Chapter 1

General Introduction
• **INCOMPLETE AND COMPLETE METAMORPHOSIS.**

The body of an insect is completely covered by an exoskeleton (external skeleton) called cuticle, constructed from layers of proteins and chitin. It protects the animal and provides points of attachment for the muscles that move the appendages. In order to grow, an insect must occasionally shed its old exoskeleton and secrete a larger one. This process called moulting, is energetically expensive and leaves the animal temporarily vulnerable to predators and other dangers.

Some insects develop by passing through a series of successive moults. With each moult they get to look more like the adult, but there is no striking change in appearance at any one moult. The phenomenon is named incomplete metamorphosis and these insects are called hemimetabolous. The grasshopper is an example of an insect that matures in this fashion (Figure 1a). In contrast, many other insects pass through successive stages that are quite different from the adult form. From the egg hatches a larva, which grows and moult several times until the last larval stage. Then, the last moult forms a pupa, which does not move or feed. During pupation, all larval structures are broken down and used as raw materials in the adult development. Each part of the adult (legs, wings, eyes, etc…) develops from groups of cells, called imaginal discs (Figure 2), which remain more or less quiescent during the larval period. Finally, the adult emerges from the pupa. This phenomenon is named complete metamorphosis, and the insects undergoing a complete metamorphosis are called holometabolous. Butterflies, like the giant silkworm (Figure 1b), are generally cited as an example to present the large differences between the larval forms, the caterpillars, and the flying adults. *Drosophila melanogaster* also belongs to the holometabolous insects (See Figure 3 for the different life stages of *D. melanogaster*).

Moulting and metamorphosis are under endocrine control involving the brain and the associated glands innervated by the brain (*corpora allata* and *corpora cardiaca*) together with the prothoracic glands, and the two major hormones, ecdysone and juvenile hormone (Figure 4).
• **AN EMBRYO ENJOYING AN INDEPENDENT LIFE.**

From earlier times, the complete metamorphosis of holometabolous insects, with their different juvenile and adult stages, has been regarded with a curious interest. The different life stages exhibit different forms and exploit different habitats. For Aristotle the embryonic life of insects continues until the formation of the perfect adult insect, or imago: “the larva while it is yet in growth”, he writes, “is a soft egg”. For Harvey (1651), the eggs of insects contain too few reserves and the embryo has to leave the egg to complete its development. A larval stage is needed to store resources. Then, when there is enough reserve, the larva turns back to a new form of “egg”, called pupa, from which the imago will hatch. The same idea was retained by Ramdohr (1811) when he called the caterpillar a “moving, growing and feeding egg”, and by Viallanes (1882) when he wrote “those sorts of eggs that are called nymphs or pupae”.

• **DARWIN AND THE NATURAL SELECTION THEORY.**

In the middle of the 19th century, Darwin expressed in “On the origin of species by means of natural selection” (1859) a new theory to explain the mechanism of evolution of life forms. This theory is based on the idea that those individuals that are best adapted to their environment have a higher fitness and leave more descendants to the next generation. However, Darwin had no notion of the mechanism of genetics as expressed in Mendel’s laws of inheritance. The blending inheritance model that Darwin applied leads to a strong reduction of genetic variation in the course of generations, and hampers evolutionary change. The rediscovery of Mendel’s laws of inheritance, the work of Morgan in the beginning of the 20th century on the chromosomes of the fruitfly *D. melanogaster*, and afterwards the development of population genetics (Fischer, Haldane, Wright in the early 1930’s), specified the genetic mechanisms behind the process of evolution.

The evolution of complete metamorphosis in holometabolous insects is viewed today in the light of the natural selection theory. The story starts with the acquisition of the ability to fly (Kukolova-Peck, 1978) in hemimetabolous insects. Flight allows insects to evade predators, find new food resources and disperse rapidly, three major advantages.
However, because they typically maintain a similar body plan throughout their life, the immature and adult stages of most hemimetabolous insects live in the same habitat and utilize the same food. Consequently, there is a potential for competition between the needs for growth and the needs for reproduction (Truman and Riddiford, 2002) between the juvenile stages and the adult stage of hemimetabolous insects.

For holometabolous insects, the juvenile stages, which do not reproduce, have often evolved in a way to limit the risks of predation by living in specific and safer environments inside fruits (fruit flies), inside beans (bruchus), or even inside the body of other insects in the case of parasitoid wasps like *Nasonia*. Holometabolous insects are able to exploit a large diversity of resources during their life because of the complete modification of life stages. Furthermore, the pupal stage is generally able to resist for a long time during unfavorable conditions without feeding.

- **Appearance of the Complete Metamorphosis.**

Complete metamorphosis presented a large new potential for innovations and adaptations, and gave clearly a major advantage to the holometabolous insects, which are now the most diverse group of organisms and constitute about 90% of all insect species. Though we have a good understanding of the advantages of the large difference between larval and adult stages of holometabolous insects in the light of evolution, still the question remains which mechanisms are involved in the appearance of complete metamorphosis. In other terms, how did the three-part life cycle of holometabolous insects (larva, pupa and adult) evolve from the nymph and adult life stages of ancestral insects?

One view initially proposed by Berlese (1913) and later elaborated by Imms (1937) makes a clear distinction between the nymphs of hemimetabolous insects and the larvae of holometabolous insects. They noted a similarity between the larval body forms and the morphological transitions seen during embryogenesis of hemimetabolous insects. They proposed that the holometabolous larva arose from a premature completion of embryonic developments. As the larva became the major feeding stage, the number of nymphal instars was reduced to a single instar that became the pupa.
A second hypothesis, originally proposed by Poyarkoff (1914) and extended by Hinton (1963), is generally accepted (Sehnal et al., 1996). According to this hypothesis, nymphal and larval stages were initially equivalent. The start of niche separation between juvenile and adult forms leaded to an increasing gap in morphology and gave rise to the evolution of a transitional stage between larva and adult: the pupa.

However, Truman and Riddiford (1999, 2002) have developed a new hypothesis based on recent works on endocrine and developmental data, which is more consistent with some of the views of Berlese. They suggested that the pronymph of hemimetabolous insects, which ends the embryonic period just before the first-stage nymph, appears to be the basis of the holometabolous larva. In other terms, this last hypothesis is more or less consistent with the idea of Aristotle about the larva: an embryo enjoying an independent life.

**ENVIRONMENTAL STRESS AND EVOLUTION IN HOLOMETABOLOUS INSECTS.**

Environmental stress, defined as an environmental factor that reduces Darwinian fitness, appears to play a major role in evolution (Calow and Berry, 1989; Hoffmann and Parsons, 1991; Bijlsma and Loeschcke, 1997a). Such an environmental component is often irregular in its occurrence and intensity, and can lead to a high selection pressure. Even if the event is rare, the effects on the population can be strong and lead to rapid phenotypic and genotypic changes. For example, in the finch *Geospiza fortis* on the Galapagos Islands, only 15% of the birds survived a severe drought period in 1977 (Boag and Grant, 1981). The birds with the larger beaks survived best, and the selection intensities were among the highest recorded for a vertebrate population. The understanding of the effects of such environmental stress on populations may become very important not only to understand the mechanisms of evolution, but also to find applications in conservation biology (Bijlsma et al., 1997).

Holometabolous insects live generally in different habitats according to their life stage. Larva and adult have a different shape and often have even a different alimentary regime. However, it is still only one individual with, of course, only one genome. A
single environmental stress may thus affect differently each life stage, and the adaptation to a particular stress may be specific to a particular life stage (Krebs and Loeschcke, 1995; Loeschcke and Krebs, 1996). Alternatively, an increase in tolerance to a stress in one life stage may be correlated to a similar increase in an other life-stage because the same mechanism is involved. The analysis of the genetic effects of stress acting in different life stages is the main topic of this thesis.

• **Drosophila melanogaster as a model organism.**

The fruitfly *Drosophila melanogaster* is one of the most extensively studied animals in genetics, including population genetics. *D. melanogaster* is a small fly and is very easy to culture in large numbers under laboratory conditions. The generation time is quite short, less than two weeks at 25°C, which allows long-term experiments. As all holometabolous insects, the fruitfly has a complete metamorphosis which divides its life in two major phases: the larval stage which is the growing phase (Figure 3), and the adult stage which is the reproductive phase. *D. melanogaster* is thus the perfect animal model to study the different effects of a single environmental stress on both larval and adult stages. Furthermore, many studies have been performed in *D. melanogaster* with respect to alcohol tolerance (van Delden, 1982; David, 1988; Geer et al., 1990; van Delden and Kamping, 1997; Ashburner, 1998).

• **Alcohol resistance in Drosophila melanogaster.**

The fruitfly *D. melanogaster* provides us an excellent model system to study the effects of environmental stress on both juvenile and adult stages. The feeding substrate in the wild of this species is constituted by decaying fruits which can contain considerable concentrations of ethanol due to fermentation (McKenzie and McKechnie, 1979; Gibson et al., 1981). Females lay their eggs and larvae grow and feed on this medium. Both juvenile (larval) and adult stages exploit more or less the same habitat and may be in contact with toxic concentrations of ethanol. However, because the larvae are bound to their site, they cannot escape and have to cope with ethanol. At low concentrations ethanol can be used as a food component, but at higher concentrations it becomes toxic.
The flying adults on the contrary are very mobile and may always move to a feeding or an egg-laying site with a lower level of alcohol. Boulétreau and David (1981) hypothesized that the stay of adults on fermenting fruits is too short to require a special metabolic adaptation and concluded that the selective pressure mediated by ethanol occurs mainly during the juvenile stages. Several studies suggested that the life stages of the fruitfly were substantially variable in their sensitivity to toxic concentrations of alcohol (Middleton and Kacser, 1983; Hoffmann and McKechnie, 1991; Freriksen et al., 1994).

Alcohol tolerance in D. melanogaster presents an additional advantage to study our question: it has been one of the centers of interest in the selectionist-neutralist controversy in evolutionary biology (see Brookfield and Sharp, 1994) and has been intensively studied in the last 30 years (see van Delden, 1982; Geer et al., 1990; van Delden and Kamping, 1997; Ashburner, 1998). In the selectionist point of view, the large extent of allozyme polymorphisms (see Lewontin and Hubby, 1966; Lewontin, 1974; Powell, 1975; Nevo, 1978; Brown, 1979; David and Capy, 1988) is maintained by natural selection (Ayala, 1972, 2000) by means of balancing selection (through higher fitness of heterozygotes, frequency dependent selection or variable selection in time or place). On the contrary, the neutralist hypothesis (Kimura, 1968; 1983; 1991; Kimura and Otha, 1971) states that the observed variation is mainly a product of mutation and drift of selectively neutral genes. As the enzyme alcohol dehydrogenase (ADH) plays an important role in alcohol tolerance, the Adh polymorphism in relation with alcohol tolerance in D. melanogaster provided an excellent opportunity to study the controversy between selectionists and neutralists (see next paragraphs).
ALCOHOL DEHYDROGENASE AND ALCOHOL RESISTANCE IN *D. MELANOGASTER*

Alcohol dehydrogenase (ADH) is a key enzyme in the metabolic pathway of alcohols in *D. melanogaster* (Figure 5). More than 90% of the conversion of ingested ethanol to acetaldehyde is mediated by ADH (Heinstra *et al*., 1989; Geer *et al*., 1993), while acetaldehyde is converted to acetate also by ADH in larvae, and by acetaldehyde dehydrogenase (ALDH) in adults (Heinstra *et al*., 1983, 1989; Eisses *et al*., 1985; Leal and Barbancho, 1992). ADH is essential to the fly to survive in ethanol environments, as has been shown by the sensitivity of Adh null mutants (David *et al*., 1976; Kamping and van Delden, 1978; van Delden and Kamping, 1988; Geer *et al*, 1990; Bijlsma and Bijlsma-Meeles, 1991).

THE ALCOHOL DEHYDROGENASE (ADH) POLYMORPHISM.

The Adh polymorphism was first described by Johnson and Denniston (1964). Natural populations of *D. melanogaster* contain two common electrophoretic alleles: the fast (AdhF) and the slow (AdhS) allele. The two corresponding enzymes differ in only a single amino acid. At position 192, a lysine in ADH S is substituted by a threonine in ADH F. This modification leads to an clear difference in the catalytic efficiency between ADH S and ADH F. For both juvenile and adult stages, individuals homozygous for AdhF exhibit a three to four fold higher in vitro ADH activity compared to AdhS individuals of the same life stage. AdhSF heterozygotes show an intermediate ADH activity. Laurie-Ahlberg (1985) showed that the difference in ADH activity is partly due to a difference in the concentration of the ADH protein, and partly to a difference in catalytic efficiency. This difference in enzyme activity is generally associated with a difference in alcohol tolerance. AdhFF has a better egg-to-adult survival and adult survival in presence of ethanol compared to AdhSS, while the heterozygotes AdhSF exhibit generally an intermediate survival (Oakeshott, 1976; Kamping and van Delden, 1978; van Delden *et al*., 1978; Vigue *et al*., 1982; Dorado and Barbancho, 1984; van’t Land, 1997, Oppentocht, 2001). In sites with high ethanol concentrations, the AdhF allele gives an advantage, consequently a small-scale geographic variation in Adh allele frequencies may be observed between wineries and surrounding areas (summary in van Delden, 1982), with higher AdhF frequencies in wineries (Gibson and Wilks, 1988;
McKenzie et al., 1994). The recently obtained structural information on ADH (Benach et al., 2000) could provide new elements for a better understanding of the mechanisms underlying molecular evolution and population genetics. However, apart from ADH it appears that also other factors contribute to alcohol resistance.

- **THE LATITUDINAL CLINE OF THE ADH POLYMORPHISM IN D. MELANOGASTER.**

In the case of geographic clines, in which the frequency of a specific allele is linked to a geographic factor such as altitude or latitude, natural selection may be inferred (Hoffmann and Parsons, 1991). Many morphological or stress resistance traits vary with latitude in an identical way on several continents (Hallas et al., 2002). Although the adaptative nature of such latitudinal clines is generally implied, the exact nature of selection is often obscure.

The geographical distribution of Adh allele frequencies is characterized by the occurrence of such a latitudinal cline, which has been found for both Northern and Southern hemispheres. In all cases, the frequency of AdhS is high at the equator and declines with increasing latitude (David, 1982; Oakeshott et al., 1982; Cohan and Graf, 1985; David et al., 1986; van’t Land et al., 1993, 1995; Parkash and Shamina, 1994; Bénassi and Veuille, 1995; Bubli et al., 1996; van’t Land, 1997; van't Land et al., 2000). However, it is not evident that alcohol tolerance, in relation with ADH activity, is the cause of this cline. Adh is linked to two other polymorphisms, the alpha-glycerophosphate dehydrogenase (αGpdh) allozyme polymorphism and the inversion In(2L)t polymorphism, both located, like Adh, on the left arm of the second chromosome in D. melanogaster (Figure 6). Recent investigations suggested that the inversion In(2L)t associated with AdhS, plays a dominant role in resistance to high temperatures and is (partly) responsible for the Adh latitudinal cline (van Delden and Kamping, 1997; Kamping and van Delden, 1999; Kamping, 2000).
Chapter 1

- **TWO SEPARATE PROMOTERS FOR THE SAME GENE.**

Although alcohol may not be the environmental factor responsible for the *Adh* latitudinal cline, it is certainly an environmental stress which has different effects on *D. melanogaster* according to the life stage. *Adh* is effectively a key enzyme for alcohol detoxification, but the impact of alcohol may depend on the life stage. The presence of two promoters separated by about 700 base pairs (Benyajati *et al.*, 1983) may be a consequence of this difference according to the life stage. Effectively, *Adh* expression during the larval stages is mainly mediated by the proximal promoter, while in adults the distal promoter is primarily used (Savakis *et al.*, 1986).

The presence of ethanol in the food increases the ADH activity level in larvae (McKechnie and Geer, 1984; Kerver and van Delden, 1985), because of an increase in transcriptional level from the proximal promoter (Geer *et al.*, 1988, Kapoun *et al.*, 1990). The presence of ethanol induces also the transcription from the distal promoter, but to a lower extent. This transcriptional difference between juvenile and adult stages may be related to a difference in the selection pressure from alcohol, and may reflect an adaptation specific to the life stage. The induction of the proximal transcript may allow the larvae to a short-term adaptation when alcohol is present in their feeding site from which they cannot escape, furthermore the energetic costs to produce ADH when alcohol is absent will be limited. This is not a superficial saving of energy as ADH can make up to one per cent of the total soluble protein in larvae (Chambers, 1988, 1991). On the contrary, as the flying adults are always able to escape when the alcohol concentration is too high, this long term adaptation is not essential and the induction of the distal transcript will be limited.

The topic of this thesis is how natural selection acts on the different life stages of a holometabolous insect. An attempt is made to reveal the underlying genetic mechanisms.
• **OUTLINE OF THE THESIS.**

As explained previously, an environmental stress can affect insects differently according to the life stage. The selection pressures of alcohol are different for the larvae of *D. melanogaster* which are bounded to their substrate, and for the adults free to fly to an other site. An artificial selection experiment is a relevant method to study the effects of an environmental stress. It allows to select the individuals with the higher resistance, and then, generation after generation, it may lead to the increase of the frequency in the population of alleles involved in the resistance to the stress, until eventually their fixation.

To investigate if mechanisms of resistance specific to a life stage exist, three different procedures of selection have been followed. In the first one (ADU), individuals were selected only at the adult stage, while during the juvenile stages the individuals were never in contact with alcohol. In the second selection procedure (LAR), on the contrary, the juvenile stages were fed and grown in an alcohol-supplemented medium, and the adults were kept on standard medium without ethanol. Finally, in the third selection procedure (WHO), the individuals were continuously kept on ethanol medium during their complete life cycle. The initial population was kept on standard medium and used as a control (CON) for the experiments. As the different effects of the two common alleles of *Adh* on ethanol tolerance are already well known, and because we did not want to select just the *Adh*<sup>F</sup> allele in the three different selection procedures, which can be expected when the selection starts in a polymorphic population, we decided to perform all selection procedures in duplicate, starting with two populations homozygous either for *Adh*<sup>S</sup> or for *Adh*<sup>F</sup>. The hypothesis is that the life stage specific selection will lead to life stage specific adaptation. Though it is clear that the *Adh* gene is involved in alcohol resistance, the presence of other genes associated with resistance after longterm exposure to ethanol will be investigated in relation to life stage and *Adh* genotype.

**Chapter 2** presents direct and indirect responses for all selected lines after 20 generations of selection. For the direct responses, we examined both egg-to-adult and adult survival on ethanol medium. It shows a clear response in adult tolerance for the lines selected at the adult stage (ADU), and an increase in egg-to-adult survival for the lines selected at the juvenile stages (LAR) for both *Adh* genotypes. For the indirect
responses, we examined several traits that are known to be related to the alcoholic stress (ADH activity) or related to other environmental stresses (developmental time, body weight, and protein content). Each response is discussed according to the life stage, the Adh genotype and the sex for the adults.

In Chapter 3 we focus on the response in survival for both life stages after 40 generations of selection, in parallel with the response in Adh expression and ADH activity. We compare the increase in alcohol tolerance and ADH activity for both adult and juvenile stages after 20 and 40 generations of selection procedure. The role of ADH in the increase in survival specific to the life stage is discussed.

Chapter 4 evaluates the relative importance of induction of Adh expression by ethanol and ADH activity in adults and larvae derived from the various selection procedures after 40 generations of selection. Induction in the juvenile life stages is compared with induction in adults, and its role in alcohol tolerance according to the life stage is discussed.

Chapter 5 presents the chromosomal localization of loci involved in the increase in ethanol tolerance in two selected lines, one selected for increased adult tolerance and homozygous for Adh^F (ADU-FF), and the other selected for increased juvenile tolerance and homozygous for Adh^S (LAR-SS). The number of loci involved and the role of each chromosome in alcohol tolerance in relation with the Adh gene, located on the second chromosome in D. melanogaster, is discussed.
General introduction

Figure 1: Comparison of the life histories of an insect with incomplete metamorphosis, the grasshopper (left), and an insect with a complete metamorphosis, the butterfly giant silkworm (right).
Figure 2: Schematic representation of the larval organisation and the location of the different imaginal discs in *Drosophila melanogaster*. Discs and their corresponding derivatives are connected by lines (Modified from Wildermuth, 1970).
Figure 3: Egg, first, second and third instar larvae, and the late pupa of *Drosophila melanogaster*. The bar indicates 1 mm (After Ransom, 1982).
Figure 4: Classical scheme of the hormonal control of moulting and metamorphosis in a holometabolous insect (After Rees, 1977).
**Figure 5**: Metabolic pathway of ethanol and acetic acid degradation. ADH - alcohol dehydrogenase; ALDH - Aldehyde dehydrogenase; ACS - acetyl CoA synthetase; CS - citrate synthetase; CL - citrate lyase; OA – oxaloacetate (After Oppentocht, 2001).
Figure 6: Schematic representation of the second chromosome of *Drosophila melanogaster*, with the positions of the inversion *In(2L)t*, the Adh locus and the αGpdh locus. The numbers represent map positions (in cMorgan) of the enzyme loci, and polytene chromosome band positions for the inversion. The Adh locus is located within the chromosome band 35B3 (Woodruff and Ashburner, 1979), and the αGpdh locus within the chromosome band 26A (Cook *et al.*, 1986) (After van’t Land, 1997).