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THE EFFECTS OF NUTRITIONAL ENVIRONMENT ON INBREEDING DEPRESSION AND THE EXPRESSION OF CONDITION-DEPENDENT TRAITS

by

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LIST OF ORIGINAL ARTICLES

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1. INTRODUCTION

1.1. Inbreeding effects in naturally outbreeding species

All populations of normally outbreeding species contain a load of rare partially recessive alleles that reduce fitness when homozygous (Frankham et al. 2010). Since inbreeding increases levels of homozygosity the hidden genetic load carried by a population is exposed by mating among relatives (Frankham et al. 2010). Despite the evolved behavioral and genetic mechanisms to avoid inbreeding, mating among relatives occurs regularly in many populations of wild species (Pusey & Wolf 1996; Crnokrak & Roff 1999; Hedrick & Kalinowski 2000; Keller & Waller 2002; O'Grady et al. 2006; Frankham et al. 2010). The negative effects of incestuous mating on individual performance and population persistence can be devastating (Keller & Waller 2002; Frankham 2005; Kristensen & Sørensen 2005; Reed et al. 2007). Apart from the negative effects on the mean values of many fitness-related traits, inbreeding leads to changes in the distribution of genetic variance. Ultimately, in small and isolated populations inbreeding and genetic drift tend to decrease genetic variability (Kristensen & Sørensen 2005; Buskirk & Willi 2006). The extent to which genetic diversity is lost and characters displaced away from the selective optima are important for individual fitness as well as for population dynamics and viability (Buskirk & Willi 2006; Willi et al. 2006; Hughes et al. 2008; Vandewoestijne et al. 2008; Reed et al. 2007). Because genetic diversity is required for populations to evolve in response to environmental changes and because heterozygosity levels are linked directly to reduced population fitness via inbreeding depression, genetic diversity is one of the three levels of biodiversity that the World Conservation Union (IUCN) has recommended for conservation (Reed & Frankham 2003; Frankham et al. 2010).

Inbreeding depression occurs when offspring produced by the mating of close relatives show reduced trait values (Wright et al. 2008). Two hypotheses have been advanced to account for the existence of inbreeding depression. According to the overdominance hypothesis inbreeding depression is due to the general superiority of heterozygotes over homozygotes, whereas the partial dominance hypothesis posits that inbreeding depression results from the increased expression of deleterious recessive or partially recessive alleles that are masked in heterozygotes but are exposed in homozygotes (Charlesworth & Charlesworth 1987, 1999; Roff 2002a; Charlesworth & Willis 2009; Kristensen et al. 2010). Which of the two hypotheses underlies the cause of inbreeding depression is still open to debate, although the partial dominance hypothesis has replaced the once more popular overdominance hypothesis, and is now the most favored in explaining inbreeding depression at least for most fitness traits (Charlesworth & Charlesworth 1987, 1999; Barrett & Charlesworth 1991; Dudash & Carr 1998; Roff 2002a; Wright et al. 2008 but see e.g. Karkainen et al. 1999; Li et al. 2001; see also Willis 1999; Frankham et al. 2001; Crnokrak & Barrett 2002; Radwan 2003; Swindell & Bouzat 2006a,b). Because the

strength of inbreeding depression depends on the genetic load carried by a population, inbreeding depression may not always be visible in inbred individuals, and even within populations it may be environmentally-dependent (Crnokrak & Roff 1999; Armbruster & Reed 2005; Szulkin & Sheldon 2007).

1.1.1. Trait and environment specific consequences of inbreeding

Although inbreeding generally reduces fitness its magnitude and effects can be highly variable depending on the trait (Roff 1998; DeRose & Roff 1999; Wright et al. 2008; Mikkelsen et al. 2010), environment (Armbruster & Reed 2005; Kristensen & Sørensen 2005; Liao & Reed 2009; Kristensen et al. 2010), history of inbreeding (Bijlsma et al. 2000; Kristensen et al. 2003; Reed et al. 2003; Pedersen et al. 2005; Demontis et al. 2009), genetic makeup of a population (Reed et al. 2003, 2007; Vandewoestijne et al. 2008; Bijlsma et al. 2010) and selection (Bijlsma et al. 1999; Whitlock 2002; Swindell & Bouzat 2006c; Leberg & Firmin 2008; Demontis et al. 2009). The effect of inbreeding on a given trait depends upon the proportion of directional dominance in it (Roff 1997; Roff & Emerson 2006). Because traits under weak selection are expected to show less directional dominance than traits that are under stronger selection (Lynch & Walsh 1998) inbreeding depression is expected to be more pronounced for life-history traits than for traits not closely related to fitness (Roff 1997; Roff 1998; DeRose & Roff 1999; Roff & Emerson 2006; Wright et al. 2008). The influence of environmental variation on the magnitude of inbreeding depression has gained a lot of attention in the past few years. Because of their decreased overall fitness and genetic variability inbred individuals are expected to be more sensitive to changing environmental conditions than their outbred conspecifics (Bijlsma et al. 1999; 2000; Dahgaard & Hoffmann 2000; Kristensen et al. 2003, 2010; Reed et al. 2003; Vermeulen & Bijlsma 2004; Armbruster & Reed 2005; Kristensen & Sørensen 2005; Swindell & Bouzat 2006c; Liao & Reed 2009). Furthermore, most studies report more pronounced inbreeding depression under novel and stressful conditions (Armbruster & Reed 2005; Kristensen & Sørensen 2005; Liao & Reed 2009; Kristensen et al. 2010).

Armbruster and Reed (2005) reviewed the literature on the relationship between the magnitude of inbreeding depression and environmental stress and although in 76 % of the reviewed cases inbreeding depression was found to be greater under stressful conditions the authors emphasized the importance of noting the large number of cases in which inbreeding depression was not found to increase. Moreover, in the common fruit fly, *Drosophila melanogaster*, low levels of inbreeding depression in resistance to a number of stress factors were recently reported (Mikkelsen et al. 2010). Organisms use such methods as increased expression of stress proteins and changes in metabolism and hormone concentration to cope with environmental stress (Hoffmann & Parsons 1991; Sørensen et al. 2003). Genes being differentially expressed between inbred and outbred lines have been shown to include an overrepresentation of those associated with metabolism, stress and defense suggesting that inbreeding induces some of the

same responses as environmental stress (Kristensen et al. 2002, 2005; Pedersen et al. 2005; Ayroles et al. 2009; Paige 2010). It has been suggested that the deleterious effects of inbreeding could, at least to some extent, be ameliorated by a set of genes that respond to inbreeding (Vermeulen et al. 2008). For instance, up-regulation of genes coding for antibacterial peptides in an inbred population could play a role in explaining those observations in which better disease resistance is found among inbred compared to outbred populations (Kristensen et al. 2003). On the other hand, inbred individuals have been suggested to have the option of devoting more resources to stress resistance than outbred individuals as a consequence of their reduced investment into other traits. For example, inbred crickets, *Gryllodes sigillatus*, have been suggested to have the option of devoting more resources to cellular immunity than outbred individuals due to their reduced reproductive effort (Gershman et al. 2010). Furthermore, the intrinsic difference in the amount of energy spent on courting between inbred and outbred male bruchid beetles, *Callosobruchus maculates*, has been suggested as an explanation for the increased lifespan in response to inbreeding (Bilde et al. 2009). Hence, although inbreeding generally reduces fitness, its effects can be highly trait and/or environment specific.

1.2. Early-life nutrition and adult performance

Phenotypic development is the result of a complex interplay between the genetic architecture of an organism and the environment it experiences during development. Depending on the environmental conditions a given genotype can hence give rise to a variety of phenotypes (West-Eberhard 2003). At present there is a great interest in the extent to which environmentally induced phenotypic change is adaptive (Monaghan 2008). Predictive adaptive responses are defined as changes that take place during development in response to environmental cues, but where the advantage of the induced phenotype is not evident until later in life (Gluckman et al. 2005; Monaghan 2008). Whether such phenotypic changes are beneficial depends on how closely the conditions experienced during development predict those later in life (Monaghan 2008; Saastamoinen et al. 2010). However, although phenotypic plasticity can be adaptive, it need not be. For example, where low resource availability gives rise to a low-quality individual, development of the optimum phenotype is simply constrained by environmental effects (Monaghan 2008).

The immediate negative effects of adverse environmental conditions on individual fitness are well documented in the ecological literature. The impact of early-life nutrition in determining life-history variation in organisms is also widely recognized (Metcalfe & Monaghan 2001; Mitchell & Read 2005; Taborsky 2006; Andersen et al. 2010). In general, whereas diet restriction and mild starvation are often associated with increased longevity and stress tolerance (Bubli et al. 1998; Wenzel 2006; Burger et al. 2007; Smith et al. 2007) poor nutrition during early development is usually associated with negative

effects on many adult traits such as body size, survival, secondary sexual trait expression, stress and disease resistance (Lindström 1999; Metcalfe & Monaghan 2001; Lummaa & Clutton-Brock 2002; Gluckman & Hanson 2004; Waterland & Jirtle 2004; Mitchell & Read 2005; Taborsky 2006; McGraw et al. 2007; Andersen et al. 2010). Laboratory experiments with *D. melanogaster* that have manipulated the protein availability (yeast concentration) in the larval growth media have demonstrated the effect of poor early nutrition on several morphological and postcopulatory traits (Bubliy et al. 2000; Amitin & Pitnick 2007; McGraw et al. 2007), stress resistance (Andersen et al. 2010) and immune gene expression (Fellous & Lazzaro 2010). Even if an organism appears to recover from the nutritional deprivation when food conditions subsequently improve, nutritional deficits experienced during early development may still have permanent effects on the adult individual and even on its offspring (Metcalfe & Monaghan 2001; Ali et al. 2003; Vijendravarma et al. 2010). Moreover, although compensatory intake can bring quick benefits, the attempt to compensate for a bad start may itself be associated with a variety of costs, which are not well documented (Lindström 1999; Metcalfe & Monaghan, 2001; Ali et al., 2003). The complex effect of diet on individual performance is further demonstrated by the growing number of studies demonstrating interaction between parental and offspring nutrition in determining offspring performance (Prasad et al. 2003; Mitchell & Read 2005; Bonduriansky & Head 2007; Grech et al. 2007; Donelson et al. 2009; Frost et al. 2010; Vijendravarma et al. 2010).

1.2.1. Transgenerational effects of parental nutrition

In addition to direct environmental effects current and past environmental conditions experienced by other individuals, often the parent(s), may be important in shaping an organism's phenotype (Mousseau & Fox 1998). Parental effect is defined as any effect on offspring phenotype that is not determined by the offspring's DNA but instead is brought about by the genotype or environmental experience of its parents (Youngson & Whitelaw 2008; Bonduriansky & Day 2009). Parents that acquire high condition from a resource-rich environment may benefit by transferring their condition to their offspring, which due to their higher quality will do better under any environmental conditions than offspring of poor-quality parents (Mousseau & Fox 1998; Vijendravarma et al. 2010). On the other hand, parents may also respond to environmental cues in ways that enhance offspring performance under particular environmental circumstances. Under this scenario, offspring will do best in an environment similar to that experienced by their parents (Mousseau & Fox 1998; Badyaev & Uller 2009). Because mothers tend to invest more resources in production and/or care of offspring maternal effects are often considered more important than paternal effects (Ridley 1978; Tallamy 1984; Zeh & Smith 1985; Mousseau & Fox 1998; Magiafoglou & Hoffmann 2003). However, because only a few studies have actually tested for environmentally induced paternal effects in species where males make no obvious material contribution to offspring, the effect of the paternal environment or the potential for joint effects of both parental environments

on offspring performance remain poorly understood in such species (Bonduriansky & Head 2007).

Variation in parental nutrient provisioning is considered important in determining progeny phenotype (Bonduriansky & Day 2009). The effect of maternal nutrient provisioning on offspring condition and life-history has been documented for a number of species including many insects (Mousseau & Dingle 1991; Rossiter 1996; Mousseau & Fox 1998; Bounduriansky & Day 2009). Although paternal effects have been reported in species where males contribute to offspring care or provide females with nutrition or other substances that can be transferred to eggs/embryos by the female (Dussourd et al. 1988; Rossiter 1996; Smedley & Eisner 1996; Hunt & Simmons 2000; Gillott 2003; Guzman-Novoa et al. 2005; García-González & Simmons 2005; 2007; Ivy 2007; Bonduriansky & Day 2009) parental effects are often assumed to be mediated solely by the mother when males do not partake in progeny care in the conventional sense (Bonduriansky & Head 2007; Ivy 2007; Curley et al. 2011). One such species where males make no obvious material contribution to offspring is *D. melanogaster* (Markow & Ankney 1984). Even though it is used extensively for studies of nutrition-related life-history trade-offs relatively little is known about cross-generational dietary effects in this species (Prasad et al. 2003). *D. melanogaster* females raised on poor larval food have been found to lay heavier eggs than females raised on standard food, which could indicate enhanced egg provisioning by poorly fed mothers (Prasad et al. 2003; Vijendravarma et al. 2010). In those species that lack parental care, egg or newborn size can be used as an estimate of parental provisioning (Roff 2002b). Moreover, according to a study by Vijendravarma et al. (2010) *D. melanogaster* raised on poor food developed faster and were lighter if their mothers also developed on poor food. No effect of maternal diet on development time and body size was detected when the offspring were raised on standard food (Vijendravarma et al. 2010). The results of these and other studies indicate a role for maternal experiences in determining how offspring respond to current environmental conditions (Prasad et al. 2003; Mitchell & Read 2005; Bonduriansky & Head 2007; Grech et al. 2007; Donelson et al. 2009; Frost et al. 2010; Vijendravarma et al. 2010). Although paternal effects have been demonstrated in *D. melanogaster* (Giesel 1988; Huey et al. 1995; Watson & Hoffmann 1995; Crill et al. 1996) no studies have investigated male-mediated transgenerational effects of diet in this species. In mice and in the fly *Telostylinus angusticollis* dietary effects of both mothers and fathers have been shown to be transmissible to the next generation (Bonduriansky & Head 2007; Curley et al. 2011).

1.3. Cost of immunity

Susceptibility to pathogens and genetic variation in disease resistance is assumed to persist in nature because of the high costs associated with immunity (Sheldon & Verhulst 1996; Schmid-Hempel 2003). In terms of resource investment disease resistance is a

costly function. Costs of resistance come in three forms (Schulenburg et al. 2009). Costs involved in maintaining the immune system are related to investments made into the infrastructure of the system and keeping the system at a given level of readiness in the absence of infection; costs of deployment arise from using the immune system (Sheldon & Verhulst 1996; Siva-Jothy et al. 2005; Sadd & Schmid-Hempel 2009a; Schulenburg et al. 2009). Whereas the latter form of costs is only paid when the individual is infected, the former form of costs is paid irrespective of infection. The third form of costs is associated with immunopathology – i.e. tissue damage caused by the immune system (Sadd & Siva-Jothy 2006; Schulenburg et al. 2009). To come up with the costs associated with disease resistance organisms make trade-offs between immune function and other life-history traits (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Moret & Schmid-Hempel 2000; McKean et al. 2008; Ye et al. 2009; Bascuñán-García et al. 2010; van der Most et al. 2011). Consequently, assuming energy and resources are limiting factors, once used in disease resistance, the energy and resources are no longer available for other functions which may have fitness consequences to the individual.

Costs of immunological deployment are readily measured as a change in fitness following immunological challenge (Schmid-Hempel 2003; Siva-Jothy et al. 2005). To demonstrate the costs of immunological maintenance is somewhat more difficult (Lochmiller & Deerenberg 2000). Råberg et al. (2002) studied the costs of immunological maintenance by comparing the basal metabolic rates of normal and lymphocyte deficient knockout mice (mice without adaptive, but with innate immunity) and found deficient mice having higher metabolic rates than normal mice, indicating that an optimal combination of innate and adaptive immunity could save energy. Because invertebrates lack the adaptive defense system, the constraints set by maintenance costs are assumed to be different in invertebrates (Schmid-Hempel 2003). The approach mostly used for identifying costs of immunological maintenance in invertebrates involves artificial selection. *D. melanogaster* lines selected for increased resistance and compared, in the absence of infection, with the appropriate control lines in a range of fitness parameters have been widely employed in this context (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Ye et al. 2009; see also Hoang 2001; McKean et al. 2008).

Although it is generally recognized that immunity is costly, we still know relatively little about how these costs are distributed among different compartments of the immune system. The cellular immune responses have been suggested as being more effective in cleaning bacterial infections than the humoral responses (Haine et al. 2008), which suggests different costs for the two arms of the innate immunity. In studies that have investigated maintenance costs of immunological defense it is in most cases not possible to differentiate the costs of antibacterial defense from those of cellular defense (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Hoang 2001; McKean et al. 2008; Ye et al. 2009). Furthermore, resource availability can play an important role in determining the strength and direction of trade-offs between immunity and other life history components (McKean et al. 2008). When resources are not limiting organisms can compensate extra demands by increasing the intake of resources, and hence, costs of

immunity are often detected first when conditions deteriorate (Moret & Schmid-Hempel 2000; Hoang 2001; Schmid-Hempel 2003; McKean et al. 2008).

1.4. Immune function in *Drosophila*

In contrast to vertebrates that have both an acquired and an innate system of defense, invertebrates rely on innate immune reactions for defense against infection (Gillespie et al. 1997). A key feature of the adaptive immunity is immunological memory. In vertebrates the development of B and T cells into memory cells provides a mechanistic basis for immune memory. As no such cells exist in invertebrates, it has long been controversial whether something functionally akin to the vertebrate acquired immunity could exist in invertebrates (Sadd & Schmid-Hempel 2009b). Although increased protection against microparasitic infection functionally equivalent to the acquired response of vertebrates has now been demonstrated in some invertebrate species, extensive homology between vertebrates and invertebrates has only been found for the innate defense system (Little et al. 2005; Sadd & Schmid-Hempel 2007). Unlike in vertebrates, the mechanism underlying invertebrate immunological memory is not yet understood (see e.g. Kurtz & Armitage 2006).

The first and critical step in the initiation of an immune response is the recognition of the invading pathogen. Most of the systemic response of insects is activated by pattern-recognition receptors that recognize infectious agents (Broderick et al. 2009). Once pathogens are recognized a variety of defense reactions can be activated either directly, as in the case of phagocytosis and melanization, or indirectly through intracellular immune-signaling pathways that initiate the transcriptional activation of appropriate antimicrobial peptides (AMPs) (Das et al. 2009 and the references therein). Innate immunity of insects is divided into two major reaction types: humoral and cellular reactions. Whereas immunocytes perform the major cell-mediated immune functions (e.g. phagocytosis, melanization-encapsulation and nodulation) that act as a first line defense, humoral factors, characterized by the inducible expression of a large array of AMPs, are considered to function secondarily to eliminate those infectious agents that survive the constitutive immune response (Gupta 2001, 2002; Haine et al. 2008). In *Drosophila* the production of AMPs is regulated by two signaling pathways, Toll and Imd (Lemaitre et al. 1995). Both signaling cascades lead to nuclear localization of an NF-κB family transcription factor Dif/Dorsal or Relish, consequently leading to expression of AMP genes and to the production of AMPs (Leclerc & Reichhart 2004; Royet et al. 2005). The Imd pathway branches into two distinct sub pathways of which one leads to transcription of AMP genes via Relish while the other, JNK signaling, has a role in cellular immune responses and in the stress response (Park et al. 2004; Royet et al. 2005). In general, microbial pathogens such as fungi and bacteria are tackled by the humoral immune system. Whereas immune response to Gram-negative bacteria is primarily mediated via the Imd pathway, the Toll pathway reacts to fungi and Gram-positive bacteria (Leclerc

& Reichhart 2004; Royet et al. 2005). Despite its lack of antibody-mediated defense mechanisms akin to those found in vertebrates the innate immune system of insects is quite specific in its antimicrobial action (Das et al. 2009). The cellular immune system plays a role against microbial pathogens via phagocytosis, but is also used against macro-parasites such as parasitoids which are too large to be phagocytosed. Intruders are encapsulated by a two-stage process consisting of envelopment of the parasite by hemocytes, followed by the deposition of melanin (Gillespie et al. 1997; Gupta 2001).

In vertebrates offspring can inherit maternal immune function through antibodies (Grindstaff et al. 2003). Similar phenomena have recently been observed among invertebrates (Little et al. 2003; Sadd et al. 2005; Moret 2006; Sadd & Schmid-Hempel 2007). Whereas in vertebrates the mechanism underlying transgenerational immunity is clear, the mechanism behind the phenomena in invertebrates has yet to be uncovered. In transgenerational immunity, both the mother and her environment may influence the phenotype of the offspring. For example, female *Daphnia* that reproduced under poor nutritional conditions were found to produce offspring that were more resistant to a bacterial pathogen than offspring of mothers that reproduced in a high-food environment (Mitchell & Read 2005). The ways in which invertebrate offspring resistance relates to aspects of parental experience other than pathogen pre-exposure have not been systemically investigated (Miller et al. 2009). Moreover, with a notable exception (Roth et al. 2010), studies on trans-generational priming have thus far focused on a transfer via the mother. Using the red flour beetle, *Tribolium castaneum*, Roth et al. (2010) challenged the traditional view that males provide only genes to their offspring in species without parental care by demonstrating that trans-generational immune priming can occur also through fathers. If trans-generational immune priming takes place via both parents as observed in the study by Roth et al. (2010), information about pathogens in the environment of both parents could be transferred to the offspring and consequently, the protection offspring receives from its parents may even be more than additive and hence, result in offspring better adapted to the local conditions (Roth et al. 2010).

2. AIMS OF THE THESIS

This thesis investigates the condition dependent effects of inbreeding and the expression of condition-dependent traits. In studies **I** and **II** condition-dependent effects of immunological maintenance were investigated by creating lines of *Drosophila melanogaster* that differed in their antibacterial innate immune response. In study **I** costs of immunological maintenance on survival were investigated by following the survival of the flies under starved and fed conditions. In study **II** the effects of immunological maintenance on male attractiveness were investigated by comparing the mating success of wild type (wt) and immunodeficient mutant flies. The possible effect of early nutrition in shaping the response in study **II** was assessed by repeating the study with flies reared under both poor and standard nutritional conditions. In study **III** the effects of inbreeding on adult survival under starved and fed conditions were investigated. In study **IV** the effects of early nutrition on the magnitude of inbreeding depression in development time, adult body size and adult resistance to the bacterium *Serratia marcescens* were investigated. Finally, in study **V** the possible transgenerational effects of parental early nutrition on offspring development time, adult body size and adult susceptibility to the bacterium *S. marcescens* were examined. Both maternal and paternal dietary effects as well as their interaction on offspring raised themselves under standard nutritional conditions were tested.

If a trait is costly to produce and/or maintain I hypothesize the costs to be more pronounced under conditions in which resources are limiting (**I**, **II**). Moreover, I expect more erratic consequences of inbreeding when combined with the effects of nutritional stress (**III**, **IV**). Finally, I anticipate the effects of early nutrition to be transmitted to the next generation (**V**).

3. MATERIALS AND METHODS

More detailed descriptions of materials and methods can be found in the original articles.

3.1. Study species

All studies were conducted on the common fruit fly, *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae).

3.1.1. *D. melanogaster* stocks and husbandry

Flies used in studies **III**, **IV** and **V** were collected from a laboratory base population of *D. melanogaster* that originates from approximately 500 females collected by baits from an apple grove at Lappi in Southern Finland in September 2006. These wild caught females were also used to create the inbred and outbred lines of flies needed in study **III** (see section 3.1.2. Inbred and outbred flies). After their establishment in the laboratory the stock was expanded and maintained in large glass jars at room temperature (23 ± 1 °C) under continuous light with a standing adult population of several thousand individuals. *D. melanogaster* larvae were reared on: 10 g agar, 80 g cornmeal, 20 g brewer's yeast, 1.5 dl syrup, 10 ml nipagin, 1 L water diet and adult flies were fed with baker's yeast. Oregon R. flies (*D. melanogaster* laboratory strain) that were needed in study **II** were maintained in large glass jars at 22 °C in a 12L:12D light regime. Oregon R. larvae were reared on: 10 g agar, 60 g potato muss powder, 11 g baker's yeast, 60 g syrup, 8.5 ml nipagin, 1 L water diet and adults were fed with baker's yeast. In studies **II**, **IV** and **V**, in which larvae were reared under both standard and poor nutritional conditions the poor food environment refers to conditions in which the amount of baker's yeast was reduced to 1/10 (study **II**) and that of brewer's yeast (studies **IV** and **V**) to 1/8 of the standard amount. Ice and CO_2 were used in handling the flies.

3.1.2. Inbred and outbred flies

Inbred and outbred flies used in studies **III** and **IV** were generated by following the crossing design of Roff (1998, 2002a; see also Wright et al. 2008). First, females from the stock (study **IV**) / wild caught females (study **III**) were allowed to lay eggs in baker's yeast supplemented vials (one female in each vial). Upon eclosion to the adult stage the next generation flies were collected as virgin and male–female pairs were set up to construct full-sibling families (16 full-sibling families in study **IV**, 20 full-sibling families in study **III**). These families were then grouped into pairs (ten pairs in study **III**, eight pairs in study **IV**) and adults were crossed as shown in figure 1 − from each group, two inbred families were formed by full-sib mating, and two outbred families were formed by reciprocal matings of a male and a female from each family within the

group. The advantage of the breeding design is that, within each group, there is an equal representation of alleles, only their combinations changing (Roff 1998). In study **IV** only the first generation progeny of these matings were used. In study **III** the maintenance of the lines was continued for approximately 30 generations before the experiments commenced. Full-sibling mating was used to continue the inbred lines, the outbred lines were continued by mating a female from an outbred line with a randomly chosen male from the base population. The crossed lines used in study **III** were constructed by crossing separate, randomly chosen inbred lines (only the first generation progeny of the crossed lines was used).

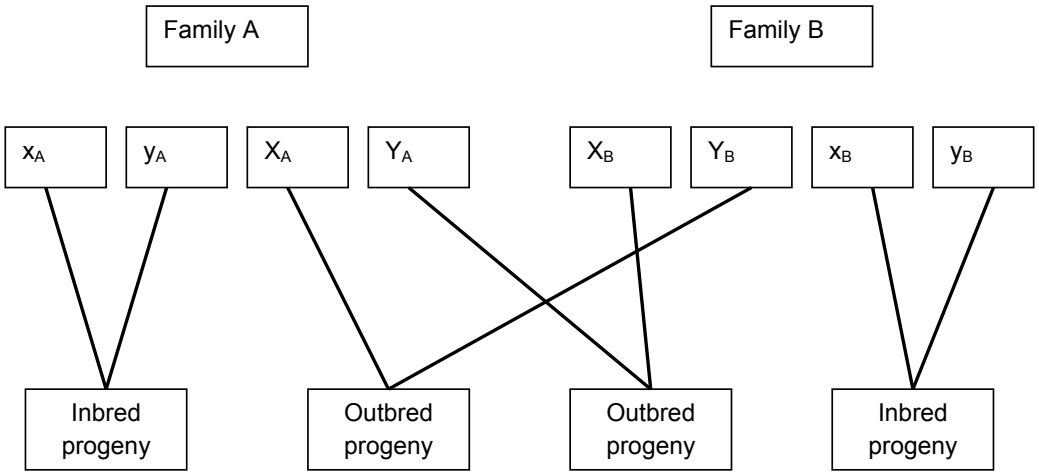

Figure 1. Schematic illustration of the breeding design for a single 'group': X, x, female; Y, y, male; upper case indicates crosses between the two families, A and B, to produce outbred progeny, and lower case indicates brother–sister matings, producing inbred progeny (studies **III** and **IV**).

3.1.3. Immunodeficient *Relish* mutant flies

Flies (*D. melanogaster*) used in studies **I** and **II** were created by crossing flies deficient in the functional Relish protein (*Relish* E20 mutant flies) with wt Oregon R. flies. The outcrossing was done because mutant flies (*Relish* E20) may differ not only in the ability to produce AMPs in response to microbial pathogens but also in other life history traits compared to normal wt flies (Oregon R.) and, hence, normal flies from these outcrosses provide a better control for the experiments. To produce flies that are homozygous for either the Oregon R. wt allele or the *Relish* E20 deletion mutation (i.e. differ in their antibacterial innate immunity due to differences in the expression of the *Relish* gene) twelve Oregon R. × *Relish* E20 breeding pairs were set up. Each pair constituted a line that was maintained as an inbred line by full sib mating. In the first generation the amount of lines was tripled by setting up three full-sib pairs per line, subsequently the amount of lines was doubled in the second and in the third generations in the same

manner (see figure 2). After fifteen generations of full-sib mating, four sisters and three of their brothers were allowed to continue a line to reduce the probability of accidental loss of a line.

According to Hedengren et al. (1999) immunocompromised *Relish* mutants die within 17 hours when infected with approximately 2×10^5 of *Enterobacter cloacae* –bacteria, whereas wt flies generally survive this treatment. To measure the strength of immunity towards the bacterium *E. cloacae* flies were anaesthetized with CO_2, placed on ice, and the thoraces of individual flies pierced with a 0.1-mm pin dipped in a suspension of an overnight culture of the bacteria on LB-agar plates. Flies that were alive 24 hours after the infection were regarded as representing lines with normal wt immunity; the ones dead as representing lines with impaired immunity.

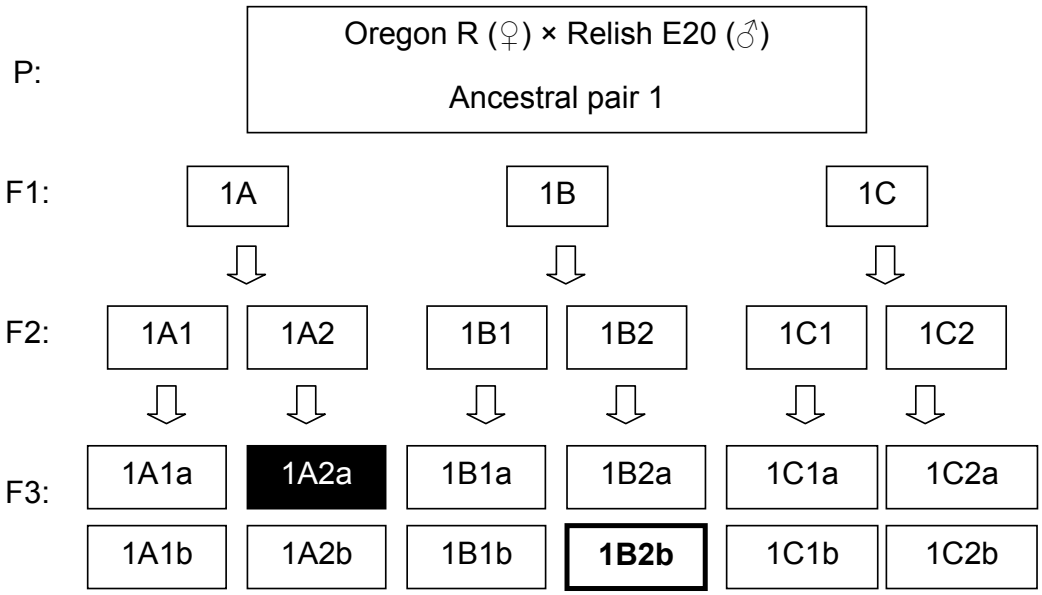

Figure 2. Schematic illustration of the crossing scheme used to create the experimental lines used in studies **I** and **II** (one of the twelve ancestral pairs is given as an example). Wild type line with normal immunity (white colored text) was chosen a closely related pair with impaired immunity (bold text). Both lines descended from the same ancestral breeding pair (ancestral pair 1).

3.2. Experimental procedures

3.2.1. Lifespan and starvation resistance

In study **I** costs associated with maintaining a normally functioning immune system were investigated by following the survival of wt and *Relish* mutant flies under starved and fed conditions. In study **III** the effect of inbreeding on adult survival was investigated

by comparing the abilities of inbred and outbred flies to survive under starved and fed conditions. In the survival assay each fly was provided with a 30 ml vial that contained either no food (starved vials) or *ad libitum* access to yeast (food vials). In the starved vials a 1 cm thick moist cotton ball was placed in the vial to ensure access to water, the non-starved vials contained 10-15 ml standard food with baker's yeast on top. Vials were capped with cotton plugs so that the flies had space to move freely. The survival of the flies in starved vials was scored every two hours. The survival of the flies that were fed was checked once a day and every two weeks these flies were tipped into fresh food vials. Consequently, the survival was determined as the time from the placement of a fly in the assay vial to its death.

3.2.2. Development time and adult body size

The effect of early nutrition on the magnitude of inbreeding depression in development time and the effect of parental early nutrition on offspring development time were investigated in studies **IV** and **V**, respectively. Development time was determined as the length of time between oviposition and adult eclosion. To measure development time parents were allowed to interact with each other and lay eggs for 24 hours in 30 ml vials. The following day eggs were harvested and transferred into fresh vials at a density of 20 eggs per vial. The vials were placed at 22 °C in a 12L: 12D light regime and checked for emerged adults 2-3 times a day until eclosion ceased.

In studies **I**, **II**, **IV** and **V** thorax length, an estimate of adult body size, was measured under a light microscope using an ocular micrometer.

3.2.3. Mate choice assay

To assess the effect of *Relish* genotype on male mating success (study **II)** a *Relish* mutant and its wt relative were allowed to compete for a wt Oregon R. female in a 30 ml vial for two hours. The vial was capped with a cotton plug so that the flies were able to move freely. The time taken for one of the males to start copulating with the female was recorded and the winner was identified. Males that did not mate within the time period of two hours were considered as having both lost the trial. To identify the males, they were marked with black dots on either the right or the left wing. The marking was interchanged between *Relish* mutant and wt flies in an effort to mark an equal number of wt and mutant flies on a particular wing. The marking was accomplished approximately 24 hours before the competition start. The trials were conducted at room temperature (23 ± 1 °C). A similar assay has previously been used by e.g. Rolff and Kraaijeveld (2003).

3.2.4. Pathogen resistance

The effect of early nutrition on the magnitude of inbreeding depression in adult pathogen resistance (study **IV**) and the transgenerational effect of parental early nutrition on

offspring disease resistance (study **V**) were assessed using a host resistance test, in which the likelihood of survival against *Serratia marcescens* infection was measured. *S. marcescens* (a Gram-negative entomopathogenic bacterium) is found worldwide, and it is known to be pathogenic to over 70 species of insects, including *D. melanogaster* (Flyg et al. 1980). The outline of the bacterial infection follows the assay used by Lazzaro et al. (2004, 2006).

The immunity assay was performed on adult, virgin flies aged between 4-7 days (post eclosion). To measure the strength of immunity towards the bacterium flies were anesthetized with CO_2, placed on ice, and the thoraces of individual flies pierced with a 0.1 mm pin dipped in a suspension of an overnight culture of the bacteria in liquid broth (OD_{590} = 0.039, LB = 10 g tryptone, 5 g yeast extract and 10 g NaCl, 1L water). In study **IV** control flies were pricked with a pin dipped in liquid broth. Because studies **I**, **II** and **IV** had shown that flies only pricked with a pin (**I**, **II**) or with a pin dipped in liquid broth survive the assay period, in study **V** the control flies were only transferred into fresh food vials, i.e. they were not sham infected. After infection/sham infection flies were placed on fresh food and housed either individually (**IV**) or in same sex groups of 2-5 individuals (**V**) at room temperature (23 ± 1 °C). In study **IV**, in which both survival and survival time were measured the survival of the flies was scored every three hours; in study **V**, in which only survival was measured the survival was scored twice daily. Individuals that survived five days were considered to have survived the infection.

3.2.5. Inbreeding depression and heritability

In study **IV**, in which the effect of early nutritional environment on the magnitude of inbreeding depression in development time, adult body size and pathogen resistance was analyzed, the amount of inbreeding depression (δ) was estimated as $\delta = 100\ (1 - X_1/X_2)$. In the equation X_1 is the trait value diminished by inbreeding and X_2 is the outbred trait value. If the analyzed traits show inbreeding depression one would expect development time to increase and adult body size and pathogen resistance to decrease. Whereas for the last two traits δ could be calculated in the usual manner for development time the calculation needed to be reversed − i.e. X_1 was set as the outbred trait value, X_2 as the inbred trait value. Furthermore, in study **IV** the response of heritability to early nutrition (poor vs. standard) was assessed for development time and adult body size. Trait heritability (h^2) was defined as the proportion of total phenotypic variation (V_P) due to additive genetic variation (V_A): $h^2 = V_A/V_P$.

4. RESULTS AND DISCUSSION

4.1. Condition-dependent effects of immunological maintenance

Costs associated with the maintenance of the humoral arm of the innate immune system were investigated in studies **I** and **II**. In study **I** in which the effect of immunological maintenance on survival was studied by following the survival of wt and *Relish* mutant flies under both starved and fed conditions immunodeficient mutant flies were found to survive longer under starvation conditions than wt flies; when food was provided *ad libitum* the opposite was found (figure 3). In study **II** the effect of immunological maintenance on male attractiveness was investigated by comparing the mating success of wt and mutant flies. The possible effect of the larval nutritional environment in determining the strength of the potential trade-off between male mating success and immune function was assessed by repeating the study with flies reared under both poor and standard nutrition. When raised on poor food immunocompromised *Relish* mutants were somewhat more successful in the competition for mates than wt flies. When the flies were raised on standard food no difference in mating success between the two genotypes was observed. Support for the trade-off between immunological maintenance and traits associated with male mating success has previously been provided by McKean and Nunney (2008) who selected *D. melanogaster* for greater sexual competitiveness and showed the selected lines having reduced immune function. On the other hand, studies that have manipulated resistance instead of sexually selected traits appear to question the existence of this trade-off (Rolff & Kraaijeveld 2003; Ye et al. 2009). The contradicting results obtained from different studies appear to support the notion that different forms of defense bear different costs and are linked with different fitness-related traits.

The fact that costs of immunological maintenance were detected only among individuals subjected to adverse nutritional conditions demonstrates that the effects of immunological maintenance are condition-dependent. To come up with the costs of maintaining and/or using the immune system organisms make trade-offs between immune function and other fitness related traits (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Moret & Schmid-Hempel 2000; McKean et al. 2008; Ye et al. 2009; Bascuñán-García et al. 2010; van der Most et al. 2011). Because under conditions in which resources are not limiting organisms can increase the intake of resources to compensate for any extra demands, costs of immunity are often detected first when conditions deteriorate (Moret & Schmid-Hempel 2000; Hoang 2001; Schmid-Hempel 2003; McKean et al. 2008). The results of studies **I** and **II** demonstrate the importance of environmental variation in the study of evolutionary trade-offs and stress the importance of considering the possible effects of the early-life environment on adult life-history trade-offs. Moreover, studies **I** and **II** are the first attempts to demonstrate costs associated with the maintenance of a particular compartment of the innate immune system, the antibacterial defense system.

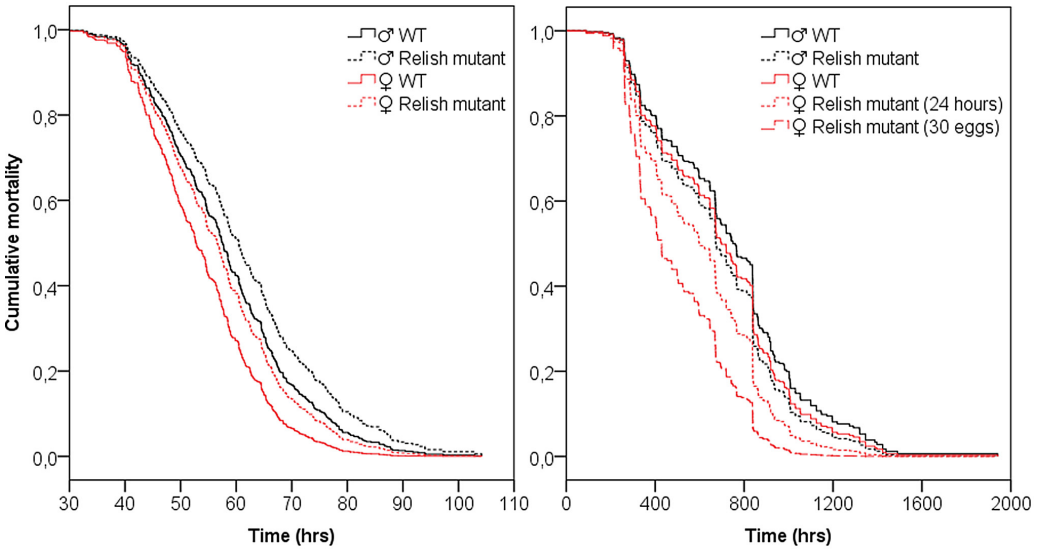

Figure 3. Cumulative mortality of immunodeficient *Relish* mutant and wt flies under starved (left) and non-starved environment (right). *Relish* mutant flies are more short-lived when food is provided *ad libitum*, but more long-lived under starvation conditions compared to wt flies (for more details see study **I**).

4.2. Trait specific effects of inbreeding

In study **III** the effects of inbreeding on adult survival on life-span and starvation resistance were investigated. According to the results inbreeding reduced the mean time of survival under fed conditions but had no effect on survival under starved conditions (figure 4). Because of their decreased overall fitness and genetic variability inbred individuals are expected to be more sensitive to changing environmental conditions than their outbred conspecifics (Bijlsma et al. 1999; 2000; Dahgaard & Hoffmann 2000; Kristensen et al. 2003, 2010; Reed et al. 2003; Armbruster & Reed 2005; Kristensen & Sørensen 2005; Swindell & Bouzat 2006c; Liao & Reed 2009). This has also been suggested to decrease survival and lifespan under most circumstances (Vermeulen & Bijlsma 2004). Most studies report more pronounced inbreeding depression under novel and stressful conditions (Armbruster & Reed 2005; Kristensen & Sørensen 2005; Liao & Reed 2009; Kristensen et al. 2010). Armbruster and Reed (2005) reviewed the literature on the relationship between the magnitude of inbreeding depression and environmental stress and found inbreeding depression in 76 % of the reviewed cases greater under stressful conditions (in 48 % of the cases the increase was found significant). However, the authors emphasized the importance of noting the large number of instances in which inbreeding depression was not found to increase. Moreover, in a recently published study Mikkelsen et al. (2010) report strong trait specific consequences of inbreeding and generally low levels of inbreeding depression on resistance to such stress factors

as heat, cold and desiccation in *D. melanogaster*. Whereas evidence for the deleterious effects of inbreeding on lifespan has been previously provided, the effects of inbreeding on starvation resistance remain largely unexplored (Sverdlov & Wool 1975; Hoffmann et al. 2001).

The results of study **III** indicate highly trait specific consequences of inbreeding. The effect of inbreeding on a given trait depends upon the proportion of directional dominance in that trait (Roff 1997; Roff & Emerson 2006). In general, traits under weak selection are expected to show less directional dominance than traits that are under stronger selection (Lynch & Walsh 1998). The results of study **III** demonstrate that whereas directional dominance is observed for lifespan no directional dominance is observed for starvation resistance (figure 4). Consequently, because the flies in our experimental set up were normally maintained under ample food conditions in the laboratory little selection may have operated at loci controlling starvation resistance, which could explain the absence of inbreeding depression in that trait. Inbreeding effects on starvation resistance within wild populations may turn out to be rather different from those documented among laboratory adapted populations because in the wild populations are more likely to face periods of food scarcity.

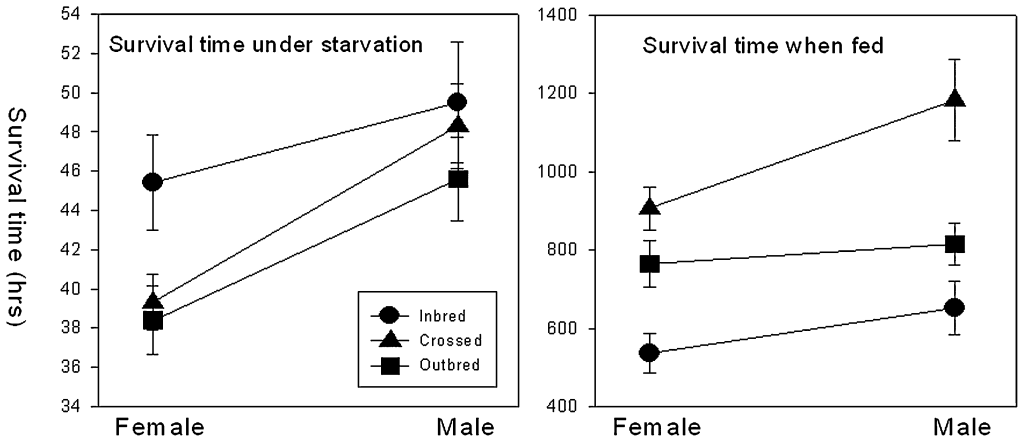

Figure 4. Mean survival times (±1 SE) under fed and starvation conditions (study **III**).

4.3. The magnitude of inbreeding depression is not widely affected by early nutrition

In study **IV** the effects of early nutrition (poor vs. standard) on the magnitude of inbreeding depression in development time, adult body size and adult resistance to the bacterium *S. marcescens* were investigated. According to the results early nutritional environment had no effect on the magnitude of inbreeding depression in development time or adult body size but may have played a small role in adult resistance to the bacterial infection (figure

5). Whereas the bacterial disease was the most important factor explaining survival, sex and food quality were the most important factors explaining development time and adult body size. In general, food restriction improved 5-day survival, which agrees with the classical finding of increased longevity under calorie restriction (Bubli et al. 1998; Wenzel 2006; Burger et al. 2007; Smith et al. 2007). Moreover, the observed better 5-day survival of the outbred individuals compared with that of the inbred individuals agrees with the classical finding of increased effects of inbreeding under stressful conditions (figure 4) (Armbruster & Reed 2005; Kristensen & Sørensen 2005; Liao & Reed 2009; Kristensen et al. 2010; but see Mikkelsen et al. 2010; study **III**). There are no previous studies investigating the combined effect of early nutrition and inbreeding on individual performance.

The observed low values of inbreeding depression in both development time and adult body size suggest little directional dominance for these traits. Large variation among the inbreeding depression values for 5-day survival and survival time indicate that some of the inbred lines were as good at withstanding infection as were the outbred lines whereas other inbred lines did worse or even better than some of the outbred lines. Among vertebrates there is an abundance of evidence that inbreeding compromises the resistance of species to parasites and pathogens (Keller & Waller 2002). Contrary to studies on vertebrates, studies on invertebrates are not consistent with the observation that inbreeding compromises resistance to infectious agents (Stevens et al. 1997; Gerloff et al. 2003; Calleri et al. 2006; Rantala & Roff 2006; Gershman et al. 2010). Individuals used in the foundation of the lines obviously carried different alleles (beneficial or deleterious) that contributed to the observed variation in the inbreeding depression values for survival and survival time. The results demonstrate that some populations can retain high pathogen resistance to a particular pathogen following population bottlenecks whereas others cannot.

The estimates for heritabilities of development time in the poor food environment were significantly larger than those measured in the standard food environment, whereas no difference in the variation in the heritability of adult body size under the two food treatments was detected. The measured heritability difference in development time was primarily because of a decrease in the additive genetic variance under ‘‘unfavourable’’ conditions. The basis for increased genetic variance often observed during stress is a debated topic (Sørensen et al. 2003). Several hypotheses have been invoked to explain heritability differences between environments. The predictions that arise from these hypotheses are variable (Hoffmann & Merilä 1999). Because study **IV** was not designed to identify the mechanisms behind the observed differences in heritabilities between the two treatments, the results only add to the growing body of literature that heritabilities are not constant but vary with environmental conditions.

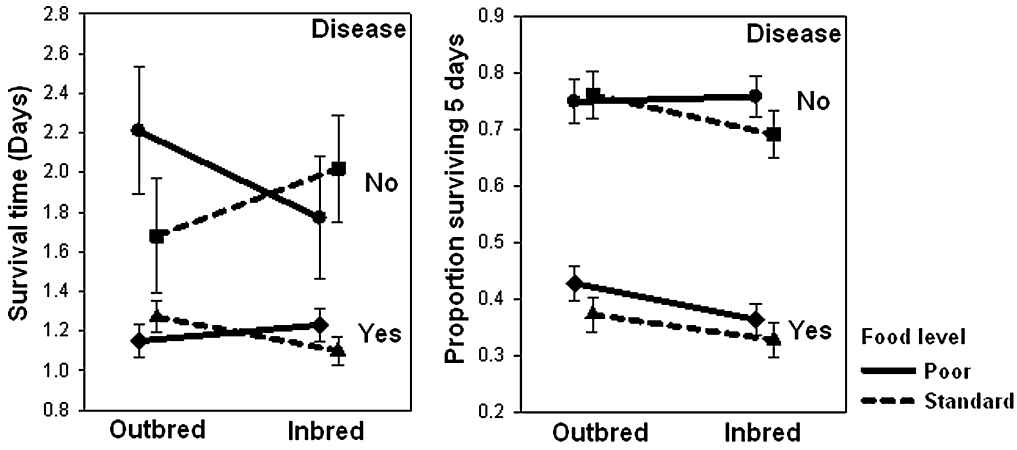

Figure 5. Mean proportional survival and mean survival time of those flies that did not survive the five day assay period as a function of breeding type, food level, and disease treatment (study **IV**).

4.4. Maternal and paternal environments interact in their effect on offspring performance

In study **V**, in which the effect of parental early nutrition on offspring development time, adult body size and adult resistance to the bacterium *S. marcescens* was studied, flies for the parental generation were raised on either poor or standard diet and then mated in the four possible sex-by-parental diet crosses. Female flies that were raised on poor food as larvae produced larger offspring than females that were raised on standard food. Furthermore, male progeny sired by fathers that were raised on poor food were larger than male progeny sired by males raised on standard food. No effect of paternal diet on adult body size of the female offspring was detected. Egg-to-adult development times were shortest for offspring whose one parent was raised on standard and the other parent on poor food (P-S, S-P) and longest for offspring whose both parents were raised on poor food (P-P). Offspring whose parents were raised on standard food (S-S) had intermediate development times (figure 6). No evidence for transgenerational effects of parental larval diet on offspring disease resistance was found.

Since in *D. melanogaster* body size increases with development time (Roff 2002b) it is possible that the larger size of offspring whose parents were raised on poor food reflects a trade-off with the slower development of these offspring. Hence, by directly affecting one of the two traits, development time or adult size, parental nutrition could have caused indirect changes in the other trait. Parental dietary effects would hence seem to involve both adaptive as well as maladaptive effects on offspring performance. The results of the present study could suggest that under appropriate nutritional conditions

an individual's life-history strategy may, to some extent, be determined by the nutritional history of its parents. Consequently, when raised under standard nutritional conditions offspring whose parents were raised on standard food would develop faster but be smaller as adults than offspring whose parents were raised on poor food; offspring whose parents have a history of malnourishment would have the opposite strategy. Which of the two life-history strategies is most beneficial under the given circumstances cannot be identified by our experimental setup. By comparing development times of offspring whose parents both had experienced poor nutritional conditions as larvae (P-P) with those whose parents were raised on standard food (S-S) it would appear that parents transferred their condition to their offspring. However, because the shortest development times were found among offspring whose one parent was raised on standard and the other parent on poor food (P-S, S-P) the mechanistic basis appear more complicated than that.

In species, such as *D. melanogaster*, where males do not partake in progeny care in the conventional sense parental effects are often assumed to be mediated solely by the mother (Markow & Ankney 1984; Bonduriansky & Head 2007; Ivy 2007; Curley et al. 2011). While study **V** demonstrates the importance of not only considering the relative contributions each parental sex has on progeny performance but also the potential interactions that may exist among the sexes it does not address the underlying modes of action. In general, whereas maternal effects comprise a number of phenomena (Mosseau & Fox 1998; Wolf & Wade 2009) the possible factors contributing to paternal effects are less clear. Because incorporation of nutrients from the male ejaculate does not occur in *D. melanogaster* (Markow & Ankney 1984), differential female investment in reproduction based on the perceived quality of the mate or alternatively, variation in the ability of males to manipulate female reproductive investment could play a role in paternal transmission of, in this case, dietary effects (for similar reasoning see Pischedda et al. 2011). On the other hand, if variation is directly caused by males it could occur via variation in male seminal proteins (Pischedda et al. 2011; Chapman 2001; Findlay et al. 2008). Epigenetic modifications of sperm DNA could also have a role in mediating transgenerational parental effects (Curley et al. 2011). Whatever the mechanism will turn out to be, the emerging evidence supporting the occurrence of paternal effects in species with no paternal care indicate the possibility that also paternal experience may be translated into variation in offspring fitness.

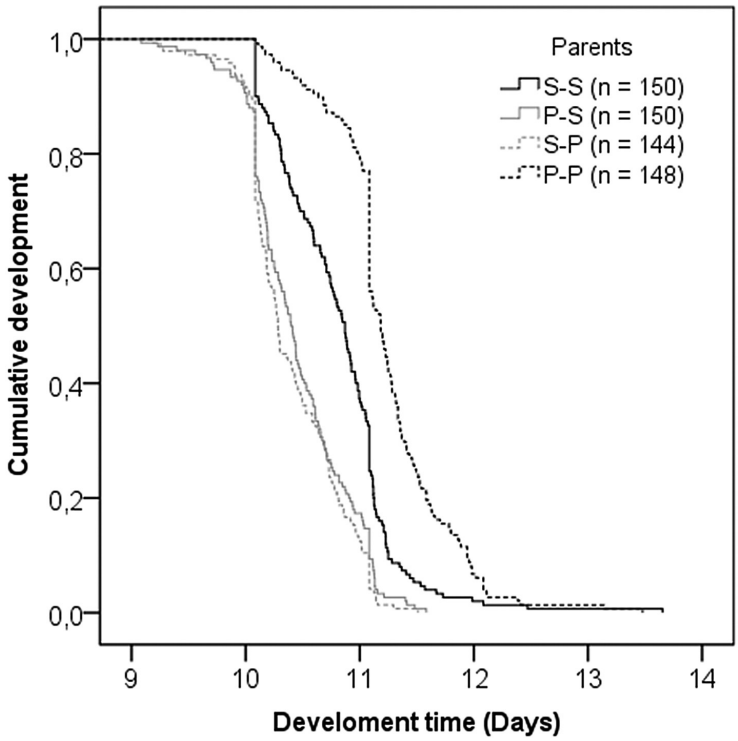

Figure 6. Cumulative development times of offspring (data pooled across sexes). The progeny of P-P parents had the longest development times, those of S-S intermediate development times and those of S-P and P-S parents had the shortest development times. All comparisons were statistically significant except for that between the progeny of S-P and P-S parents. Curves were calculated using the Kaplan-Mayer survival analysis (study **V**).

5. CONCLUDING REMARKS

Although the ability to conserve energy is important to any organism at risk of experiencing food scarcity, the ability to save energy becomes fundamental during actual episodes of food shortage. Under caloric restriction limiting energy usage and shifting metabolism toward oxidation of stored nutrients take place (Kersten et al. 2010). Under conditions of resource limitation organisms need to distribute limited resources between various competing traits based on their relative importance. Under such adverse environmental conditions only those individuals in good conditions can afford to spend resources on those traits ranked as less important. To come up with the costs of maintaining and/or using the immune system organisms make trade-offs between immune function and other fitness related traits (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Moret & Schmid-Hempel 2000; McKean et al. 2008; Ye et al. 2009; Bascuñán-García et al. 2010; van der Most et al. 2011; studies **I** and **II**). As the results of this thesis and those of previous studies demonstrate such resource-based trade-offs may be detected first when conditions deteriorate (Moret & Schmid-Hempel 2000; Hoang 2001; Schmid-Hempel 2003; McKean et al. 2008; studies **I** and **II**). Moreover, the results of this thesis stress the importance of considering the possible effects of the early-life environment on adult life-history trade-offs (study **II)**. Although it is generally recognized that immunity is costly, not much is known about how these costs are distributed among different compartments of the immune system. Relish is a key factor in the induction of an entire set of antibacterial as well as antifungal peptides with no known effects on cellular immune reactions (Hedengren et al. 1999). Using genetically modified *D. melanogaster* Libert et al. (2006) demonstrated, by overexpressing the putative pathogen receptor molecule PGRP-LE, that chronic activation of innate immunity pathways reduces lifespan in this species. The reduced longevity was shown to be due to continued activation of the NF-κB factor Relish suggesting the presence of a physiological cost for enhanced antimicrobial immunity and a trade-off between resistance and longevity. In studies investigating maintenance costs of immunological defense it is in most cases not possible to differentiate the costs of antibacterial defense from those of cellular defense (Kraaijeveld & Godfray 1997; Fellowes et al. 1998; Ye et al. 2009; Hoang 2001; McKean et al. 2008). Studies **I** and **II** are the first attempts to estimate costs associated with the maintenance of the antimicrobial defense system.

In the wild many species have to cope with periodical malnutrition or starvation and even those animals with seemingly abundant food supplies may be limited by the availability of specific nutrients (Raubenheimer & Simpson 1999; Harbison et al. 2004; Rion & Kawecki 2007; Andersen et al. 2010). Nevertheless the effects of inbreeding on individual performance under dietary restricted conditions remain largely unexplored. Whereas evidence for the deleterious effects of inbreeding on lifespan has been previously provided, only a few studies have investigated effects of inbreeding on survival under starved conditions (Sverdlov & Wool 1975; Hoffmann et al. 2001). A number of studies

report more pronounced inbreeding depression under novel and stressful conditions (Armbruster & Reed 2005; Kristensen & Sørensen 2005; Liao & Reed 2009; Kristensen et al. 2010). According to the results of this thesis (study **III)** inbreeding reduced survival under fed but not under starved conditions. Although the mechanisms behind the observed results cannot be identified by the experimental design used in study **III**, the results demonstrate highly trait specific consequences of inbreeding. Because the flies in our experimental set up were normally maintained under ample food conditions in the laboratory little selection may have operated at loci controlling starvation resistance. Inbreeding effects on starvation resistance within wild populations may hence turn out to be rather different from those documented among laboratory adapted populations because in the wild the trait is more likely to be under selection as wild populations are more likely to face periods of food scarcity.

Whereas the immediate negative effects of adverse nutritional conditions are generally well documented in the ecological literature, the understanding of the importance of early-nutrition on individual performance has emerged more recently (Lindström 1999; Metcalfe & Monaghan 2001; Lummaa & Clutton-Brock 2002; Gluckman & Hanson 2004; Waterland & Jirtle 2004; Mitchell & Read 2005; Taborsky 2006; McGraw et al. 2007; Andersen et al. 2010). To our knowledge, there are no previous studies investigating the combined effect of poor early nutrition and inbreeding on organism performance. Although the interaction between inbreeding depression and early nutritional environment may have had a small role in adult survival and resistance to *S. marcescens* infection, in general the findings of this thesis provide little evidence that the magnitude of inbreeding depression is influenced by early nutrition (study **IV**). We studied the relationship between inbreeding and one component of invertebrate immunity. Since different components do not necessarily show correlated responses (Adamo 2004), it would be of interest to investigate the combined effects of inbreeding and poor early nutrition on other aspects of immunity.

Furthermore, environmental conditions experienced by parents are increasingly recognized to affect offspring performance. Past environmental conditions, especially those experienced by the mother, are considered important in shaping offspring phenotype, and recently, they have been shown to play an important role in determining the way offspring respond to current environmental conditions (Prasad et al. 2003; Mitchell & Read 2005; Bonduriansky & Head 2007; Grech et al. 2007; Donelson et al. 2009; Frost et al. 2010; Vijendravarma et al. 2010). Variation in parental nutrient provisioning is considered particularly important in shaping offspring phenotype (Bonduriansky & Day 2009). The extent to which maternal environment influences offspring phenotype and fitness is considered to determine whether such effects themselves will be acted on by natural selection (Mousseau & Fox 1998). The existence of paternal effects indicates that paternal experience may also be translated into variation in offspring fitness. In addition to their practical significance such effects would have important theoretical implications in the field of quantitative genetics for their potential to inflate estimates of additive genetic variance (Friberg et al. 2011). The emerging evidence supporting the

occurrence of paternal effects in species with no paternal care suggests that sire effects are more common than hitherto thought. By comparing development times of offspring whose parents both had experienced poor nutritional conditions as larvae with those whose parents were raised on standard food it would appear that parents transferred their condition to their offspring. However, because the shortest development times were found among offspring whose one parent was raised on standard and the other parent on poor food the mechanistic basis appear more complicated than that (study **V**). Whether parental effects are independent of the mate, or whether parental effects change depending on the combination of the parental genotypes need further investigation.

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