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THE EFFECTS OF COPPER EXPOSURE ON LIFE-HISTORY TRAITS IN INSECTS

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The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6659-2 (PRINT)

ISBN 978-951-29-6660-8 (PDF)

ISSN 0082-6979 (PRINT)

ISSN 2343-3183 (ONLINE)

Painosalama Oy - Turku, Finland 2016

**”When the last tree is cut down, the last fish eaten, and the last stream poisoned,
you will realize that you cannot eat money...”**

– Native American saying

(Oxford Proverbs, 1983 H. Wasserman America Born and Reborn 277)

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ABSTRACT

Human-caused environmental pollution is a fast-growing and devastating threat that organisms have to face and tolerate. The primary aim of this thesis was to experimentally study the direct and trans-generational effects, costs and possible trade-offs of developmental copper exposure on traits expressed in later life. I have used two insect species, a blow fly, *Protophormia terraenovae*, and the common fruit fly, *Drosophila melanogaster*, as model species.

First, I studied the potential interaction effect between two different environmental conditions on condition-dependent traits such as immune function. I found that the effects of copper on immunity and other life-history traits are temperature-dependent. In addition, females and males responded differently to environmental stressors, suggesting that the costs of copper exposure are compensated from distinct sources.

Environmental conditions experienced by the parental generation had effects on their progeny. I found that direct exposure to copper activated the immune system of the parental generation, but more importantly, this activation was trans-generationally transmitted to their offspring, even if the copper exposure was no longer apparent. Maintenance of unnecessarily high immune functions consumes valuable energy and these costs can be detected under conditions where individuals are not able to compensate the costs by increasing their resource intake. I explored the potential costs and trade-offs caused by the elevated immune functions under starvation conditions. Individuals exposed to copper survived longer under starvation, suggesting that the costs are compensated between other traits than starvation resistance.

Direct exposure to copper impaired female fecundity in the parental generation, and this trait was trans-generationally transmitted to their offspring. More specifically, individuals whose parents were exposed to copper expressed reduced fecundity under uncontaminated conditions. Conversely, if the offspring condition corresponded to the parental condition, no difference in fecundity was discovered. Results show that exposure to copper has trans-generational effects on fecundity but short-term exposure might generate acclimatization to the prevailing conditions. Lastly, I explored the potential mechanism for compensating the costs of copper exposure by measuring the metabolic rate. Males exposed to copper had a lower metabolic rate, whereas no difference among females was detected.

To conclude, the effects of environmental pollution on different traits can be trans-generationally transmitted to the following generations, indicating that environmental pollutants can have far reaching effects on animal populations, including humans. Indeed, the effects of pollutants on animal populations may remain, even if the pollutant itself is no longer apparent. Secondly, short-term exposure may generate acclimatization to prevailing conditions, which might in turn incorporate expenses, and animals might suffer reduced fecundity under uncontaminated conditions. Further, I discovered a difference in resource allocation in response to environmental stressors between sexes. To summarize, pollutants may have more complex and far reaching effects on animal populations than previously considered. I hope that with this thesis I will draw attention to the fast increasing concerns of environmental pollution. Even though the deleterious effects of many pollutants have been recognized, growing numbers of new chemicals are being emitted, with their effects on animal populations, and on us, being yet mainly unknown.

TIIVISTELMÄ

Ihmisen aiheuttama ympäristön saastuminen on vakava uhka eliöille. Väitöskirjassani olen selvittänyt kokeellisesti varhaisten kehitysvaiheiden kokeman kuparialtistuksen aiheuttamia suoria vaikutuksia yksilöihin sekä mahdollisia seuraaviin sukupolviin ulottuvia vaikutuksia. Tutkin myös kuparin aiheuttamia kustannuksia sekä mahdollisia allokaatiokustannuksia myöhemmin elämässä esiintyvissä ominaisuuksissa. Olen tutkimuksissani käyttänyt mallina kahta hyönteislajia: raatokärpäsiin kuuluva *Protophormia terraenovae* -lajia ja banaanikärpästä (*Drosophila melanogaster*).

Tutkiessani kahden ympäristöolosuhteen – lämpötilan ja raskasmetallialtistuksen – välisiä interaktioita havaitsin, että kuparin vaikutukset kunnosta riippuviin ominaisuuksiin, kuten immuniteettiin olivat lämpötilariippuvaisia. Lisäksi koiraat ja naaraat reagoivat eri tavalla ympäristön aiheuttamaan stressiin. Tämä on todennäköisesti seurausta siitä, että koiraat ja naaraat kompensoivat stressitekijöiden aiheuttamia kustannuksia eri tavalla.

Vanhempien kokemat ympäristöolot saattavat vaikuttaa niiden saamien jälkeläisten ominaisuuksiin. Tutkimuksessani kuparialtistus aktivoi immuunipuolustuksen, ja tämä vaikutus siirtyi seuraavaan sukupolveen, vaikka jälkeläiset olivat kasvaneet saasteettomissa olosuhteissa. Tarpeettomasti aktivoituneen immuniteetin ylläpito aiheuttaa energiataloudellisia kustannuksia, mikä on mahdollista havaita olosuhteissa, joissa yksilöt eivät pysty kasvattamaan kompensatorista resurssien sisäänottoa. Kuparille altistetut yksilöt selviytyivät paremmin ravinnottomassa ympäristössä, joten immuuniaktivaatiosta koituneet allokaatiokustannukset kompensoidaan todennäköisesti muuta reittiä pitkin.

Kuparialtistus heikensi naaraiden fekunditeettiä eli jälkeläistuottoa, ja lisäksi vanhempien kokema kuparialtistus vaikutti niiden tuottamien jälkeläisten fekunditeettiin. Yksilöt joiden vanhemmat oli altistettu kuparille, ilmensivät heikentynyttä fekunditeettiä saasteettomissa olosuhteissa. Mikäli jälkeläiset pidettiin samoissa olosuhteissa kuin niiden vanhemmat, ei muutoksia fekunditeetissä esiintynyt. Muutokset fekunditeetissä ovat todennäköisesti seurausta kuparialtistuksen aiheuttamista suorista kustannuksista sekä immuuniaktivaatiosta koituvasta kuormituksesta. Lopuksi selvitin voisiko kustannuksia kompensoida metaboliamuutoksen avulla, jota on pidetty yhtenä keinona epäedullisissa olosuhteissa. Kuparille altistettujen koiraiden metaboliantaso oli alhaisempi, sen sijaan vastaavaa eroa ei ollut havaittavissa naarailla.

Yhteenvetona voin todeta, että vanhempien kokema kuparialtistus vaikuttaa seuraaviin sukupolviin, minkä perusteella vaikutukset eliöihin – ihmiset mukaan lukien – ovat kauaskantoisia. Saasteiden vaikutukset eliöissä voivat säilyä, vaikka saastetta ei ympäristössä enää esiintyisikään. Kuparialtistus aiheuttaa allokaatiokustannuksia, jotka sukupuolten välillä näyttäisivät kompensoituvan eri tavalla. Mukautumista vallitseviin olosuhteisiin voi esiintyä lyhytaikaisen altistuksen seurauksena, mutta se saattaa heikentää fekunditeettiä saasteettomissa olosuhteissa. Väitöskirjani osoittaa, että saasteiden vaikutukset eivät ole yksiselitteisiä, ja vaikutukset ympäristössä ovat kauaskantoisempia kuin aiemmin on ajateltu. Toivon, että väitöskirjani herättää huomiota alati pahenevaan ympäristön saastumiseen. Vaikka monien saasteiden haitalliset vaikutukset on tunnistettu, kasvava määrä kemikaaleja päätyy luontoon, ja näiden kemikaalien vaikutukset eliöihin ja ihmisiin ovat pääosin tuntemattomia.

LIST OF ORIGINAL PAPERS

This thesis is based on the following publications and manuscripts, hereafter referred to in the text by their Roman numerals:

- I Pölkki M, Kangassalo K and Rantala MJ. 2014. Effect of interaction between temperature conditions and copper exposure on immune defense and other life-history traits of the blow fly *Protophormia terraenovae*. *Environmental Science & Technology*, 48: 8793-8799.
- II Pölkki M, Kangassalo K and Rantala MJ. 2012. Transgenerational effects of heavy metal pollution on immune defense of the blow fly *Protophormia terraenovae*. *PLoS ONE*, 7(6): e38832.
- III Pölkki M, Kangassalo K and Rantala MJ. Effects of larval heavy metal exposure on survival in later life. *Manuscript is preliminary accepted for publication.*
- IV Pölkki M and Rantala MJ. Intra- and trans-generational fitness costs of larval copper exposure and evidence for short-term acclimatization - do parents prepare their offspring? *Manuscript.*
- V Pölkki M, Valtonen TM, Mattila A, Saastamoinen M and Rantala MJ. Decreased metabolic rate: a mechanism to compensate the costs of heavy metal exposure? *Submitted manuscript.*

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1. INTRODUCTION

1.1 Ecological effects of environmental pollution

Anthropogenic environmental pollution is considered one of the most grievous threats to organisms and ecosystems. Despite emission limitations, environmental regulations and improvements generated by new innovations in technology, environmental pollution is still a severe problem especially in the vicinity of industrial areas, refineries, mining industry and urban areas (Kozlov and Zvereva 2007, Fonseca et al. 2013, Krishna et al. 2013), and even in agricultural soil (Wong et al. 2002, Nicholson et al. 2003, Szolnoki et al. 2013). Environmental pollutants can roughly be divided into three major groups: organic compounds, organometals and heavy metals (Widdows and Donkin 1991). Of these, heavy metals are known to be transferred from the ambient environment to plants and from plants to consumers in food webs (Beyer et al. 1985, Hunter et al. 1987a, b, Heliövaara et al. 1987, 1990, Rabitsch 1995a, Zvereva et al. 2003). Also, they have been discovered to accumulate in plants, various invertebrate and vertebrate species (Beyer et al. 1985, Rabitsch 1995a, see also Borowska et al. 2004). Exposure alters species composition (Spurgeon et al. 1994) as well as abundance (Eeva et al. 1997, 2002) and consequently heavy metals can have a direct impact or indirect influence on the functioning of ecosystems (Spurgeon et al. 1994, Eeva et al. 1997, 2002, 2012). Furthermore, recent discoveries imply that toxicants might move between ecosystems from aquatic sediments to land ecosystems carried by insect-eating animals (Lilley et al. 2012, Buckland-Nicks et al. 2014).

Exposure to harmful compounds is a major risk and a widespread problem that organisms have to face and tolerate, if not to go extinct locally. It has generally been thought that the amount of toxic compounds may increase with trophic level, reaching notably high concentrations in higher consumers (e.g. Laskowski 1991). The main exposure routes into organisms include food, the body surface, water and air (Hunter et al. 1987c). However, when it comes to the emanation of heavy metals, it is ambiguous. There are differences in accumulation rates between species (Andrews et al. 1984, Beyer et al. 1985, Hunter et al. 1987b, Rabitsch 1995a, b, Dehn et al. 2006), trophic levels and populations (Hunter et al. 1987b, Rabitsch 1995a). Additionally, there seems to be a seasonal variation in concentrations observed in leaf foliage (Hunter et al. 1987a, Kozlov 2005) and invertebrate species (Hunter et al. 1987b, Rabitsch 1995a, b). This phenomenon can partly be explained by climatic factors such as precipitation and wind direction (Kozlov 2005), whereas in invertebrates variation in species abundance and structure, and likewise changes in age composition, might have an impact on detected concentrations (Hunter et al. 1987b). Even though the emission rates of heavy metals have decreased, concentrations in the environment and organisms might not be declining as fast (Rabitsch 1995a, Kozlov 2005), indicating that toxic compounds may remain and circulate in ecosystems for a considerably long period of time.

Heavy metals constitute various elements and are a classification for metallic pollutants. Some, like copper (Cu), have an essential role in cellular and metabolic processes (Percival 1998, Harrison et al. 2000, Walker et al. 2006), while others, like lead (Pb) or cadmium (Cd), are highly deleterious xenobiotics even in small concentrations (Laskowski 1991). Even though trace elements are needed for various vital functions (Percival 1998, Harrison et al. 2000, Walker et al. 2006), the adverse effects of their toxicity become apparent when the concentration or exposure time increases. However, vital trace elements are thought to be more easily regulated in organisms than xenobiotic metals (e.g. Hunter and Johnson 1982, Timmermans and Walker 1989, Laskowski 1991, Rabitsch 1995b, Harrison et al. 2000).

1.1.1 Effects on organisms

Organisms' ability to process harmful substances has limitations; therefore, concentrations found in individuals correlate with the dosages measured in the ambient environment (Laskowski 1991, Borowska et al. 2004). However, species have evolved several mechanisms, adaptations and habits to avoid intake of or exposure to toxic compounds. Such mechanisms include behaviour, excretion of pollutants and detoxification processes (Calow 1991, Posthuma and van Straalen 1993). Mobile organisms can easily migrate elsewhere or simply reduce the intake of toxic compounds by avoiding contaminated nourishment through food selection (Zidar et al. 2004). When toxic compounds have entered the body, trace elements can be excreted via secretion mechanisms (Posthuma and van Straalen 1993). For example, even if insect larvae contain high amounts of copper, concentrations measured in adults are less acute, suggesting that they have specific excretion abilities or ability to handle surplus copper. Also, dosages found in larval skins and pupae are relatively low, supporting the existence of an efficient excretion process during larval development (Timmermans and Walker 1989).

Individuals exposed to heavy metals are shown to express higher glutathione S-transferase (GST) and esterase rates (Zvereva et al. 2003, Sun et al. 2008, Dubovskiy et al. 2011). Together with monooxygenases these enzymes belong to one of the primary detoxification compounds (Terriere 1984, see also Zvereva et al. 2003, Serebrov et al. 2006). However, they possess a wider range of functioning, including participation in defence against fungal toxins and bacteria (Serebrov et al. 2006, Dubovskiy et al. 2008). Another important group in detoxification processes are metallothioneins. These proteins are essential regulators of metal homeostasis that have the ability to bind free heavy metal ions (e.g. Kägi 1991, Balamurugan and Schaffner 2006, Egli et al. 2006). Metallothioneins are apparent in animals and can even be found in plants (Tamai et al. 1993, Egli et al. 2006). The wide abundance among species indicates that these proteins are essential in detoxification processes and that they are evolutionarily significant. For instance, MTF-1 (metal responsive transcription factor-1) mutant flies that are not able to produce metallothioneins are detected to have impaired tolerance to heavy metals (Egli et al. 2003).

Exposure to heavy metals is found to increase the production of heat shock proteins (HSP) (Sanders et al. 1995, Karouna-Renier and Zehr 2003), which were first discovered in insects (Ritossa 1962). One reason why they are particularly interesting is that they seem to respond to a variety of environmental stressors that activate the excretion of these proteins (Sørensen et al. 2003). Additionally, they are highly conserved across animal species (Sanders et al. 1995, Santoro 2000, Sørensen et al. 2003). HSPs can be found across a range of organisms, from single-celled organisms to humans (Santoro 2000). Also, they are thought to play an important role in the immune responses (Srivastava 2002, Wallin et al. 2002, Robert 2003, Tsan and Gao 2004) as immunoregulators (Pockley 2003). This conjecture has gained indirect support in experimental studies, in which heavy metal exposure was detected to alter hemocyte activity, which possesses an important role in the immune function of invertebrates (Olabarrieta et al. 2001, Borowska et al. 2004, Borowska and Pyza 2011). It has been addressed that a signalling linkage exists between HSPs and the immune system, whereby an increase in HSP levels may indicate damage or some other kind of cellular stress to the immune system (Wallin et al. 2002). Furthermore, HSPs are thought to be connected to reproductive success through infection-related activation (Neuer et al. 2000). Despite the evolutionary importance of HSP against stress, the line between advantage and cost is fairly thin. Increased HSP rate is found to be costly already at a low level (reviewed by Sørensen et al. 2003), indicating that they may cause trade-specific costs. Above described impacts are considered to be an outcome of several factors, such as concentration of the toxins, route of exposure and other environmental and conditional factors (Olabarrieta et al. 2001, Dehn et al. 2006), which is why predicting the consequences may be complex.

Previous findings from wildlife species indicate a connection between immune functions and environmental pollutants (reviewed by Galloway and Depledge 2001). Moreover, the costs related to exposure to environmental stress or toxicants have been acknowledged in the past. In the wild, individuals exposed to pollutants suffer from immunosuppression and as a consequence of increased susceptibility to diseases they may carry a heavier pathogen load (de Swart et al. 1996), suggesting that exposure to harmful compounds might lead to trade-offs in immunity and detoxification processes. Since the handling and detoxification of the disadvantageous compounds in body tissues consumes energy and causes stress (e.g. Calow 1991) it may result in energy deficits; therefore, less energy might remain for growth, reproduction and maintaining immune functions against pathogens, for example. There are several known direct effects on numerous condition-dependent traits, which are discussed below.

1.2 Direct effects of the ambient environment on condition-dependent traits

Direct exposure to heavy metal pollutants causes immediate and diverse harmful effects on various condition-dependent traits. These effects are most likely due to the above mentioned costs caused by detoxification processes, cellular stress and immediate adverse effects of precarious compounds (Calow 1991, reviewed by Posthuma and van

Straalen 1993). Indeed, individuals exposed to heavy metals during their development reach a smaller size (Simkiss et al. 1993, Spurgeon et al. 1994), have lower energy reserves (Donker 1992), develop more slowly (Simkiss et al. 1993) and suffer from a higher mortality rate (Spurgeon et al. 1994, Zidar et al. 2004, Al-Momani and Massadeh 2005) as well as reduced fecundity (Moe et al. 2001), reduced reproductive success (Spurgeon et al. 1994) and reduced longevity (Moe et al. 2001) compared with individuals growing under unpolluted conditions. According to recent discoveries, heavy metal exposure affects individuals' ability to defend themselves against pathogens and parasitoids, or more precisely, it alters the activation rate of the immune defence system. Past studies have detected changes in the encapsulation response against an artificial parasite (van Ooik et al. 2007, 2008, Dubovskiy et al. 2011) and real pathogens, such as fungi and bacterial infections (Dubovskiy et al. 2011). Interestingly, low concentrations of heavy metals seem to activate the immune defence system, whereas higher amounts have an opposite impact on immunity (Sorvari et al. 2007, van Ooik et al. 2008). Heavy metal concentrations used in the studies mentioned above were partly derived from observed maximum levels occurring naturally in contaminated areas and were measured either from birch leaves or from individuals that were gathered from a polluted region (Sorvari et al. 2007, van Ooik et al. 2007, 2008). It has been thought that the ability to tolerate heavy metals is related to the genetic background of a species (Dallinger and Höckner 2013). Even though direct exposure has immediate disadvantageous impacts on various traits, long-term exposure might generate metal-tolerant phenotypes through selection towards tolerant genotypes, which are better adapted to existing conditions (see Beeby and Richmond 1987, Greville and Morgan 1991). This could partly explain why individuals from polluted areas are found to be more successful in heavy metal-containing conditions than individuals from unpolluted areas (van Ooik and Rantala 2010).

Favourable living conditions are a prerequisite for life. As discussed above, environmental conditions encountered by individuals shape the existence and phenotype of organisms. One of the most crucial abiotic environmental conditions is temperature, which sets the limits of the existence of life (Begon et al. 2006). Temperature conditions have a strong impact, especially on ectothermic animals (Stevenson 1985). Experienced temperature conditions have an effect, for instance, on achieved individual size and development time (Grassberger and Reiter 2001, 2002, Tarone et al. 2011). Under warmer temperature conditions individuals develop faster (e.g. Grassberger and Reiter 2002) until a certain limit (Tarone et al. 2011). However, there are differences between distinct species and populations (Grassberger and Reiter 2002). Earlier discoveries imply that the temperature conditions individuals faced during their early development might have an impact on the expressed immune system activity, for example. A short-term exposure to the lethal temperature of 43 °C or development under a mild heat-shock temperature of 38 °C has been shown to strengthen pathogen resistance of the greater wax moth *Galleria mellonella* larvae (Wojda and Jakubowicz 2007, Wojda et al. 2009). However, the simultaneous effect of two distinct environmental conditions on life-history traits is still not well understood, although the growing concern over human-caused climate change has recently focused attention onto this field (Kimberly and Salice 2013).

1.2.1 Trans-generational effects

According to growing evidence from empirical studies, environmental conditions encountered by parental generations may have an influence on the life-history traits expressed by their offspring (Little et al. 2003, Magiafoglou and Hoffmann 2003, Mitchell and Read 2005, Sadd et al. 2005, Bonduriansky and Head 2007, Sadd and Schmid-Hempel 2007). Such parental effects are not determined by the offspring's genotype but are instead brought through by the genotype or environment faced by the parental generation (Youngson and Whitelaw 2008, Bonduriansky and Day 2009). Trans-generational effects are mediated, for instance, by transmission of epigenetic, nutritional, environmental and behavioural variation (Bonduriansky and Day 2009). One such effect stems from variations in the environment, in which the availability of nutrient supplies causes variation in parental provisioning. In resource-rich environments parents may improve offspring fitness if their acquired condition is transmitted to their offspring (Mousseau and Fox 1998, see also Bonduriansky and Head 2007), whereas according to the parental stress hypothesis an unsuitable environment may result in reduced offspring quality (Mousseau and Fox 1998, Badyaev and Uller 2009, Vijendravarma et al. 2010). In contrast, the adaptive parental effects hypothesis predicts that parents may produce offspring that will do best in those particular conditions that their parents experienced. One suggested mechanism is that parents produce less offspring but invest more per individual instead (Mousseau and Fox 1998, Vijendravarma et al. 2010). However, there also exists non-genetic transmission mechanisms, such as trans-generational environmental epigenetics, which encompasses phenotypic changes caused by the environmental conditions experienced by the parental generation (Skinner and Guerrero-Bosagna 2009). These modifications are trans-genetically inherited without altering coding DNA and are transmitted to the next generation and expressed by the progeny through non-genetic mechanisms (Uller 2008, Youngson and Whitelaw 2008). Epigenetic effects are understood as those molecular operators in the immediate vicinity of DNA which are able to control the activity state of the genome. Such operators are, for instance, modifications in histone or chromatin structure, DNA methylation and non-coding RNA (McGowan et al. 2008, Skinner and Guerrero-Bosagna 2009). Even though the field has received significant attention recently, the idea was originally proposed in the 1940s (Waddington 1942). Epigenetic effects have recently become the subject of a great and growing interest in the study of vertebrates, particularly rats (Anway et al. 2005, Anway and Skinner 2006) and mice (e.g. Dolinoy et al. 2006, 2007), but in invertebrates they have received less attention so far.

1.3 Costs of immunity and other life-history traits

The classic hypothesis of immune-mediated sexual selection was originally presented by Hamilton and Zuk (1982), where ornaments are suggested to be indicators for superior genetic resistance against parasites. According to the immune handicap hypothesis, the honesty of these traits is derived from the assumption that only individuals of high quality are able to maintain enhanced immunity and express exaggerated secondary

sexual ornaments (Folstad and Karter 1992). Males that can express both are preferred by females (e.g. Ahtiainen et al. 2006), suggesting that these traits are under intense selection and that they are crucial for survival.

One of the fundamental factors affecting survival is the immune defence system (Lochmiller and Deerenberg 2000, Sadd and Schmid-Hempel 2009). A common assumption in ecological immunology is that immune responses are costly for hosts (Rolff and Siva-Jothy 2003, Sadd and Schmid-Hempel 2009). However, even though there is a price to be paid, excessively parsimonious defences will in the worst case, when the host loses the battle, lead to death (Rolff and Siva-Jothy 2003). The price that hosts have to pay in case of a pathogen attack not only includes the direct harm caused by the intruder but also the actual costs yielded from the usage and maintenance of the immune defence system (Moret and Schmid-Hempel 2000). These assets spent on defence cannot be claimed back and used elsewhere. The life of organisms consists of a constant balancing of the costs required for immune defence and the demands of reproduction and other traits. Thus, decreased immune function may only be beneficial when survival is not threatened by pathogens (Hanssen et al. 2004).

In the case that available resources are limited, maintaining a good immune defence system comes at an even greater price. The true nature of expenses emerges under stressful conditions which differ from an individual's optimum and which might set limits for reproduction, growth and eventually for survival (Begon et al. 2006). Occasional scarcity in resource abundance is common under natural conditions that organisms have to cope with. This is one reason why the ability to survive under an environmental stressor is considered to be under intense selection (Rion and Kawecki 2007). It seems that mild stress can expand lifespan, whereas harsh conditions reduce it (see Vermeulen and Loeschke 2007). However, it has also been shown that diet has an effect on longevity in a variety of species (e.g. Houthoofd et al. 2003, Lin et al. 2004, Mair et al. 2004), being firstly observed in rodents (McCay et al. 1935). Individuals reared under restricted food conditions have a longer lifespan, even though the direct mechanisms involved have remained obscure (Piper et al. 2005, Pletcher et al. 2005, Tatar 2011).

It has been addressed that under some stressful conditions individuals may preserve resources and reduce exposure time to a disadvantageous condition by diminishing their metabolic rate (Hoffmann and Parsons 1989, see also Bublly and Loeschke 2005). Support arises from a discovered linkage between improved starvation resistance and stress tolerance. Selection lines towards improved starvation resistance have revealed connections between the selected trait (starvation resistance) and other traits. It has been shown that individuals with better starvation resistance have higher lipid content (Chippindale et al. 1996, Harshman and Schmid 1998, Harshman et al. 1999a, Schwasinger-Schmidt et al. 2012), a lower metabolic rate (Harshman et al. 1999a) and a better ability to tolerate other stresses, like chemicals that are a potential source for oxygen radicals (Harshman et al. 1999b). Interestingly, selection towards higher parasitoid resistance actually impaired survival when food availability was limited and competition was high (Fellowes et al. 1998). Recent empirical studies indicate also trade-

offs between immune function and reproduction (Moret and Schmid-Hempel 2001, Gwynn et al. 2005, Zerofsky et al. 2005, Luong and Polak 2007), suggesting that there might be costs of immune functions to other traits. Usually the correlations found between immunity and other life-history traits are negative (Lazzaro and Little 2009), which highly supports the hypothesis of trade-offs between costly life-history traits (Sheldon and Verhulst 1996). However, genes related to immunity undergo rapid evolution, suggesting that they are under selection and shaped by an intense force of host-parasite co-evolution (Lazzaro and Little 2009), which is why host-parasitoid interactions are based on a large genetic pool and variation (Sorci et al. 1997, Tinsley et al. 2006).

1.4 Insects as model organisms

The majority of animals constitute insects and all ecosystems are highly dependent on them, which is why they function as important ecological models. Since their maintenance under laboratory conditions for several generations is relatively easy, insects being space, time and cost efficient, they have become one of the most important model organisms for experimentally testing ecological and evolutionary hypotheses. Diverse species of insects are widely used in laboratories for scientific purposes. As discussed above, the basic structures of the main detoxification, stress tolerance and immunological components (see below) are somewhat similar to those of other organisms. In humans and flies signalling pathways responsible for the upregulation of the innate immune system share some similarities (Silverman and Maniatis 2001). In general, insects lack or have a limited state of parental care, which is why they are an excellent model group for testing trans-generational effects. Male parental care is usually concluded after copulation and females abandon the offspring instantaneously after egg laying (Alcock 2010). Because of the similarities with other groups, results can be partly generalized to other organisms with fewer ethical concerns, which make insects ideal study organisms.

1.4.1 The immune defence system in insects

In order to defend themselves against pathogens, parasites and parasitoids, organisms have developed numerous different defence mechanisms against their enemies during their long shared evolution, co-evolution (Futuyma 2006, Schulenburg et al. 2009). Simultaneously, and as a result of co-evolution, pathogens, parasites and parasitoids have evolved mechanisms to crack their hosts' defences (Godfray 1994). Animals have two main defence mechanisms against the attacks of foreign intruders. Of these, acquired immunity is the prevalent defence system among vertebrates, whereas immunity in invertebrates is grounded in the innate immune system (e.g. Hoffmann 2003). Overall, the primary functions of the defence mechanisms in these two animal groups are somewhat similar (Hoffmann 1995, Vilmos and Kurucz 1998, Hoffmann 2003, Royet et al. 2005, Libert et al. 2006). Acquired immune responses may also exist in invertebrates in some form according to recent findings (e.g. Kurtz 2004), but so far the exact mechanism has remained unknown.

In brief summary, the main mechanism of insects' innate immune system consists of two dissimilar clusters: the cellular and the humoral defence mechanism (Gillespie et al. 1997, Gupta 2001, 2002). Under pathogen attack, the latter is thought to function as a second line of defence, i.e., as a major defence shield against small particles and unicellular pathogens (Gupta 2001). The humoral defence mechanism segregates a variety of circulating proteins and antimicrobial peptides (AMPs) (Hoffmann 1995, Gillespie et al. 1997, Gupta 2001, 2002, Hoffmann 2003), which are used to destroy intruders. The cellular branch, on the other hand, is the first line of defence and is activated when the attacker is larger in size, a multicellular organism (Gillespie et al. 1997, Gupta 2001, 2002), a virus (Washburn et al. 1996) or has already entered into the body cavities (Leclerc and Reichhart 2004). Cellular responses consist of encapsulation, nodulation and phagocytosis (Gillespie et al. 1997, Gupta 2002). Of these, the encapsulation response is mainly responsible for eliminating parasitoids (Ivory et al. 2008) but may also act against other pathogens. During the encapsulation process, plasmatocytes and lamellocytes (Ivory et al. 2008) encapsulate identified novel particles and destroy them (Strand and Pech 1995). In species such as *Drosophila melanogaster*, the majority of circulating hemocytes, up to 95 %, are plasmatocytes, whereas the remaining 5 % minority consists of crystal cells (Borowska and Pyza 2011). Their function rests in melanization of novel pathogens and they also play an important role in wound healing (Hultmark 1993, Borowska and Pyza 2011). Even though defence mechanisms are effective weapons against intruders, activation of the immune system (e.g. Moret and Schmid-Hempel 2000) and its maintenance in the absence of pathogens has expenses (e.g. Valtonen et al. 2010a), which are suggested to result from the physiological cost of producing unnecessarily high levels of antimicrobial peptides, as well as cellular stress like tissue damage (Zerofsky et al. 2005, Schulenburg et al. 2009). There are, however, other theories for the ultimate causes of the observed costs (see e.g. Libert et al. 2006). Nevertheless, the costs may be compensated to some degree by increasing the energy intake, in cases where the availability of resources is limited, complex trade-offs between costly life-history traits may occur.

2. AIMS OF THE THESIS

In my doctoral thesis, the primary aim was to experimentally investigate intra- and trans-generational effects, costs and potential trade-offs of larval heavy metal exposure on different life-history traits in adulthood by using insects as model organisms. In my studies, I have principally used a sub-lethal concentration of heavy metals to avoid mortality-related selection towards improved tolerance. Previously, direct impacts of heavy metal exposure on a variety of traits have received plenty of interest and are fairly well recognized; however, the potential trans-generational effects, costs and trade-offs between different traits are not well known. Furthermore, the potential latent effects as well as interaction effects of heavy metal exposure and other environmental factors, such as temperature, need further study. In my first study (**I**), I therefore examine the effects of the interaction between heavy metal exposure and temperature conditions on different life-history traits.

Based on the increasing evidence supporting the fact that the parental environment might have an effect on distinct condition-dependent traits expressed by their offspring, one could also expect that heavy metal exposure experienced by the parental generation might have an influence on the traits expressed by their progeny. However, there are no previous studies to test this conjecture in insects. In my next study (**II**), I address the question: does parental exposure to heavy metal have an influence on the innate immune system of their offspring even when the exposure is no longer effective? To answer this question, I firstly explored the direct effects of heavy metal exposure by testing the immunity of the parental generation exposed to heavy metal. Next, I investigated the possibility that the expressed traits of the parental generation in the form of immune activation might be trans-generationally transmitted to the progeny.

In the following studies (**III-V**), I have concentrated on disentangling the costs and poorly known trade-offs of heavy metal exposure between distinct life-history traits. As has been described so far, exposure to low amounts of toxicants enhances immune responses (**II**). However, up-regulated immune activation may cause direct expenses that can be detected under conditions in which individuals are not able to compensate for their loss. Study **III** examines potential survival-related costs of heavy metal exposure under starvation conditions by using four increasing concentrations of heavy metal. Additionally, I determine the lifespan, immediate costs of exposure on larval mortality rate, adult size and development time.

Based on the assumption that heavy metal exposure increases energy demand, I hypothesize that exposure-related costs might reduce reproductive success through resource allocation and that the costs may reach to the next generations. This can directly affect the parental generation, but the costs might also be trans-generationally transmitted to the next generation (**II**). Study **IV** explores fitness-related costs by gathering evidence on whether direct exposure to heavy metals has an effect on the fecundity of the parental generation and their offspring. I also examine the possibility of acclimatization to the prevailing conditions after short-term exposure. The effects of heavy metal exposure on

fecundity are poorly understood at present, and to the best of my knowledge there are no previous studies that have considered either the potential trans-generational effects or short-term acclimatization through phenotypic plasticity.

It has been proposed that low metabolic rate might be an advantage under some disadvantageous conditions. In order to understand the potential mechanisms behind previous studies (**II**, **III**), in study **V** I ask the question: does exposure to a sub-lethal concentration of heavy metals have an influence on the metabolic rate? By reducing their metabolic rate, individuals might compensate for the costs of toxic compounds and free up valuable resources for the maintenance of other traits. The effects of heavy metal exposure on metabolic rate have thus far not been extensively studied. I expect metabolic rate to be lower among individuals exposed to heavy metals.

3. MATERIALS AND METHODS

More detailed descriptions of the experiments and maintenance of the laboratory stock populations can be found in the original papers (I-V).

3.1 Model species

I have conducted my thesis using two different study organisms. In the first two studies (I, II) a blow fly, *Protophormia terraenovae* (Diptera: Calliphoridae; Robineau-Desvoidy, 1830), was used as a model species and experiments III-V were accomplished using the common fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae; Meigen, 1830), as a study species. The main reason why I decided to use two different model species rests on the basic structure of the experimental design and on the ecological, behavioural and morphological differences between these two species. I consider both species to be highly suitable models for my doctoral thesis for a number of reasons, explained below.

P. terraenovae is one of the cold-tolerant fly species, having a holarctic distribution. Maggots of this species feed mostly on decaying carcasses, whereas adults nourish mainly on nectar as well as on the fluids of decomposing carcasses to acquire proteins (Richard and David 1997). The species is relatively large in size (larger than a common house fly). Despite this large size, generation length is short. Development time of *P. terraenovae* at the temperature of 35 °C is 9.19 ± 0.3 days (Grassberger and Reiter 2002). As Grassberger and Reiter's study has shown, this species can be reared under a variety of different temperature conditions. The main advantage of using the blow fly as a model is its size, which is why it is easy to handle and a convenient species for the immune assay conducted in this thesis (I, II).

Thus, in studies that demand high volume and pair mating under restricted space, the common fruit fly appears to be a more appropriate model organism. *D. melanogaster* flies are widely used laboratory animals in scientific studies. The species is relatively small in size, which is why *D. melanogaster* is an appropriate model and an ideal species in studies in which a large number of individuals (thousands of individuals) are needed and space is limited (III-V). Both *P. terraenovae* and *D. melanogaster* can be maintained on an artificial diet, to which a heavy metal supplement is easy to add. Both species were kept under controlled laboratory conditions for several generations before conducting the experiments.

3.1.1 Origin and maintenance of the blow fly stock

More than 600 adult *P. terraenovae* flies were captured to establish a laboratory stock population. The flies were caught in Turku, in south-western Finland, during the summer of 2009 and maintained in several large (70 litre) plastic cages under controlled temperature conditions of 23 ± 1 °C and under constant light (24 L: 0 D). This light

condition corresponds to the natural light conditions in Finland during midsummer. Each cage was supplied with adult nourishment, dry powder food (ingredients 1: 1: 1: semolina, sugar and baby's milk formula, containing 10 % of dry yeast) and fresh water *ad libitum*. Maggots were reared separately in several 2 litre boxes in plastic containers and fed with cat food (Pirkka® beef pâté). Each box contained a layer of sawdust on the bottom for pupation.

3.1.2 Origin and maintenance of the common fruit fly stock

To form a laboratory population, *D. melanogaster* adults were collected from Rauma, Finland, during the autumn of 2006. The flies have since been maintained under controlled laboratory conditions. The stock contains thousands of individuals, kept in several 2 litre glass jars under controlled temperature conditions of 23 ± 1 °C and a constant light regime (III) or under a 12 L: 12 D photoperiod (IV, V). Jars contained 200 ml of larval medium for maggots (ingredients: 1 l water, 20 g brewer's yeast, 150 g syrup, 80 g semolina and 10 g agar, supplemented with 7.5 ml of nipagin), with yeast culture on top for adult flies.

3.2 Breeding designs and treatments

In order to study the effects of heavy metal exposure during early larval developmental phases on different adult traits, eggs or adult flies were obtained from the laboratory stocks. In all studies, individuals for the experiments were gathered either by placing an oviposition stimulus into the stocks for adults to lay eggs on (I, II) or by collecting virgin, newly hatched adults whose eggs were used in the experiments (III-V).

In study I, I tested the influence of two different environmental conditions on life-history traits. In this study, individuals exposed to heavy metal and uncontaminated control individuals were reared under three distinct temperature conditions of 23, 28 and 33 °C in order to examine the potential interaction effects of temperature conditions and heavy metal exposure.

In studies II and IV, I tested the potential trans-generational effects of heavy metals on the offspring of the parental generation. Individuals from the same parental treatment group were allowed to copulate to generate the next generation. Traits were measured from the parental generation and from their offspring. Additionally, in study IV, I examined the presence of short-term (two generations) acclimatization due to phenotypic plasticity in reaction to heavy metal exposure. For the experiment, the parental generation was fed either with uncontaminated larval medium or food supplemented with heavy metal. Individuals were allowed to copulate within their own treatment group and the next generation of both groups was either reared on contaminated or uncontaminated larval medium. The treatment group reared only on uncontaminated larval food functioned as a control to which the other treatments were compared.

3.2.1 Selected heavy metal and concentrations

Of the heavy metals, I decided to use copper (Cu) to demonstrate the possible effects of heavy metal exposure on distinct traits. In all studies, individuals were continuously exposed to copper through the larval diet during their early developmental phases and traits were measured from adult flies. Larvae were either reared on uncontaminated (control) larval food or on food supplemented with copper sulphate (CuSO₄, Sigma Aldrich® 10.00 g, standard solution for 1000 ml) (Egli et al. 2006, van Ooik et al. 2008).

Perhaps one of the most examined heavy metals in scientific publications is copper (Laskowski 1991). As formerly shown, high concentrations of copper may have a significant impact on mortality rate (Karouna-Renier and Zehr 2003), whereupon surviving individuals might be selected to better tolerate contaminated environmental conditions. To avoid selection towards higher copper tolerance through increased larval mortality and to avoid the impact of uneven individual density (Ireland and Turner 2006), I have used mainly sub-lethal dosages. I preliminary tested the copper concentration used in studies **I** and **II**. A copper concentration of 200 µg/g did not have a significant effect on mortality of the blow fly *P. terraenovae* but instead had an effect on immunity, so it was used in the experiments. In studies **IV** and **V**, the utilised copper concentration was based on the results of study **III**, in which I used four different increasing dosages of copper (50, 100, 200 and 300 µg/g) and an uncontaminated control to determine the effects of copper on survival-related costs. Of these, the concentration of 50 µg/g did not have an effect on the mortality of larvae of *D. melanogaster* and was selected for the later experiments (**IV**, **V**). To put the selected dosages into a perspective with those observed in nature, the concentrations found nearby emission sources can reach notably higher levels. Rabitsch (1995a) reported an average soil copper concentration of 1319.6 µg/g at a distance of 300 meters from an emission source. Amounts measured in adult Formicidae reached 47.1 µg/g of copper (median) (Rabitsch 1995a). Based on this, the concentrations selected for my studies are likely to exist in nature.

For exposure time, I have chosen continuous exposure to copper throughout the larval development. Adult flies were, without exception, fed with uncontaminated food in order to avoid instant effects. Since adults are more mobile and their diet is likely to consist of a variety of food sources, I consider larval exposure to be ecologically more relevant (Shorrocks 1975). It has been shown that exposure experienced as a larva might no longer be apparent in adulthood, since copper can be quite effectively removed from body tissues (Timmermans and Walker 1989). In all the experiments I have used an uncontaminated control group as a treatment control, whose food contained the same amount of distilled water as the contaminated groups' food contained copper solution.

3.3 Life-history traits under study

3.3.1 Immune assay

In the first two experiments, I tested the intra- (**I**, **II**) and trans-generational effects (**III**) of heavy metal exposure on the innate immune system of the blow fly *P. terraenovae*. Furthermore, I examined the potential interaction effects of two distinct environmental factors on immunity (**I**) by assessing the encapsulation response. Insects' innate immune response can be determined in various reliable ways (Lawniczak et al. 2006). A frequently used method in immunological studies is measuring the strength of cellular responses by delineating the encapsulation rate against a novel particle (e.g. König and Schmid-Hempel 1995, Rantala et al. 2002, 2003, Ahtiainen et al. 2004, Kortet et al. 2007). This method aims to artificially mimic a parasitoid larva or egg that activates the immune defence system of the host (Gillespie et al. 1997). One advantage of using an artificial antigen is that the distraction of complex chemical communication between a host and its pathogen is lacking (Gillespie et al. 1997, Ivory et al. 2008), which enables research attention to concentrate solely on the immediate effects of the encapsulation process itself. The complex process commences when the immune system of the host recognizes a foreign particle (Gillespie et al. 1997, Gupta 2002, Schmid-Hempel 2005, Ivory et al. 2008). Consequently, the intruder is capsulated by layers of hemocytes that contain dark melanin (Hultmark 1993, Gillespie et al. 1997, Schmid-Hempel 2005). The shade of darkness of a capsulated artificial implant can be interpreted by estimating the strength of the encapsulation response during a preliminarily tested time period. Previously, this method has been demonstrated to be repeatable (Rantala and Roff 2006) and to be a sufficient and reliable method to determine individuals' ability to defend themselves against foreign intruders (Rantala and Roff 2006, Smilanich et al. 2009). Based on these arguments, I consider this method to be an adequate measure for assessing immunity in my thesis.

I have determined the encapsulation response of *P. terraenovae* by using nylon monofilament implants to artificially simulate a novel intruder (see above). To do so, monofilament strings (diameter 0.18 mm) were rubbed with sand paper and knotted. 2 + 1 mm long pieces were cut, so that between the longer end (placed into the insect) and the shorter end (for handling the implant) there was a knot (see figure 1 below). On the left side of the thorax, the chitin was pierced by a sterile needle and a sterile implant was placed into the fly. For the placement and removal of implants, flies were anesthetized with carbon dioxide. Flies' immune system was allowed to react to the foreign particle for four hours. This time period was preliminarily tested to have the highest variation in encapsulation rate in this species, and therefore it was the most relevant time interval to use in the experiments. Removed implants were stored by freezing. Encapsulation rate was later assessed from digital photographs taken through a light microscope from two different directions. The melanin content of the implants was analysed with the Image J program (Image J 1.42, National Institute of Mental Health, Bethesda, MD, USA) that measures the average darkness of the reflected grey of each picture. The mean values were scaled so that the highest darkness was equal to the most intense encapsulation rate (an artificial unit) by subtracting the values from a control (a clean implant) (Rantala et al. 2002, Kortet et al. 2007).

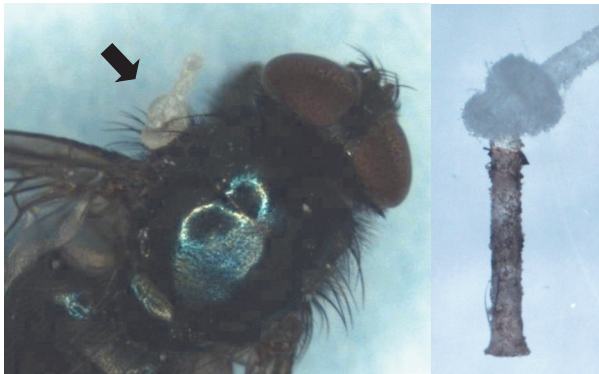


Figure 1. The blow fly *P. terraenovae* with an inserted nylon monofilament implant on the left side of the thorax (picture on the left). Monofilament implant after 4 hours of reaction time inside a fly, encapsulated with dark melanin containing hemocytes (picture on the right).

3.3.2 *Survival under starvation and lifespan*

When testing the costs and trade-offs between life-history traits it is crucial to pay attention to the experimental conditions. One way to control the conditions is to use an enclosed system, in which compensatory resource intake does not occur, such as diapausing insect pupae (Freitak et al. 2003). Another admissible procedure to examine expenses is to maintain individuals without access to nourishment, i.e., by keeping them under starved conditions (Moret and Schmid-Hempel 2000). Starvation is an especially harsh but simultaneously straightforward method to explore costs under a condition in which individuals are not able to compensate for loss of energy through nourishment (Moret and Schmid-Hempel 2000). To examine the potential survival-related costs of heavy metal exposure in study **III**, half of the adult flies from different copper concentrations and from uncontaminated (control) food were randomly selected to either feed *ad libitum* in order to assess lifespan, or to be kept under food-deprived conditions to determine survival under starvation conditions. The starvation group had constant access to water through a moist cotton ball. The non-starved group was shifted into fresh food vials (consisting of larval medium and yeast culture) once a week to maintain suitable food conditions for attaining reliable lifespan measurements. In the starved group, survival was checked every two hours and in the non-starved group once a day, until the last fly had died (Valtonen et al. 2010a, 2011).

3.3.3 *Fecundity*

Intra- and trans-generational fitness-related costs of heavy metal exposure on fecundity through larval diet were examined in study **IV**. Fecundity was determined by counting the eggs produced by each female (Simmons and Bradley 1997). It is, however, important to distinguish the difference between fertility and fecundity. The latter comprehends the number of offspring produced per individual, whereas the former is derived from the number of viable offspring (Awmack and Leather 2002). To test potential fecundity, randomly selected male and female pairs from the same treatment condition (uncontaminated or contaminated) were allowed to mate. The male was removed from the vial (Okada et al. 2011) and the female was allowed to lay eggs for 24 h (Simmons and Bradley 1997) and the eggs were counted under a light microscope.

Unlike in the study of Simmons and Bradley (1997), here, removal of the male was made without anesthetization. The amount of CO₂ is difficult to control, which might have had an impact on the results.

I consider a 24-hour oviposition time to be a reliable measure of fecundity for the study question of my thesis. Even though there might be some variation caused by age, early fecundity is thought to predict future reproductive success (e.g. Awmack and Leather 2002). Fertility during early stages is likely to be ecologically important (Begon et al. 2006). Nevertheless, based on the reasons mentioned above, I believe that my results provide a viable estimate for expected reproductive success.

3.3.4 *Standard metabolic rate (SMR)*

In study V, I tested the potential mechanism behind the observed enhanced immune activation (I, II) and starvation resistance (III) by measuring the standard metabolic rate (henceforth referred to as SMR) of uncontaminated and copper-contaminated individuals. Since the development time of contaminated individuals is prolonged, eggs for this treatment were harvested 24 hours earlier than eggs for the uncontaminated treatment (Vijendravarma et al. 2010), so that the SMR measurements could be conducted at the same time. Unlike studies I-IV, which were carried out wholly at the University of Turku, the SMR assay of study V was conducted at the Lammi Biological Station. Produced CO₂ levels were measured using flow-through respirometry in which filtered air (dry and CO₂-free) was pumped into a measurement chambers to delineate the standard metabolic rate of the treatment groups (Niitepöld 2010). The SMR was measured from adult *Drosophila* flies. Prior to the measurements, flies were covered with a black cloth and allowed to calm down for a predesignated time of 2 min. Each run was repeated twice in order to obtain a reliable SMR. The produced CO₂ level was analyzed using Li-Cor 6251 (LiCor, Lincoln, USA) (Rogowitz and Chappel 2000, Niitepöld 2010, Piironen et al. 2010). Since variation during the first run was fractionally higher, the mean values of the second measurement were used to ensure trustworthy results.

3.3.5 *Assay of condition-dependent traits*

As estimates of condition-dependent traits I used the measurements of body size, described below, and development time. Size was assessed either by measuring dry body mass (I, II) or thorax length (III-V). To measure the dry body mass of *P. terraenovae* flies, they were dried in 60 °C for the preliminary tested time of 24 hours and weighed to the nearest 0.1 mg. The thorax length of *D. melanogaster* flies was measured using an ocular measure under a light microscope. Received values were scaled to millimetres (mm). Both measures of size have been used in previous studies (e.g. Simmons and Bradley 1997, Bublik et al. 2000, Valtonen et al. 2012), so I considered them to be reliable measures to estimate individual size.

Development time was determined by counting the time elapsed between oviposition and adult eclosion. Hatching events were recorded either daily or several times a day,

depending on the species. Previously, copulation has been shown to have an effect on female immune gene expression (Fedorka et al. 2007) and individuals' ability to resist pathogens (Valtonen et al. 2010b), which is why ensuring virginity is essential in immunological studies. Adult *Drosophila* flies may mature as early as eight hours after eclosion, depending on developmental conditions (Ashburner 1989), which is why hatched flies were collected several times per day during the light period in studies **III-V**. In studies **I** and **II**, blow flies, which are larger in size and require more time for maturation (Wolff and Hansson 2005), were collected once a day to obtain virgin flies.

Given that starvation resistance (e.g. Chippindale et al. 1996, Harshman et al. 1999a), longevity (Simmons and Bradley 1997) as well as metabolic rate (Kleiber 1947, Nespolo et al. 2003) and fecundity (Honěk 1993) correlate with individuals' size, this was taken into account when conducting statistical analyses by correcting these variables for variation in size. To do so, I have regressed the variables against individual size (Saastamoinen and Rantala 2013).

4. RESULTS AND DISCUSSION

4.1 Interaction between two distinct environmental conditions on life-history traits (I)

The growing concern over climate change has drawn attention to the potential interaction effects between temperature conditions and other environmental stressors (Kimberly and Salice 2013). In study I, my aim was to concentrate on potential latent effects, which are so far poorly understood. To do so, I examined the interaction between larval copper exposure and constant temperature conditions on innate immune system function, body mass and development time of adult flies. This study demonstrates that temperature conditions have an impact on immune system activation measured as encapsulation response (Figure 2a), and that the effects of larval copper exposure on adult immunity and other traits are temperature-dependent. More importantly, I found a sex difference in immune defence under distinct temperatures. Under higher temperatures, males had a notably stronger immunity than females (Figure 2a), suggesting that there might be a difference in the detoxification process and/or metabolic rate between sexes (see V). Similar results considering a sex difference in immune defence have been found in earlier studies (e.g. Gray 1998, Rantala and Roff 2006), which supports the possibility of a sex difference in resource allocation between costly features. Additionally, temperature condition had an effect on the magnitude of copper exposure. Copper exposure had a stronger impact on immunity in lower temperatures compared with higher temperature conditions. Previously, laboratory selection experiments have revealed that cold-adapted individuals express higher mass specific metabolic rates than individuals from higher temperature conditions (Berrigan and Partridge 1997). Based on this, one could think that individuals might be able to handle toxic compounds more effectively under higher temperatures, whereas under colder conditions less energy is available for the costly detoxification processes (Posthuma and van Straalen 1993). On the contrary, fresh water snails (*Physa pomilia*) were more sensitive to cadmium under higher temperature (Kimberly and Salice 2013). However, compared to cadmium copper is considered to be more easily regulated in body tissues (Balamurugan and Schaffner 2006). In general, individuals' ability to respond to a stressor depends on their developmental stage, exposure time, strength of the stressor and the individual's overall condition (Truscott and White 1990, Abdel-Lateif et al. 1998, Moe et al. 2001, Dehn et al. 2006, Kimberly and Salice 2013). Nevertheless, temperature is an important environmental factor especially among ectothermic organisms.

I also found that temperature had a significant effect on other life-history traits such as body mass and development time. Under higher temperatures individuals were larger in size (Figure 2b) and developed faster than those at a lower temperature. Slower development time indicates the costs of the detoxification process (reviewed by Posthuma and van Straalen 1993), but it has also been thought that in a colder environment more energy is needed for maintaining vital processes and body temperature

(Stevenson 1985). Results indicate that trade-offs between costly traits exist to compensate for energetic costs of living under unfavourable environmental conditions. To conclude, results of this study suggest that the ability to regulate toxic compounds is indeed temperature-dependent, and sexes may respond differently to such changes in environmental conditions.

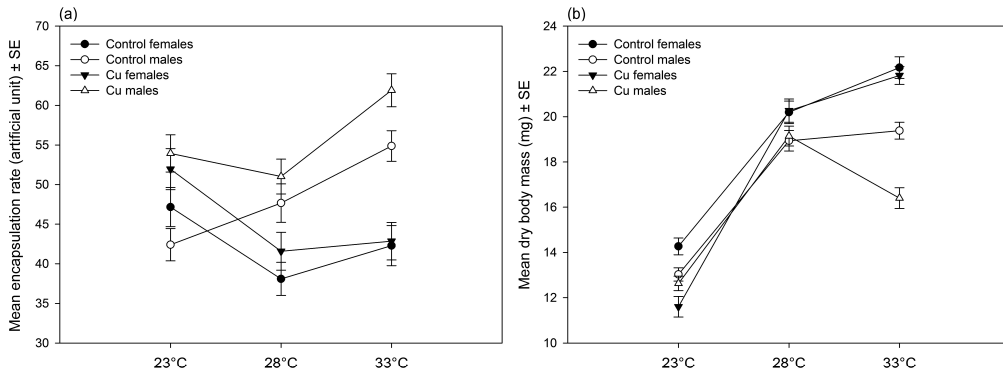


Figure 2. (a) Mean encapsulation rate (an artificial unit) and (b) dry body mass (mg) of males and females exposed to a sub-lethal concentration of copper (triangles) or reared on uncontaminated control food (circles). Individuals from both treatments were kept under constant temperatures of 23, 28 and 33 °C. Standard errors (\pm SE) are presented.

4.2 Trans-generational effects of copper exposure (II)

Here, I demonstrate that direct exposure to a sub-lethal concentration of copper through the larval diet enhanced the immunity of adults and, more specifically, exposure had a trans-generational effect on the innate immune defence system. The observed up-regulated immune activation might be a consequence of an increased number of hemocytes (Coles et al. 1995) and granulocytes in the hemolymph, which has previously been detected in Mollusca species exposed to metals (Ruddell and Rains 1975, Russo and Lagadic 2004). The number of hemocytes in the hemolymph has been found to be correlated to the magnitude of the encapsulation response (e.g. Rantala et al. 2000) and, particularly, hemocytes have an important role in the encapsulation process (e.g. Gillespie et al. 1997).

This study is the first to demonstrate the trans-generational effects of copper exposure on immunity in insects. Parental larval exposure to copper had an intensifying trans-generational effect on their offspring's immunity, even if the progeny was raised in an uncontaminated environment. These results corroborate former experiments in which the parental environment was found to influence offspring traits through non-genetic inheritance (Magiafoglou and Hoffmann 2003). The observed up-regulated encapsulation response might be transmitted to offspring through epigenetic adjustments (Skinner and

Guerrero-Bosagna 2009), although the actual transmission mechanism was not tested here. Even though immunity is an essential defence against pathogens and a requirement for survival, the maintenance of exaggerated defence mechanisms demands resources. Up-regulated immune activation might be a prominent advantage under conditions of constant pathogen load (Sadd et al. 2005). Otherwise, the maintenance of enhanced immune responses that do not match the current infection risk might yield fitness costs, particularly in the case of resource limitations that prevent the compensation of costs.

Parental copper exposure did not have an influence on the body mass of the parental generation or their offspring, although, as shown in study **I**, this trait is highly temperature-dependent. However, the parental generation exposed to copper developed slower than uncontaminated control groups, but this trait was no longer apparent in the next generation in which both of the treatment groups were reared under uncontaminated larval food. This indicates that among the parental generation the observed differences in development time between treatment groups might be an outcome of the immediate toxic effects of copper. Previously, individuals that had been exposed to heavy metals generated larger amounts of glutathione S-transferase (GST) and esterase compounds, known as mainline detoxification enzymes (Dubovskiy et al. 2011), indicating the toxic effect of heavy metals. These kinds of detoxification processes for surplus copper require energy, which is why less energy remains for other life-history traits (Calow 1991, Posthuma and van Straalen 1993).

4.3 Potential costs to survival (III)

Individuals exposed to increasing concentrations of copper through their larval diet expressed prolonged starvation resistance as adults in the lowest (a sub-lethal dosage of 50 $\mu\text{g/g}$) and highest concentration (300 $\mu\text{g/g}$). In previous studies, maintenance of immunity has been found to be energetically costly (Valtonen et al. 2010a) and trade-offs between the expenses of this trait might affect survival under conditions in which individuals are not able to compensate for the energy loss. As shown in study **II**, exposure to a sub-lethal concentration of copper enhances innate immunity system activity, whereas former studies have demonstrated that exposure to high concentrations of this metal decreases immunity (van Ooik et al. 2008). The latter finding is consistent with our result and could explain the observed outcome in the highest copper treatment group (300 $\mu\text{g/g}$). Since immunity was not directly tested here, this presumption remains only at a speculative level. Furthermore, following the conjecture stated above, a sub-lethal exposure should have resulted in decreased starvation resistance due to the energy demands of immune activation. In addition to this, studies on *Drosophila* flies have revealed that selection towards parasite resistance impairs survival under starvation conditions (Hoang 2001), suggesting that increased energy consumption is a trade-off of up-regulated immune activation. Therefore, the observed enhanced survival under starvation conditions in the lowest copper concentration suggests resource allocation between other traits than immune response.

Previously, selection experiments have revealed a connection between body condition and starvation resistance. Those individuals selected for higher starvation resistance develop slower, are larger in size and have higher lipid content (Harshman et al. 1999a, Schwasinger-Schmidt et al. 2012). In my study, exposure to copper linearly increased development time and had an adverse effect on achieved adult size (variation in size was controlled for in statistical analyses). Even though individuals were notably smaller in size, they were able to survive longer under starvation conditions, which lead to the assumption that there might be another mechanism than size involved in the observed prolongation of starvation resistance. Selection experiments indicate that improved starvation resistance might result in impaired egg production and longer lifespan (Rion and Kavecki 2007, see also Schwasinger-Schmidt et al. 2012). Furthermore, starvation resistance is thought to be connected to a decreased metabolic rate (Harshman et al. 1999a). Despite this relationship between metabolic rate and starvation resistance, no such effect has been found related to lifespan (Partridge et al. 2005). However, in my study, even if copper exposure improved starvation resistance in some treatment groups, no effect on longevity was found. Since copper can be processed quite effectively during larval development (Timmermans and Walker 1989), adults are not likely to suffer the immediate adverse effects of copper exposure. This implies the existence of exposure-related costs during larval development, which are no longer apparent in later life. Copper might have a suppressive effect on a costly trait, which is why more resources are available for starvation resistance. This kind of survival trade-off can be detected only under environments that have limited resources and where individuals benefit by re-allocating the surplus resources towards survival. Some such down-regulated trait might be metabolic rate or fecundity, which have been tested in studies **IV** and **V** and are discussed in more detail below.

4.4 Intra- and trans-generational costs of copper exposure on fitness traits (IV)

Intra- and trans-generational effects of larval heavy metal exposure on female fecundity were tested in study **IV**. Females exposed to copper during their early developmental phase produced fewer eggs as adults compared with those reared on uncontaminated larval food, which is consistent with former studies (Spurgeon et al. 1994, Moe et al. 2001). These results follow the assumption of increased detoxification costs during early development (Calow 1991), from where the decrease in reproductive success might stem from. Detoxification processes consume valuable resources that are no longer available for such traits as ovariole development, which may also clarify the improvement in starvation resistance shown in study **III**. If females invest less resources for producing eggs, the energy can be allocated elsewhere (note that mature females lay eggs constantly, even if they are virgins), which could partly explain the improved starvation resistance observed among females. Interestingly, starvation resistance is suggested to be connected to impaired egg production (Rion and Kavecki 2007, see also Schwasinger-Schmidt et al. 2012), which is in line with study **III**.

Similar to the results presented in study **II**, ambient copper exposure experienced by the parental generation had a trans-generational effect on the fecundity of their offspring. However, the progeny suffered impaired fecundity only if the offspring larval condition differed from the conditions experienced by their parents. If the progeny was raised under similar conditions to the parental generation, no effect on fecundity was discovered. The next generation seemed to acclimatize to the prevailing conditions after short-term copper exposure (two generations), indicating possible physiological adaptations to a contaminated environment through phenotypic plasticity. Further, this might lead to the assumption that costly traits are being activated, which is advantageous only if the offspring face similar contaminated conditions than their parents. One such trait (encapsulation response) was examined in study **II**, which demonstrated the up-regulation of the immune system in the parental generation due to copper exposure and its transmission to the next generation. As discussed earlier, such immune activation (e.g. Moret and Schmid-Hempel 2000, Freitak et al. 2003) and its maintenance are costly (Valtonen et al. 2010a), which could certainly impair the fecundity in the parental generation as well as in the next generation. However, it has been shown that heavy metal exposure increases the production of detoxification enzymes (e.g. Sun et al. 2008, Dubovskiy et al. 2011), and, interestingly, these enzymes are found to be connected to the functioning and activation of the immune system (Dubovskiy et al. 2011). Therefore, I hypothesize that the observed improvement in fecundity after two generations of heavy metal exposure might be the side effect of improved detoxification processes. Under uncontaminated conditions up-regulated detoxification processes might be unnecessary and costly, but when facing the same contaminated conditions as the parental generation these traits might become advantageous (see Sadd et al. 2005). Improvements in handling the toxicants may allow more resources for the development of ovarioles and improve fecundity in adulthood. This conjecture is consistent with the adaptive parental effects hypothesis, which predicts that parents may produce offspring that are better fit to the stressful conditions experienced by the parental generation itself (Mousseau and Fox 1998, see also Vijendravarma et al. 2010). This finding may indicate non-genetic inheritance through offspring phenotype (Skinner and Guerrero-Bosagna 2009) but the exact mechanism involved was not tested here. In this context, trans-generational costs of fitness-related effects have not formerly been reported.

4.5 A potential mechanism to compensate the costs (V)

Exposure to a sub-lethal dosage of copper through larval diet decreased the standard metabolic rate (SMR) of males but not of females (**V**). Results of this study indicate that males might be more vulnerable or react faster to increased levels of environmental toxins than females, or that sexes have different ways to cope with environmental stress. However, even though the effect was observed only in males, it does not mean that such an effect might not also be apparent in females under higher copper concentrations, or in other traits like fecundity, as shown above (**IV**). Previous findings have demonstrated that improved starvation resistance is connected with diminished metabolic rate, suggesting that one potential way to tolerate stressful conditions is an alteration of the

metabolic rate (Harshman et al. 1999a), although this hypothesis has not always gained support (Harshman and Schmid 1998). However, cross-species comparisons indicate a negative linkage between metabolic rate and encapsulation response (Fellowes and Godfray 2000), which is consistent with my findings here. My results are the very first to demonstrate a potential mechanism behind observed up-regulated immune activation (**II**) and prolonged starvation resistance (**III**). In addition, results of my thesis suggest that there might be sex-dependent trade-offs between costly life-history traits, such as immune defence, female fecundity as well as immunity, and male metabolic rate.

5. CONCLUSIONS AND FUTURE PROSPECTS

The main conclusion of this thesis is that heavy metals have an influence on various life-history traits, which may cause complex trade-offs between other features throughout generations. In this thesis I have studied the effects of continuous larval copper exposure on life-history traits in adulthood stages using two Diptera species. Firstly, I discovered that copper has intra- and trans-generational effects on various traits (**I-V**). Parental exposure to copper had an effect on immune system activity and fecundity, and these changes were still apparent in the subsequent generation that was not exposed to heavy metal (**II, IV**). However, individuals may be able to acclimatize to the prevailing conditions. Secondly, I have found an interaction effect between two distinct environmental conditions: in study **I**, I show that the effects of copper exposure on immunity and other life-history traits are temperature-dependent. Finally, my studies are the very first attempts not only to distinguish the direct costs of copper (**I, II**) but also to estimate the potential trade-offs between other life-history traits to cover the expenses (**III-V**). Most importantly, my studies show a sex difference in resource allocation (**I, V**). The results suggest that not only are the costs traded differently between the sexes but also that females and males might respond differently under environmental stress. These results are summarized in greater detail below.

One main result of my thesis is that the effects of environmental pollution on different traits can be trans-generationally transmitted to the next generations. Larval exposure to a sub-lethal concentration of copper up-regulated innate immune system activity (**II**) and reduced fecundity (**IV**), and these effects remained with the next generation even if the progeny was reared under uncontaminated conditions. My results show, firstly, that the changes caused by environmental pollutants can indeed be trans-generationally transmitted to following generations. Secondly, the effects of pollutants may remain in animal populations even if the pollutant is no longer apparent. Interestingly, I discovered in study **IV** that after two generations of copper exposure individuals seemed to be acclimatizing to the prevailing conditions. Conversely, in cases where the parents were exposed to copper their offspring showed reduced fecundity even if kept under uncontaminated conditions. This finding suggests that acclimatization to the contaminated environments seems to incorporate expenses, which might reduce reproductive success under uncontaminated conditions. This is consistent with former conjectures that long-term exposure might generate metal-tolerant populations (e.g. Beeby and Richmond 1987) which are more successful under contaminated conditions (van Ooik and Rantala 2010). Nevertheless, concrete effects of heavy metal exposure on actual fitness and offspring viability are still lacking. This is likely to be a promising area for future studies.

Throughout my thesis, I have mainly used a sub-lethal concentration of copper to experimentally demonstrate the impact of larval copper exposure on different life-history traits of adults (**I, II, IV** and **V**). The dosage I used was selected purposefully so as not to have an adverse effect on the mortality rate, because mortality-related selection towards higher copper tolerance would have distorted the results. Thus, the concentration does not

correspond to the high or extreme copper levels found in nature near polluted areas (Rabitsch 1995a); therefore, the effects of higher concentrations of copper are not investigated here. Also, because of the trace element nature of copper individuals are able to regulate copper levels in body tissues to some degree (e.g. Timmermans and Walker 1989), whereas such a mechanism does not exist against many other environmental pollutants. Nevertheless, as demonstrated here, exposure to a sub-lethal concentration during larval development already has an impact on various traits in later life.

Another important finding of this thesis is the sex difference in encapsulation response under different temperature conditions (**I**) and in metabolic rate (**V**). I found that females and males responded differently to environmental stressors, suggesting that the associated costs might be compensated from distinct sources. No such sex difference was discovered in starvation resistance. Individuals exposed to a sub-lethal dosage of copper survived longer under starvation conditions (**III**), indicating that they might be able to compensate the costs of starvation resistance by re-allocating surplus resources from down-regulated traits. One such way to free up energy is the down-regulation of metabolic rate. This possibility gained support in my last experiment, in which copper exposure impaired the standard metabolic rate (**V**), but only among males. Previously, the relationship between metabolic rate and encapsulation response was thought to be negative (Fellowes and Godfray 2000). Based on this assumption, down-regulation of metabolic rate could hypothetically explain the results observed in studies **I** and **II**, in which individuals in turn expressed up-regulated immunity. Similarly, it could support the findings in study **III**, where individuals exposed to copper were able to survive longer under starvation. However, this was true only for males. Females, on the other hand, might have been saving energetic resources in form of reduced fecundity (**IV**).

To generalize the findings of this thesis, the effects of environmental stressors on various life-history traits are more likely to be a sum of several factors, rather than single segments. My results imply that the effects of heavy metal pollution on animal populations might be more complex and far-reaching than previously thought. This is something that should be considered in animal conservation programmes and risk management, i.e., when evaluating the risks of harmful compounds on animal populations, including humans. Even though the main effects of certain environmental conditions on organisms are being recognized, the potential interactive effects of distinct stressors as well as trans-generational impacts have thus far been poorly understood. In order to identify possible threats and estimate the effects of different stressors on the viability of animal populations, far more research is needed, especially in order to understand the potentially deleterious effects. One thing to keep in mind is that an alarming rate of animal populations and even species consists of small isolated groups of individuals. Because of their limited genetic diversity and small isolated nature, their ability to adapt genetically or via phenotypic plasticity to changes in environmental conditions is restricted and they are more sensitive to any kind of environmental stress (Frankham et al. 2012). Furthermore, predicting the consequences of human-caused environmental perturbations, such as climate change and pollution may be complex as they result in unpredictable interaction effects on various life-history traits.

This thesis disentangles life-history traits and trade-offs between costly features caused by larval exposure to copper from an ecological perspective. Sexes seem to respond differently towards environmental stressors, which should certainly be taken into account when conducting experiments with insects. In order to deepen our understanding, future studies should concentrate on the non-genetic factors to understand the mechanism behind trans-generational effects. Even though this thesis reveals several important life-history traits which are significant factors in the determination of individuals' overall fitness, the evolutionary meaning remains partly unknown. I hope that with this thesis I have drawn attention to the increasing concern of environmental pollution. Even though the deleterious effects of heavy metals have been recognized, growing numbers of pollutants and new compounds are being emitted, with their effects (not to mention the interaction effects between other chemicals) on organisms being largely unknown. To conclude this thesis, exposure to a sub-lethal concentration of trace metal during larval development can already have far-reaching effects on various life-history traits.

ACKNOWLEDGEMENTS

This has been a longer journey than I expected but it has finally become to its end. It has been a challenging (in a good way) and mind-blowingly interesting trip to discover the amazing world of science. I would like to express my deepest gratefulness to all those people who have been there and helped me over the past years: Thank you all!

First and foremost, I would like to thank my supervisor Markus J. Rantala for introducing me into the interesting world of insects. I am also grateful that you have given me this great opportunity to conduct my thesis and for all the valuable advice you have given during the years.

I wish to thank all my co-authors but first of all Marjo Saastamoinen and Anniina Mattila for the possibility to conduct study V at Lammi Biological Station. I further wish to thank Katariina Kangassalo, Terhi M. Valtonen, Marjo Saastamoinen and Anniina Mattila for their precious help and comments on the manuscripts. The most grateful I am to Katariina who has helped me during so many experiments. I truly appreciate your help and support! I also wish to thank all the people at the University of Turku and at the Lammi Research Station for their help and assistance during the experiments. Thank you Terhi for your help during the Lammi trip!

I wish to thank Camilla Ekblad, Serdar Dirihan, Karine Gagnon, Katariina Kangassalo and Robin Kubitzka for being my opponents and for their valuable comments during the trial defence. I also wish to thank Pekka Niemelä for his good tips for the defence. It was many years ago but still fresh in my mind. I also thank Petter Skult for kindly checking the language of the introduction and Veijo Jormalainen and Kai Norrdahl for their help along the way. Thank you to the funding sources: the Finnish Cultural Foundation, Turku University Foundation and BGG Graduate Programme.

Special thanks go to my research group and all the people in the laboratory who have helped me during the experiments; these studies would not have been possible without your assistance. Thank you all for your help! Very special and extra thanks go to Katariina, Terhi, Minna, Hanna-Kaisa, Tiia, Tapio, Ari, Ida and Samuli: thank you for your help or for just being there when I needed to chat about scientific or (mostly) not-that-scientific stuff.

Last but not least, the greatest thanks go to my family and all the loved ones. Your support and understanding during the past years has been priceless. You have always been there when I needed you the most. There are no words to describe how grateful I am to have you around! Maybe slightly far away but within reachable distance.

Helsinki, November 2016

Mari Pölkki

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