as well as

their own variation in order to determine whether virulence factors can diversify as a result of their coevolution with host targets.

Altogether, the opening of the ''black box'' containing the mechanisms underlying the variations of outcomes in host–parasitoid interactions results in the opening of an exciting area of research. It gives insights about the role of virulence factors contained in venoms or other secretions in the evolution of parasitoid host ranges, and raises also questions about the molecular basis of the specificity of these virulence factors. Hopefully, future studies on these challenging questions will include more parasitoid models and will provide interesting data about the overall evolution and diversification of parasitoids.

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