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Master thesis

The effect of multiple environmental stress on morphology of yellow dung flies (*Scathophaga stercoraria*)

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ZADÁNÍ DIPLOMOVÉ PRÁCE

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Název práce: **The effect of multiple environmental stress on morphology of yellow dung flies (*Scathophaga stercoraria*)**

Název anglicky:

Cíle práce: Based on already known fact, that plasticity of body size in insect can be caused by many different variables, from which temperature is one of the most importants, it is assumed there might be strong synergistic effects with chemical components of often used pharmaceutical treatments. That might bring risk for populations and ecosystems due to unpredictable changes in environmental conditions. For the dung fauna the common veterinary vermicide, ivermectin, is known to have a detrimental effect on many species as a toxicological stressor. Thus in the frame of the master thesis yellow dung flies will be used as a model system to investigate effects of multiple stressors temperature and presence of ivermectin. The task is to find out if there are effects on size morphology and discuss its potential consequences.

Metodika: The flies used in this study originate from a common garden experiment at Swedish University of Agricultural Sciences in Uppsala in 2010. Used individuals were treated in specific conditions during their larval stage. Full factorial design was applied using high (23°C) and low (19°C) temperature and presence and absence of ivermectin.

The master thesis consists of capturing pictures of certain body parts (wings) of used individuals and proceed morphometric analyzes using computer programe tpsUtil and tpsDig2. Suitable statistical methods will then be used to find out if there

	are effects of the treatments on the body size morphology of the model organisms, mostly working in software MorphoJ, R and Excel.
Doporučený rozsah práce:	30-40 pages
Klíčová slova:	*Scatophaga stercoraria*, wing morphology, environmental stress, phenotype plasticity, ivermectin, morphometric analysis

Doporučené zdroje informací:

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Declaration

I hereby declare that I am the sole author of this diploma thesis, which I wrote under the direction of Mgr. Filip Harabiš, Ph.D. and Doc. Ane Timenes Laugen. I duly marked out all quotations, the used literature and sources are stated in the attached list of references.

Prague, 22nd of April 2015 Kateřina Beňová

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Last but not least I would like to ask for forgiveness from all the dung flies.

Prague, 22nd of April 2015 Kateřina Beňová

Abstract

Residuals from commonly used cattle pharmaceuticals can have a detrimental effect on dung fauna species as a toxicological stressor, but very little is known about their impacts in interaction with other important environmental factors. Therefore yellow dung fly (*Scatophaga stercoraria*) was used as a model organism in common garden experiment to investigate effects of multiple stressors – temperature and presence of common veterinary vermicide ivermectin, on various measures of body size. The flies were raised in laboratory in four different treatments of low (19 °C) and high (23 °C) temperature in combination of ivermectin present and absent. As expected, it was found that flies raised in higher temperatures were smaller at eclosion than those raised in lower temperature. That might be explained by the temperature-size rule or by the fact *S. stercoraria* is rather cold tolerant species. There was larger negative effect of high temperature in wing parameteres, whereas for the total body mass there was shown stronger negative effect of ivermectin. One possible explanation for that could be certain trade-off among body parts. Nevertheless, despite the results still keep the door for further studies open, this study emphasizes the need to decrease the negative impacts on dung fauna caused by pharmaceutical products, because of its irreplaceable role as decompositors in agroecosystems.

Key words: *Scatophaga stercoraria*, morphology, multiple stressors, body size, ivermectin

Abstrakt

Reziduály farmaceutických přípravků používaných pro dobytek mohou mít škodlivý vliv na druhy koprofágní fauny, neboť se chovají jako toxikologické stresory, ale jejich vliv v rámci interakcí s dalšími důležitými environmentálními faktory je stále málo znám. Proto byla v tomto experiementu použita výkalnice hnojní (*Scatophaga stercoraria*) jako modelový organismus pro zkoumání vlivu vícenásobných stresorů – teploty a přítomnosti veterinárního antihelmintika ivermektinu, na různé měřitelné znaky velikosti těla. Výkalnice určené pro pokus byly vychovány v laboratoři ve čtyřech různě stanovených podmínkách za vyšší (19 °C) a nižší (23 °C) teploty v kombinaci s přítomností či absencí ivermektinu. Dle očekávání bylo zjištěno, že jedinci vyrůstající při vyšší teplotě byli menší než ti z teploty nižší. To lze vysvětlit základním ekologickým teplotně velikostním pravidlem nebo faktem, že se *S. stercoraria* označuje spíše jako chladnomilný druh. V rámci parametrů křídel výsledky ukázaly více negativní vliv teploty, zatímco pro váhu těla hrála zápornější roli přítomnost ivermektinu. Jedním z možných vysvětlení by mohl být určitý růstový kompromis mezi částmi těla. Přestože výsledky stále nechávají dveře otevřeny dalším výzkumům, tato práce zdůrazňuje potřebu snížit negativní dopady farmaceutických produktů na koprofágní druhy, neboť hrají nenahraditelnou roli dekompozitorů v agroekosystémech.

Klíčová slova: *Scatophaga stercoraria*, morfologie, vícenásobné stresory, velikost těla, ivermektin

Content

1. Introduction

Anthropogenic influences on the environment have increased recently, mostly in the negative direction. For example using pharmaceutical preparations which were invented to fight parasites does not only help us to keep animals healthy, but at the same time it leads to accumulation of residuals causing harm to the environment (Jochmann 2011). Common veterinary vermicide, ivermectin, is known to have a detrimental effect on many species as a toxicological stressor (Madsen et al. 1990, Jochmann 2011). Aditionally, there is an uncountable number of environmental factors which play important roles in biological processes such as development of organisms or their relationships within the food web. Temperature is one of the most important factors (Laskowski et al. 2010), having broad spectrum of consequences. For instance it has direct impact on ectotherms regarding development time (Blanckenhorn 1997a, Blanckenhorn et al. 2010), sexual behavior (Pitnick et al. 2009) or other fitness traits such like plasticity of body size (Blanckenhorn 2009).

Because it is difficult to disentangle effects of temperature and other environmental factors in the field, laboratory studies are useful tools for predicting possible impacts. Due to the option to determine specific strategy and to set up controlled conditions during the experiments we are able to enlight some of the environmental connections. Various model organisms, for instance yellow dung flies (*Scatophaga stercoraria*) (Blanckenhorn 2009, Jochmann 2011), have been used in the experiments to observe responses to certain conditions. As explanation of environmental factors there can be used different traits of organisms. For example body size, often represented by body mass or morphological characters, is an important quantitative trait with impacts on physiology and fitness of the individual (Blanckenhorn et al. 2003). Those can be analysed by geometric morphometrics, one of the modern techniques which allow us to quantify size and shape, permitting even combination with multivariate statistical approach (Klingenberg 2002).

2. Purpose and aims of the study

Despite there have been many studies on insect´s morphology focused on impacts of individual environmental factors, yet there is not enough information available about consequences of their interactions.

Therefore in the frame of this master thesis *S. stercoraria* will be used as a model system to investigate effects of multiple stressors - temperature and presence of ivermectin, with the help of morphometrics methods. The study is designated as an common garden experiment.

The null hypothesis is set up that there is no difference between individuals treated under lower (19 °C) and higher (23 °C) temperature and in addition of the effect of ivermectin.

The questions to be answered via this work are:

- Do sizes of wings of individuals differ between treatments?
- Do the weights of individuals differ between treatments?

The task is to find out if there are effects on wing size and body mass according to the certain treatments and discuss the possible reasons and potential consequences.

The thesis consists of two major parts - literature review and then the designated experiment.

3. Literature review

3.1 Dung fauna

Many different species of invertebrates, especially insects, can be found in dung. As it is claimed by Madsen et al. (1990) there is always high number of species inhabitating dung pats regardless of seasonal variation. Dung fauna plays an important role in local biodiversity and food web. Furthermore it is an important factor for dung decomposing to improve soil fertility and pasture ecosystem sustainability (Kryger et al. 2005), as well as avoiding fouling of the pastures (Jochmann 2011). The main role is played by Scarabaeid beetles, which provide many important ecological functions such as nutrient recycling, secondary seed dispersal or parasite suppresion. These promote ecosystem services like plant growth support, trophic regulation and, to a certain extent, pollination (Nichols et al. 2008). Unfortunately significant decline or even local extinctions of dung beetles have been recently reported world-wide (Nichols et al. 2008, Coggan 2012). It is assumed veterinary medication that negatively affects dung-fauna as non-target organisms (Madsen et al. 1990, Jochmann 2011) is one of the reasons. Since the dung fauna system is so unique and essential at the same time, it is of high importance to protect it to keep its life neccessary functions.

3.2 Yellow dung fly

Classification

Kingdom **Animalia (Animals)**

Phylum **Arthropoda (Arthropods)**

Subphylum **Hexapoda (Hexapods)**

Class **Insecta (Insects)**

Order **Diptera (Flies)**

No Taxon **(Calyptratae)**

Superfamily **Muscoidea**

Family **Scathophagidae (Dung Flies)**

Genus ***Scathophaga***

Species ***stercoraria*** **(Yellow / Golden Dung Fly)**

(Source: www.bugguide.net)

The yellow dung fly *Scatophaga stercoraria* is widespread cold tolerant insect species (Blanckenhorn et al. 2001, 2010). It is one of dozens of invertebrates species of dung fauna (Madsen et al. 1990), an important part of the ecosystem´s food web (OECD 2008). Because of its widespread abundance, short life-cycle, lack of obligate diapauses and simplicity both in collecting and in laboratory treating *S. stercoraria* is a good model organism (Jochmann 2011). Phenotypic plasticity in different life history traits like body size, growth or development also make it a good model for studies of environmental influence on biological processes (OECD 2008, Blanckenhorn 2009). The species has thus been subject for many studies involving mating behavior, sexual selection and reproduction, development, foraging, genetics etc. within the last four decades (Blanckenhorn et al. 2010). It has also increasingly been used for studying effects of anthropogenic disturbances such like chemical residues in dung of livestock after veterinary treatment (West and Tracy 2009, Blanckenhorn et al. 2010, Jochmann 2011).

3.2.1 Occurence

The species is widespread accross the whole northern hemisphere with preference for colder climates (Blanckenhorn et al. 2010), located often even in subarctic and also arctic zones (Gorodkov 1984). They prefer cattle dung, but they can also be found on dung of sheep, horse, deer or wild boar (Blanckenhorn et al. 2010).

Commonly the habitats are found at high altitudes and high latitudes (Blanckenhorn et al. 2010). In North-Central Europe it is assumed being the most abundant insect species of the dung fauna (Blanckenhorn et al. 2010). Population densities can be affected by many environmental factors such as climate, food, and inter- and intraspecific relationships. In warmer regions of southern Europe its distribution dicreases and the populations stay moreover just at higher elevations (e.g. Pyrenees) (Gorodkov 1984, Blanckenhorn et al. 2010). The reason is mainly the species´sensitivity to hot climate (Blanckenhorn et al. 2001), temperatures above 25º C are assumed to be lethal (Blanckenhorn 1997). That is why the flies occur also in highlands and lowlands habitats in different regimes. They show summer decline in lowlands despite the number of cattle use to be sometimes even higher there, while they keep the population density in highlands on the same level for the whole season (Blanckenhorn 1997) or they can even occur on the locality only in summer (Blanckenhorn et al. 2010). During hot temperatures in summer the flies seek for and hide in colder habitats, most likely with forests (Blanckenhorn et al. 2001, 2010). Agriculture practices is another highly important aspect in the species´occurence, since it influences the availability of food and suitable habitats (Blanckenhorn et al. 2001, 2010). Aditionally, the availability of dung pats both in space and time is important for the population dynamics (Blanckenhorn 1997).

3.2.2 Morphology

Adults of the species are about 7–13 mm long, with one pair of wings as a typical Diptera characteristic. Males are yellow and hairy and usually larger than females which is not such a typical trait in insects. The sexes also differ in colour with females being more cryptic, greyish coloured and less hairy (Blanckenhorn et al. 2010).

Figure 1 Yellow dung flies (*Scatophaga stercoraria*) while mating.
(Source: www.naturfoto.cz)

Body size of *S. stercoraria* is known to be highly influenced by many various factors such as temperature, competition or dung availability as well as location of population´s habitat (Blanckenhorn 1997). Greater size usually brings advantages within the species (Jann et al. 2000, Blanckenhorn et al. 2003). It plays an important role in mating success in males (Blanckenhorn et al. 2003, Pitnick et al. 2009). Males which succeded in copulation are usually larger regarding overall body size than the males without a partner (Blanckenhorn et al. 2003). Second case of a size advantage comes in male-male competition. As Pitnick et al. (2009) mention, males sometimes exhibit territorial behavior at low population densities. So then the larger males are the winners again (Jann et al. 2000, Blanckenhorn et al. 2003). In contrast the smaller size can be plus as well, but only if an alternative resource (like apple pomace) is available (Pitnick et al. 2009).

For females the body size affects the fecundity (Blanckenhorn 1997a ex Borgia 1981), number of eggs is often directly proportional to the size of the female (Parker 1970, Blanckenhorn et al. 2002). The same can not be claimed about the size of eggs since that is mostly affected by temperature (Blanckenhorn 1997, 2001) and not by female´s size.

3.2.3 Sexual behavior

The species shows specific sexual behaviour. Females only come to the dung pat to oviposit, in contrast to males who spend most of their time there (Parker 1970). The sex ratio on the dung pats is therefore usually very male biased, leading to high level of male sexual competition. Jann et al. (2000) observed hundreds of males on the same dung pat. Nevertheless it has been shown that in rare cases when the temperature is high enough during summer, change to female-biased sex ratio may happen since males have disproportionately higher mortality in temperatures above 25ºC (Blanckenhorn 1997).

Males typically behave aggressively while competing for gravid females on the dung pat. It is also assumed that due to intensive male competition male-biased sexual size dimorphism as well as sperm competition adaptations have evolved. Last male sperm precedence is a common rule in the species (Pitnick et al. 2009) with approximately 80% of the eggs fertilized by the last mated male (Blanckenhorn and Heyland 2004 ex Parker 1970), which probably also contributes to the violent competition and „stealing“ the females by another males followed by mating.

Copulation usually takes 20–50 minutes and it is followed by female laying eggs into the dung while male guarding her against other competitors (Parker 1978 in Blanckenhorn et al. 2010). The female leaves afterwards and the male keeps waiting for next sexual partners on the same dung pat or he switch for another, more fresh one (Blanckenhorn et al. 2010).

We see activity within the population mostly during two main flight seasons - in spring (March till June) and in autumn (September till November) (Blanckenhorn et al. 2010), even if the temperature decreases below 10 ºC (Blanckenhorn and Llaurens 2005). Nevertheless it is highly influenced by latitude and altitude, in the highlands or in the very northly placed countries the flies can reversely occur just in summer. Thus it is known there can be two or three overlapping generations under pleasant conditions, on the other hand only one or maximal two generations of flies can be able to develop in less suitable environment (Blanckenhorn et al. 2010).

The flies´ activity can probably also vary during the day. For example there are results from an observation made on swedish cattle farm close to Uppsala in summer 2013 - the flies were active early in the morning, then their activity started to decline around 10 a. m. and lasted until cca 5 p. m. when it raised up again with another peak of action around 8 p. m. It was most likely caused because of the hot, despite another factors might come into account as well (Laugen et al.-unpublished data).

3.2.4 Development and growth

As all Dipterans, *S. stercoraria* go through several life stages during its development. The lenght of total egg-to-adult development takes 17–80 days (Blanckenhorn et al. 2010, Laugen et al.-unpublished data), depending mostly on temperature (Blanckenhorn et al. 2010, Kjaersgaard et al. 2013).

Females usually lay 30–70 eggs (Blanckenhorn 1997a) - with records until even 90 eggs (Blanckenhorn et al. 2010, Laugen et al.-unpublished data) - per clutch. Number of eggs is often directly proportional to the size of the female (Parker 1970, Blanckenhorn et al. 2002) but there is no effect of male size (Blanckenhorn et al. 2002).

The egg phase takes aproximately 1–2 days. Eggs usually lie in a position partly in the dung to keep them safe against desiccation and partly protruding out to acquire oxygen, (Blanckenhorn 1997). After hatching the larvae enter the substrate of the dung which they were laid on. Larvae get oxygen while coming regularly on the surface of the dung (Blanckenhorn et al. 2010). Larvae development takes approximately 10 days and involves passing through three molts while there is no additional body mass accumulated anymore during the last days. Individuals then pupate on the encrusted dung or nearby, and pupal development takes cca 10 days before the adult flies emerge (Blanckenhorn et al. 2010). Female flies generally emerge a few days earlier than males (Blanckenhorn et al. 2010, Kjaersgaard et al. 2013), especially with increasing temperature or photoperiod (Blanckenhorn 1997).

Development time is affected by different abiotic and biotic factors such as amount of dung available, photoperiod, humidity or food availability (Blanckenhorn 1997).The most important factor is temperature (Blanckenhorn et al. 2010, Kjaersgaard et al. 2013). It also depends on the fact if the individual goes through non-direct or direct development (with or without winter diapause respectively; Blanckenhorn et al. 2010).

3.2.5 Food

Larvae of *S. stercoraria* are coprophagous (Blanckenhorn et al. 2010). Adult flies are nutritionally anautogenous (Blanckenhorn and Hosken 2003), despite they sometimes also consume nectar or fresh dung (Blanckenhorn et al. 2010). They can also be called predators (Blanckenhorn et al 2010) since they need prey for becoming sexually mature (Blanckenhorn et al. 2010).

There are different food strategies for males and females. Females forage around on the pasture, while males spend most of their time waiting on the dung for mating ocassion (Parker 1970) and just spend short amounts of time foraging.

3.3 Effects of temperature, antiparasitic drugs and their interaction

3.3.1 Temperature

Specificaly for insect temperature is assumed to be the most important factor (Blackman and Spence 1994, Laskowski et al. 2010), followed by food availability, presence or absence of predators, photoperiod, season length and humidity (Blanckenhorn 2009). Temperature directly affects body size and development time (Blanckenhorn 1997a) in insect, indirectly even many other characters such like sexual behavior and mating succes (Pitnick et al. 2009). Two of the three basic ecological rules dealing with temperature and body size called Bergmann´s, Allen´s and Gloger´s rule have been shown valid in certain cases of ectotherms as well. There is evidence that some insect species tend to grow bigger under colder conditions (Blackman and Spence 1994, Atkinson and Sibly 1997, Blanckenhorn and Llaurens 2005). This so called Bergmann´s rule, which was originally postulated for endotherms, was shown even in ectotherms, for example on four *Drosphila* species or bumble bees (Daly 1985). That „prodruding body parts“ are shorter under cooler conditions (Allen´s rule) was shown on *Aphids* (Blackman and Spence 1994) and *Drosophila* (Daly 1985). Many examples how temperature influence variable traits in species *S. stercoraria* were described previously (see chapter 3.2)

3.3.2 Ivermectin

Ivermectin is the 22,23-dihydro derivate of avermectin B1, a macrocyclic lactone produced by actinomycete *Streptomyces avermitilis* (Campbell et al. 1983). It is a semisynthetic substance obtained from *S. avermitilis´*s metabolites via chemical processes (Jochmann 2011). It has been used as antiparasitic preparation (Campbell et al. 1983) mainly for livestock (West and Tracy 2009) but also for humans (Puniamoorthy et al. 2014) for already more than twenty years. Ivermectin´s special mechanism is based on inhibiton neurotransmission channels, which exist only in invertebrates (Jochmann 2011). It was intended to target common parasitic worms found in cattle, but was not surprisingy later found to be harmful for many non-target species. Besides the lethal effects of higher doses (West and Tracy 2009), non-lethal doses prolong development time of the non-target species, which might cause then detrimental effect in time limited situations (e.g. in end of the season or during drought), and it also reduces growth of the individuals, which can lead for example to lower mating success (Blanckenhorn et al. 2013). It negatively affects especially arthropods during their molting stage and dung decomposing beetles (Blanckenhorn et al. 2013), both engaded as beneficial parts for the ecosystem (Puniamoorthy et al. 2014). Despite ivermectin does not show negative effects on all taxa, it still shows it on majority of them and its presence does not bring benefits to any taxon (Jochmann 2011).

Jochmann (2011) reviews the negative aspects of ivermectin doses commonly used in pharmaceutical preparations. Firstly, some dung breeding species of invertebrates appear to be sensitive to ivermectin, what causes decrease in their abundance. It can be responsible for elimination the presence of certain types of dung species for at least one month after application (Madsen 1990). Secondly, it seems that these species are also responsible for dung degradation. Therefore ivermectin treatment causes slowdown of dung degradation, which is an important factor for the whole dung pat microhabitat. However the rate of cow dung decompostion depends also on many other variables such like climate, season or soil type, proceeding this proces rapidly is always desirable to preserve the nitrogen fertilizing effect and to avoid fouling (Madsen et al. 1990).

West and Tracy (2009) agree on the lethal effects and they claim that the most influenced stage of the dung species is larvae during pupating. The proper reason is still in process of exploring, but the first idea is that there is a connection to phenoloxidase, enzym important for encapsulation. They showed that ivermectin treatment influenced the amount of larvae that pupated, not the amount of flies that successfully eclosed. Thus it seems that ivermectin present at the larval stage of individuals enhances their phenoloxidase activity in adult stage, or the reason might be that ivermectin selectively kills flies with low phenoloxidase activity.

Madsen et all. (1990) agree with Jochmann (2011) about the possible lethal effect on non-target organism within the dung fauna together with dung breakdown. In addition they bring up aspect considering risk of fouling valuable grasslands, which might cause not just environmental, but also practical and economic consequences.

3.3.3 Interaction of factors

Synergistics effects of two and more environmental factors are not just based on practical common sense, but quite some scientific proofs have been already brought. Laskowski et al. (2010) claim that relationship between environmental factors and chemicals is a common situation and it can vary unto complicated modifications. They came with conclusion of sure interactions between toxic chemicals and natural environmental factors in analyse containing 61 studies. Aditionally they showed that natural factors are able to modify interactions among different chemical components as far as in every second case and that also chemicals themselves can strongly react together. Another aspect is interaction between the chemicals and biotic factors as competition, predation or parasitism, which can together with reduction or removal of the species lead to negative impact on populations or possibly even to extinction of a species. The chemical compounds can also be affected by abiotic factors like temperature, humidity or light irradiation. Ivermectin for example is sensitive to UV-light, what means it is supposed to perform more persistence and efectivity in shaded environments (Jochmann 2011).

To sum up all factors combinations and interactions might lead to negative consequences extended for the whole ecosystem and it can hardly be predicted. Therefore suggestions appear for providing more explanations based on field studies made in different environments (Jochmann 2011) together with mandatory eco-toxicological tests applications (Puniamoorthy et al. 2014). For both activities yellow dung flies have been increasingly used as model organism (West and Tracy 2009). Due to its suitable characteristics such like worldwide distribution, high occurence, phenotypic plasticity, easy laboratory cultivating (Blanckenhorn 2009) and mainly larval sensitivity to chemical compounds (OECD 2008), standardized bioassay using this species was developed to test toxicity of parasiticide preparations in dung (Jochmann 2011).

3.4 Morphometrics

3.4.1 Definition, history and use

Definition of morphometrics according to Daly (1985) sounds „morphometrics is the measurement and analysis of form“, while form is considered as practically anything. History of morphometrics can be dated in the end of 20th century (Richtsmeier et al. 2005). Already since that time it has been marked as variable scientific discipline with practicall applications in anthropology, geology, cytology, entomology and other fields of study (Daly 1985). Such techniques allow us to quantify size and shape (Klingenberg 2002) as well as allometry, which is the relation between size and shape (Dujardin 2011). Since great progress has been evolved in morphometrics methods, these combine geometric concept of shape with multivariate statistical approach nowadays. Thus they permit quantitative analysis of morphological variations and also combination of developmental and morphometrics approach (Klingenberg 2002). In insect many studies using geometric morphometrics have been targeted on taxa relationships and population identifying (Albutra et al. 2012). The other main application chapter lies in studies dealing with body size, since it is asummed to be one of the most important quantitative traits which affects many other physiological and fitness characteristics (Blanckenhorn et al. 2003).

3.4.2 Methods

There are two main methods developed for morphometrics description of forms (Albutra et al. 2012), both for two and three dimensional data (Klingenberg 2002). The most prevalent way is analysis based on landmarks. Landmarks represent a set of biologically relevant corresponding points which can be recorded from a form precisely and acceptable accurated (Klingenberg 2002, Richtsmeier et al. 2005). Landmarks can be put on different points of studied object, e. g. intersection of the veins of insect wings or certain connections of mammalian mandible (Klingenberg 2002). Landmarks give us the XY coordinates of the points, therefore measurements are then provided based on their locations (Richtsmeier et al. 2005).

The second basic morphometric method is based on outline analysis. It also uses landmarks - tens or even hudreds of closely connected points are created along the outline of an investigated object to get its general shape. The analysis then extracts the margin around the specimen (Klingenberg 2002, Albutra et al. 2012).

The often used programe for geometric morphometric analyses is tpsDig, which allows us to digitize landmarks. It results into tps document containing coordinates of the samples/specimens, which can be further used in any statistical software. Another example is programe MorphoJ, in which data from tpsDig can be analysed more detailed. It has broad scale of use, it has been increasingly used for species and/or genus determination in the last years (Albutra et al. 2012).

4. Methodology

4.1 Initial field work and lab procedures

The initial generation of the flies was collected on a farm in Mjölsta, Alunda, Sweden (60^0 0′ 48,92′′ N, 17^0 59′ 26,12′′ E) in 2010. Mating pairs were collected, transferred to polypropylene tubes with filter paper smeared with dung and transported to the lab at Swedish University of Agricultural Sciences in Uppsala. When mating was finished, the males were removed. The females were allowed to lay eggs and were removed afterwards. Number of eggs in every clutch was counted through binocular microscope and carefully divided in subdivisons with 10–15 eggs each, which were then put into flasks containing approximately 90 ml of homogenized cattle dung. The dung was collected before on the same farm, where the animals had not been treated with any pharmaceutical compounds at least three months prior. It was homogenized and stored in -80^{0}C for 2 weeks to avoid presence of any higher taxa species which could be potential competitors for the flies in the experiment.

The first two generations were reared in climate chambers under controlled temperature of 20^{0}C, the third generation in 19^{0}C, with 16 h/8 h of light/dark cycle kept for all generations. After the completed development, which took cca 3 weeks under those conditions, each fly was put separately into a 250 ml flask, got water and was fed with sugar crystals and live *Drosophila* prey to become sexual matured.

4.2 Final lab procedures

To avoid extraneous environmental effects from the field environment, three generations of yellow dung flies were raised under laboratory conditions. The third generation of flies was used as a parental generation for the experimental flies. There were 40 males from different families each crossed with 3 females coming usually also from different families, mating male and female siblings was avoided (Figure 2).

The specific conditions for the experiment were chosen according to already known facts about the species. The temperature of 23^{0}C is a stressful factor causing lower survival of the individuals whereas 19^{0}C is less stressful (Laugen et al.-unpublished data). Sublethal ivermectin concentration 6,4 μg/kg FW was chosen according to Römbke et al. (2009). At first an ivermectin stock solution was prepared by dissolving 4,22 g of ivermectin powder in 100 ml of acetone. The 5 ml of the stock solution were diluted with 50 ml of acetone to get a solution with a concentration of 3,84 μg ivermectin/ml. The final 6,4 μg/kg FW ivermectin dung mixture was prepared by mixing 1 ml of previously done solution with 0,6 kg of dung. The control group of dung was prepared separately with the equivalent amount of acenote but without ivermectin. Both dung mixtures were homogenized and left to stand overnight to let the solvent evaporate before using it.

The matings were held in small transparent polypropylene tubes (28,5 x 95 mm) with thin layer of dung on a filter paper inside (Appendix 2) . The male was again removed after successful mating, the female after egg laying. Eggs were then counted and divided into subdivisons with 7 eggs per each if there were enough eggs. For this time there were 4 different treatment groups in order to assess the combined impact of temperature and ivermectin. Therefore the eggs were divided into 8 flasks with two replicates for each group (Figure 1). The flasks were labeled and stored in the climate chamber under the chosen temperature (19 or 23 °C) and light/dark cycle of 16 h/8 h. The samples were carefully observed and the emergence day and sex of the emerged individuals were recorded. Emerged flies were frozen and kept at -20 °C for future analyses.

Then 400 chosen individuals were dissected and paired morphological appendages (wings and lengs) were glued onto sheets of paper, orientated as it was on the live individual. Weight was measured before dissection and then the rest of the body was also weighted afterwards. Thus tables with samples were created, each containing information about the individuals and their dissected body parts (Appendix 1).

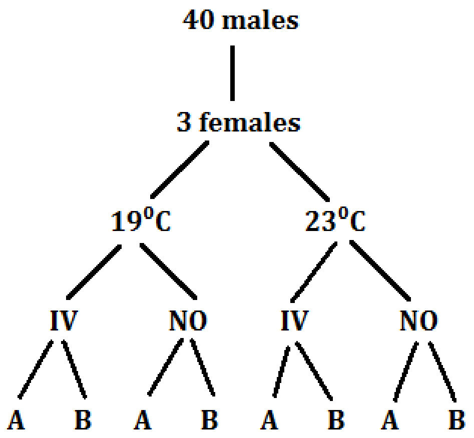

Figure 2 The scheme of the projected experiment.

4.3 Landmarks obtaining and size measurements

I captured pictures of all body parts of the chosen dissected flies with digital camera installed on a dissection stereomicroscope Nikon SMZ1500. The setting of the device was magnification 1 to make the picture as clear as possible. The images were after capturing automatically stored on the computer software delivered with the camera. I imported them later to the free software tpsDig2, which I used to add digital landmarks to the images at certain important points of the wings. It is assumed wings set an example of standard index for body size measurements in insect (Blanckenhorn et al. 2003). I chose the position of landmarks based on both common methods used in morphometrics and on consideration what kind of analyses they are suppose to later be used for. Additionally, to get the experience with placing landmarks with as less variance from the certain point as possible, I tested the repeability by measuring 15 landmarks on ten wings independently ten times each. Because it was desirable to obtain as much as possible of the size and shape of the wing, in the end I proceeded total amount of 19 landmarks for each picture (Figure 3), both on left and right wing, if there was no damage or the wing was not missing. Mostly vein intersections were used to provide reliable cues for where to put the landmarks. In the end according to Blanckenhorn´s et al. study (2003) I decided for four landmarks to be used in further measurements. After visual inspection of possible wing damages and suitable landmarks the best option seemed to be to work with landmarks number 2 and 8 to get the wing width and landmarks 5 and 19 to get the wing length. Thus two imaginatory lines - A and F appeared on the wing to illustrate the measurement intension (Figure 3).

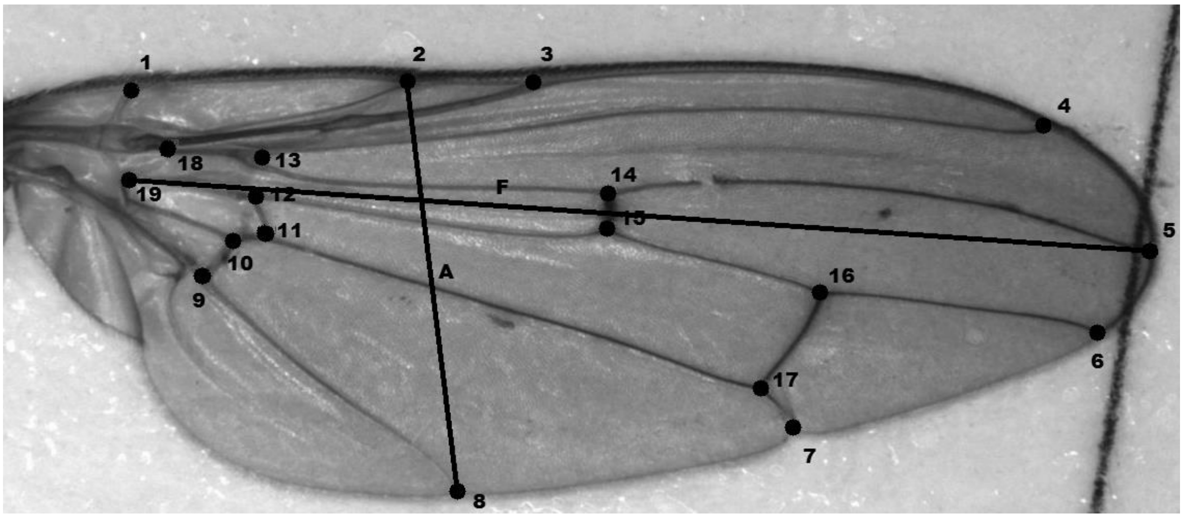

Figure 3 Wing of *S. stercoraria* with proceeded landmarks and lines for width and length measurements (Photo: K. Beňová)

Afterwards I exported the X and Y coordinates of the desirable landmarks obtained for all samples to excel to provide measurement of distances between landmarks using Euclidian equation as follows:

$$d=\sqrt{(x_2-x_1)^2+(y_2-y_1)^2}$$

where x and y are coordinates of the certain landmark. Since the result came in pixels, it was neccessary to transform the distances to milimetres by dividing *d* with the image resolution *121,48965061*.

Wing aspect as a simple measure of wing shape was calculated as wing length divided by wing width (Kjaersgaard et al. 2013).

4.4 Statistical analyses

Since the main aim was not to find out any wing assymmetry, I used only the right side wings for statistical procedures. First I visually checked the wings to avoid using indviduals with not suitable landmarks because of any damage. Therefore the amount of used individuals resulted in 381 compared to 402 initial individuals (Table 1).

Table 1 Comparison of numbers of initial data and the used ones.

Data	**Temp (°C)**	**Treatment** ***IV***	 ***NO***
Initial	***19***	86	141
	23	57	118
Used	***19***	77	139
	23	57	108

Within the dataset temperature and treatment were characterized as categorical explanatory variables, both with two levels – temperature with 19°C and 23°C, treatment with ivermectin (IV) and without ivermectin (NO). Data of wing length and width, wing aspect and wetmass were treated as continous response variables. Therefore I tested them for normality and homogenity of variance by visual inspection from histograms and using Shapiro-Wilk´s tests.

Then I analyzed the data in the statistical software R (R Core Team 2014) using generalized linear models (glm, stats library). In the full models I separately placed all the response variables into interactions always with all the explanatory variables. I created appropriate graphs and tables to visualise the results.

5. Results

5.1 The effect of temperature and ivermectin interaction

According to the normality tests the data did not show normal distribution, therefore models with gamma distribution were used for statistical analyses.

The analyses showed strong effect of temperature in all traits while ivermectin played more moderate role. Temperature was statistically significant for all traits, whereas ivermectin treatment only for wetmass values. Temperature and treatment interaction wass statistically significant for wetmass and wing width (Table 2).

Table 2 Traits of males *Scatophaga stercoraria* reared under certain treatments combining temperature (19 °C and 23 °C) and ivermectin (presence and absence).

Trait	Factor	Intercept	Deviance	Pr (>Chi)
Width (mm)	Intercept	1.206		
	Temp		0.086	***<0.001***
	IV		0.007	0.205
	Temp:IV		0.022	***0.022***
Length (mm)	Intercept	2.245		
	Temp		0.019	***<0.001***
	IV		0.010	0.086
	Temp:IV		0.007	0.139
Wing aspect	Intercept	1.020		
	Temp		0.012	***<0.001***
	IV		0.000	0.612
	Temp:IV		0.003	0.109
Wetmass (g)	Intercept	-4.081		
	Temp		6.335	***<0.001***
	IV		7.224	***<0.001***
	Temp:IV		1.717	***<0.001***

Width and length are taken for wings. Wetmass is taken as body mass before dissection.

Temp = temperature, IV = ivermectin treatment.

The number of degrees of freedom is 1 for each trait.

The significant factors are highlighted in bold.

5.2 Individual traits

5.2.1 Traits comparison

All traits except wing aspect were largest in the category of lower temperature and no ivermectin treatment 19NO (Table 3). Wing width and length had the traits characteristics very similar with the highest values in 19NO. In width it continued with 19IV, 23IV and 23NO. In length it was 19IV, 23NO and 23IV. Wing aspect reacted differently with highest values in 19IV, then 19NO, 23NO and 23IV showing the lowest values. Wetmass were highest in 19NO, followed by 23NO, 19IV and then 23IV.

Table 3 Comparison of values within individual traits of males *Scatophaga stercoraria* reared under certain treatments combining temperature (19°C and 23°C) and ivermectin (presence and absence).

Trait	**Treatment**	**Min**	**Max**	**Mean**	**± SE**
Width (mm)	19IV	2.500	3.570	3.193	0.248
	19NO	2.760	3.610	3.268	0.208
	23IV	2.500	3.510	3.163	0.017
	23NO	2.390	3.500	3.132	0.198
Length(mm)	19IV	6.260	8.890	8.048	0.558
	19NO	7.000	8.920	8.199	0.464
	23IV	6.410	8.450	7.783	0.419
	23NO	6.610	8.580	7.782	0.379
Wing aspect	19IV	2.280	2.810	2.523	0.082
	19NO	2.280	2.780	2.515	0.079
	23IV	2.210	2.670	2.473	0.079
	23NO	2.350	2.800	2.495	0.088
Wetmass (g)	19IV	0.003	0.024	0.012	0.005
	19NO	0.003	0.028	0.019	0.006
	23IV	0.003	0.020	0.012	0.004
	23NO	0.002	0.025	0.013	0.006

Width and length are taken for wings. Wetmass is taken as body mass before dissection.
Numbers 19 and 23 indicate temperature, NO means non- ivermectin treatment whereas IV means treatment with ivermectin.

Graphs comparing the mean values for all the traits can be found as Appendix 3.

5.2.2 Wing width and length

The analyse not surprisingly showed positive correlation between wing length and width (Figure 4).

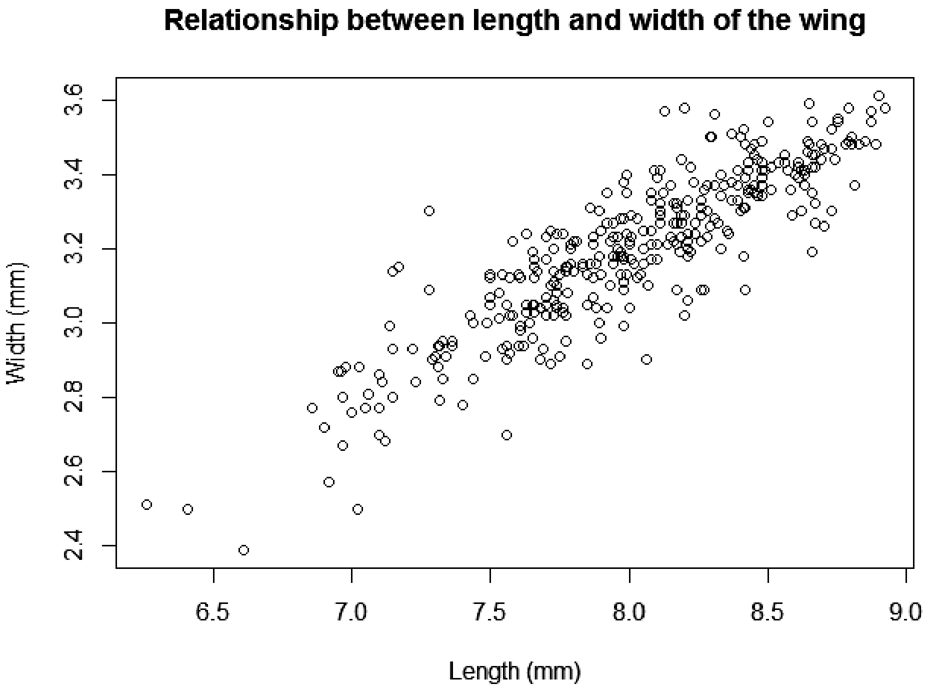

Figure 4 Positive correlaction between wing length and width.

When comparing individualy the effects of temperature and treatment, we can see just slight differences in the traits´ responses (Figure 5, 6).

In both width and length the medians showed higher values in low temperature treatment, while being highest in 19NO. Medians of treatments with higher temperature are on the same level regardless ivermectin treatment.

The lower temperature of 19°C regardless of ivermectin treatment brings in general higher values and broader data dispersion.

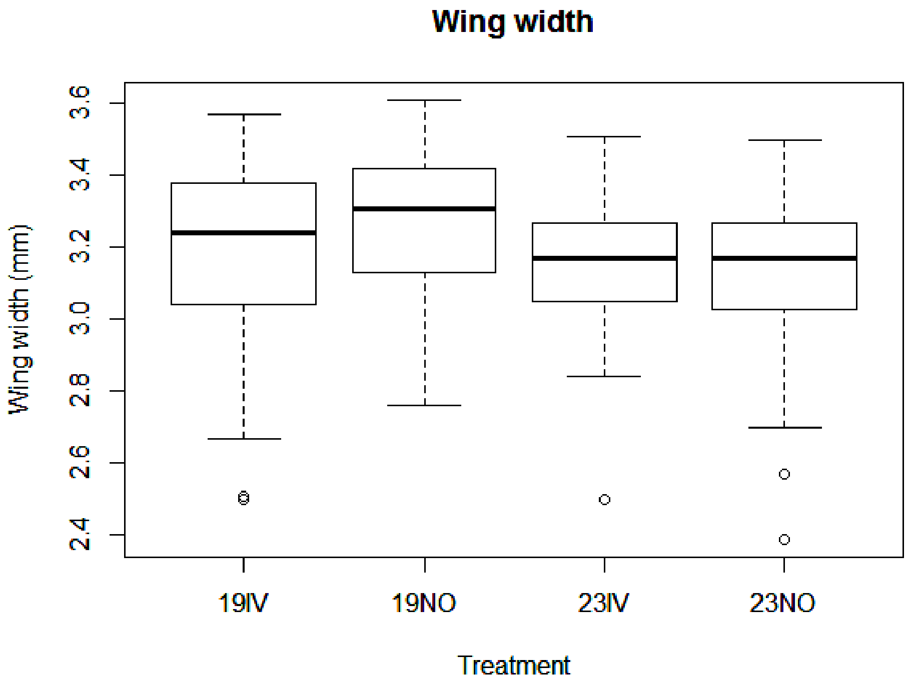

Figure 5 Comparison of width of the wing regarding individual treatments.

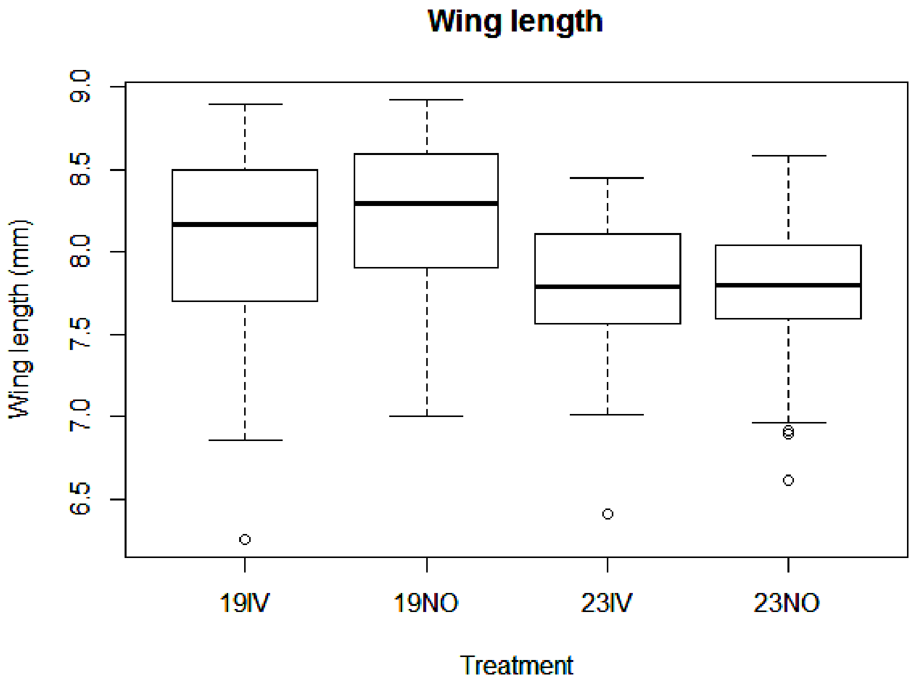

Figure 6 Comparison of length of the wing regarding individual treatments.

5.2.3 Wing aspect

The wing aspect showed the medians being more variable among all the treatment combinations. Still the values are higher in the lower 19°C temperature treatments, but the broadest variance in data is in 23NO treatment (Figure 7).

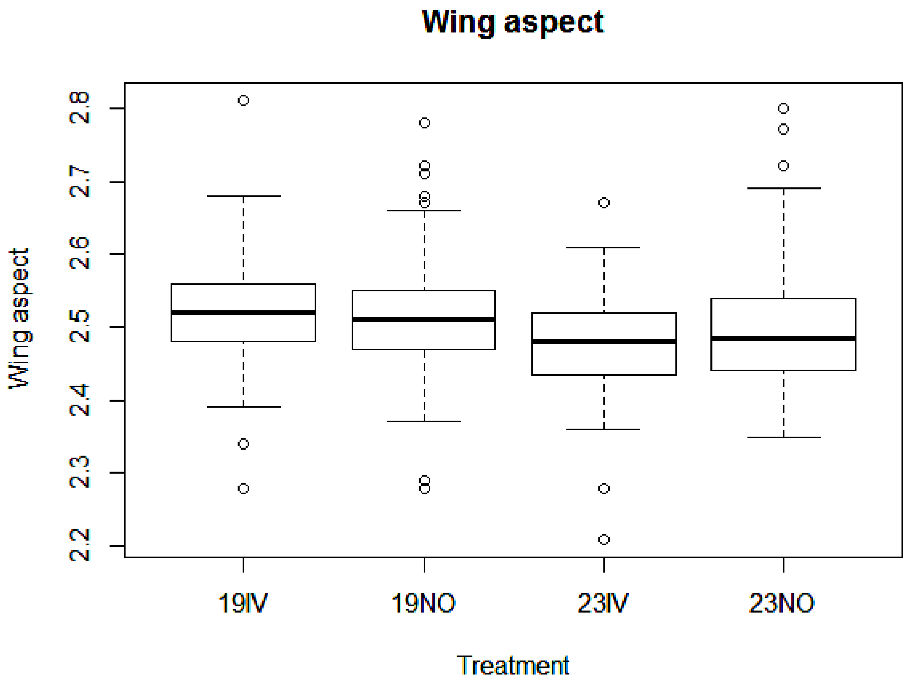

Figure 7 Comparison of wing aspect regarding individual treatments.

5.2.4 Wetmass

Compared to the previous traits, body wetmass of the individuals seems to be affected by both factors stronger (Figure 8). It showed the highest median value and also the broadest values distribution under 19NO treatment, followed by 23NO treatment. In contrast both treatments with ivermectin regardless temperature showed values with just a very slight difference.

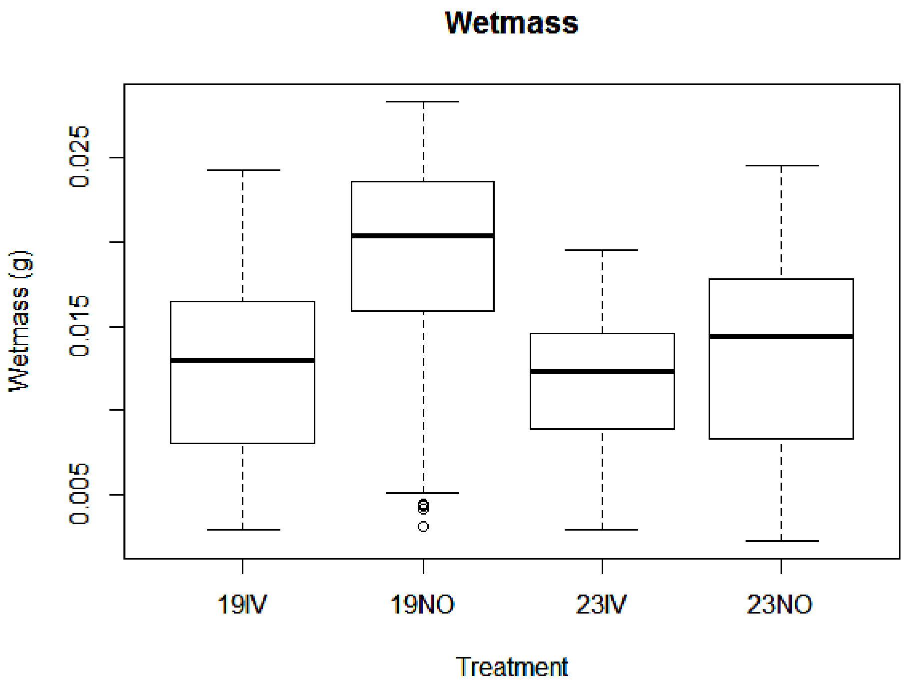

Figure 8 Comparison of body wetmass regarding individual treatments.

When plotting the temperature and ivermectin interactions in a different way, it showed the results about the body mass more clear. The flies always had lower body mass with ivermectin compared to non-ivermectin treatment, regardless of the temperature level. Nonetheless the values stayed more or less stabil despite the changed temperature, whereas in the non-ivermectin treatment the body mass showedsheer decline of individuals´ body mass in the direction from lower to higher temperature (Figure 9).

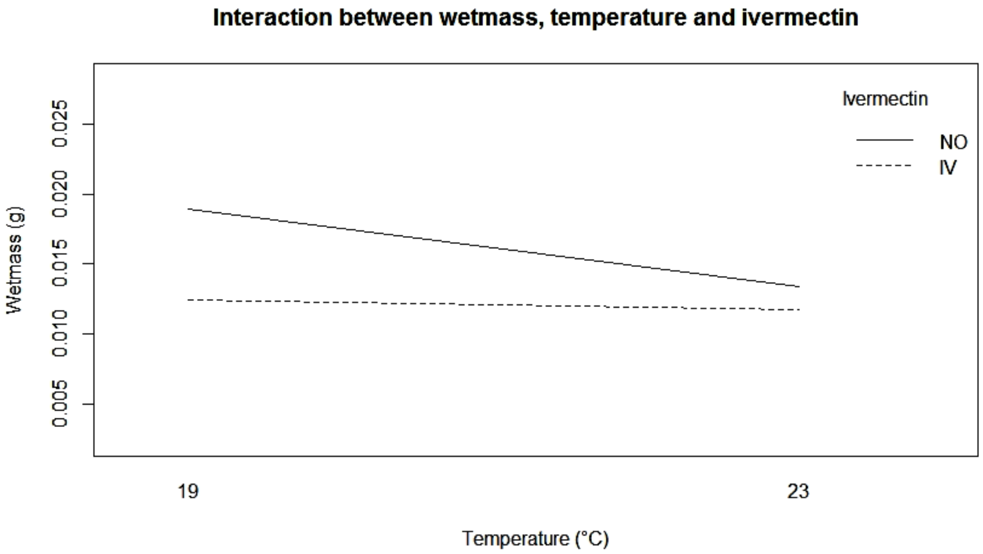

Figure 9 Comparison of temperature and ivermectin interaction effects on body wetmass.

6. Discussion

6.1 The effect of temperature and ivermectin interaction

The statistical significance of interaction between temperature and ivermectin treatment did not occur in all measured traits within the study. Nevertheless I agree with Soukup (2010), that statistical significance does not mean the same as biological importance. Therefore apart from these results some conclusions can be also made from results of individual measurements.

This particular study we can see confirmation of both statements about negative effect of stressful temperature (Blanckenhorn 1997) as well as ivermectin presence (Jochmann 2011) on the body size of yellow dung flies when the factors are separated. Although higher temperature increases both development time and growth rates, it usually results to smaller final size of the individual (Blanckenhorn 1997a). Presence of ivermectin generally increases development time and decreases body size (Jochmann 2011).

S. stercoraria is rather cold tolerant species (Blanckenhorn 1997), the growth rate is assumed maximal between 15°C and 20°C under constant laboratory conditions and the species seems to grow better under natural than laboratory conditions (Blanckenhorn 1997a). Some scientist mention that stressful for the species is temperatures above 25°C, which might be even lethal for certain life stages (Blanckenhorn 1997). According to Laugen et al. already 23°C is potentially stressful condition causing lower survival and lower female fertility. This is probably related to geographic variation in thermal adaptation, namely individuals from Central Europe can tolerate higher temperatures than those in Northern Europe (Laugen et al.-unpublished data). It has been shown in this study that even the temperature of 23°C can cause decrease of body size.

However it would have been desirable to set up more realistic temperature conditions for the experiment with fluctuating temperature with larger variance, which better corresponds to natural environment (Beasley et al. 2013). As Blanckenhorn (1997a) says individuals are able to optimise their body size according to the given temperature conditions due to adapting their development rate, for example they increase their growth rate when winter is coming. So there comes the question whether individuals treated in the lab can also expect and react on any for human unpredictable changes such like it would happen in the nature and conform to that or they are not anymore able to do that. But that is probably difficult to prove because one can never perfectly simulate the environmental conditions in the lab and field experiments in this way are not easy to maintain.

As it was showed in this study, ivermectin treatment was statistically significant only for body mass. It agrees with statements that body size declines when ivermectin is present (Blanckenhorn et al. 2013, Jochmann 2011). Nonetheless this trait has to be evaluated with caution because as Blanckenhorn et al. (2003) and Blanckenhorn and Hosken (2003) claim, wet weight in insect can be easily affected by water and food uptake and thus it is not recommended as very reliable size estimator. Characteristics concerning fitness or condition would have been neccessary to evaluate by different methods such like lipid or fat content. Despite it would have been desirable, it was not logistically possible within this project.

As it has been known for long time, the body size of adult insect is determined already during its larval stage since all the growth of individual is finished before metamorphosis (Nijhout 2003). Body size as well as size of body parts of an individual depend on both genetic and environmental factors (Nijhout 2003). Additionally, growth can also be affected by resource availability during development and in case of limited resources different tradeoffs among certain body parts might happen (Nijhout et Emlen 1998). This information can be probably applied also on the yellow dung flies and here comes the question whether flies used in this study could have experienced any kind of resource stress, which would force them to choose in which trait to invest more – to the whole body size, or to the wing size. Since they were raised up under predatory free laboratory conditions with enough food and dung storage, it seems to be probable, that they did not have to invest into any specific characteristics according to that. On the other hand because of the applied ivermectin treatment it can be seen that in wing parameteres the individuals had highest values in the treatment of lower temperature regardless ivermectin, whereas in the body mass the higher values occured firstly in the non-ivermectin treatments regardless of temperature. That might mean the flies prefer investing into the total body size than in different body parts when there is the impact of chemical compound. It can be in agreement with claiming of Blanckenhorn et al. (2003) that larger individual usually show more advantages in a question of sexual behavior and also foraging, meaning as the total body size and not only size of the wings.

The reason why the statistical significance occured only in wing width and not in wing length, although both these parameteres are presupposed being similar patterns, might be also explained by kind of trade-off. Despite the wing length and width are correlated, there might be some undiscovered trade-offs in growth of these two traits. Hypothetically if wing length would have been more important, then in case of for example limited resources it might cause some uneven growth of the wing proportions. Another reason could be the effect of possible measurements inaccuracy or quality of samples.

6.2 Individual traits

The results of this study show that individuals raised under lower temperature reached higher values in all traits concerning body size. It confirms the validity of temperature-size rule together with the known fact about *S. scatophaga* being rather cold tolerant species (Blanckenhorn et al. 2010). Negative effects of ivermectin´s presence on body size (Blanckenhorn et al. 2013) have also been proved here, it is best apparent on the mean values.

Wing aspect is counted as simple measure of wing shape and it is given when the ratio is higher, the wing is longer and narrower (Kjaersgaard et al. 2013). This study´s results regarding this trait show the highest mean values in the treatment of lower temperature but with ivermectin, which is difficult to explain. However the values for lower temperature and non-ivermectin treatment follow with the second highest values thus it can be generalized that higher temperature together regardless ivermectin has higher negative impact on this trait. These results confirm the previous investigation of Azevedo et al. (1998) on *Drosophila melanogaster* that higher wing aspect ratio is more likely under lower temperatures and it decreases when temperature during development increases. It is probably not based on genetic components and the reason is better flying possibilities for the individual (Azevedo et al. 1998).

For body mass the ivermectin influence can be considered as important factor, as it comes from results of this study, on the other hand this trait has to be handled carefully (Blanckenhorn et al. 2003). However temperature plays more fundamental role in this trait within this study, at least according to the results of the mean values where both higher and lower temperature show higher values when ivermectin is absent, whereas lower values occur in both temperature regimes with present ivermectin, as opposite to the wing responds. It might be caused by the fact that wing width and length are parameteres which do not react fast on environmental press and need some generations to evolve into different size or shape, whereas total body size is directly influenced by variable environmental factors during the individuals´ development (Blanckenhorn 1997, Blanckenhorn et Hosken 2003). Although many authors claim that large body size is advantageous for individuals (Blanckenhorn et al. 2003; Jann et al. 2000), it does not mean the individual is in good condition (Blanckenhorn et Hosken 2003).

As Blanckenhorn (1997a) claims, the effect of temperature on development and also on body size is sensitive to the way how it is expressed. The frequently cited formula that development time decreases with increasing temperature might not be a sufficient explanation anymore. It has been more frequently taken into consideration the degree-day model of individual´s development. That means amount of days at certain temperature which are above a certain development threshold (Blanckenhorn 1997a). This scheme might be suggested as useful for potential further studies within the topic of this project.

7. Conclusion

This study shows the negative impacts of temperature, ivermectin and in some cases even their interaction on traits related to body size in yellow dung flies. It shows there is higher importance of temperature than ivermectin in wing parameteres, whereas for the total body mass the absence or presence of ivermectin plays the most noted role. Body size is an important component of fitness (Blanckenhorn et al. 2003), which can be affected by various environmental factors (Blanckenhorn 1997) and correlated by temperature, development time (Blanckenhorn 1997a) and presence of chemical compounds (Jochmann 2011, Blanckenhorn et al. 2013) and interactions of all these factors (Laskowski et al. 2010).

Together with the known facts about dung fauna playing an irreplaceable role in all ecosystem´s functions (Madsen et al. 1990, Jochmann 2011) and how easily it can be affected by factors mentioned above, it should be priority for human to protect it. Some solutions how to decrease negative effect of ivermectin on the dung fauna might be start increasingly using less harmful preparations such like moxidectin (Blanckenhorn et al. 2013) or even natural products like herbs, and also to provide more exact tests of effects of pharmaceutical residuals (Jochmann 2011). Reorganization of pasture management would probably bring important changes as well. Providing information for lay public would be also desirable.

Wish for more precise information about this wide topic opens the door for further studies. From research concerning body size of organisms human could definitely obtain useful information about variable species and about environmental effects and impacts. Here just comes the question of good decision for certain projects, the reasons and possible contibutions of studies should be carefully evaluated in advance. But like in other life questions let us believe in the positive result of so called “Great turning”, respectively that we, humans, will soon learn how to become an improving element of life in the frame of the wide community on the planet Earth (Plotkin 2013).

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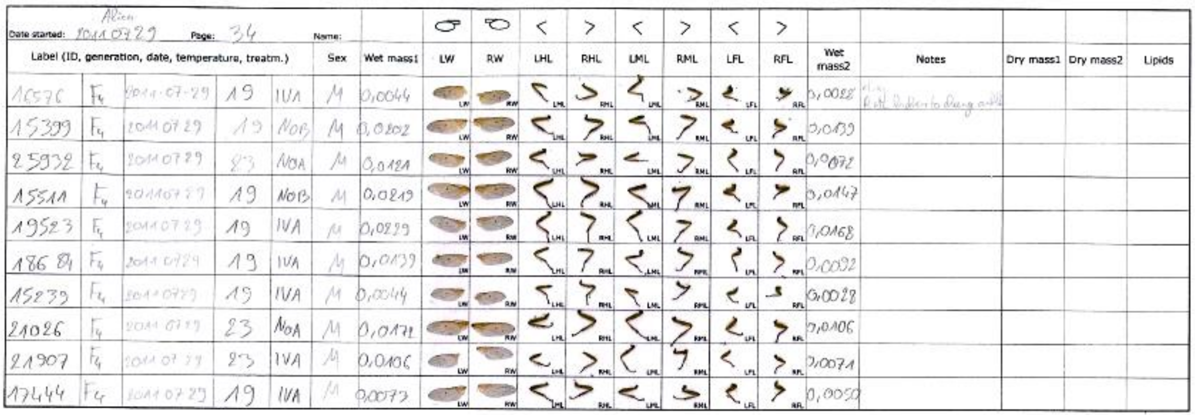
9. Appendices

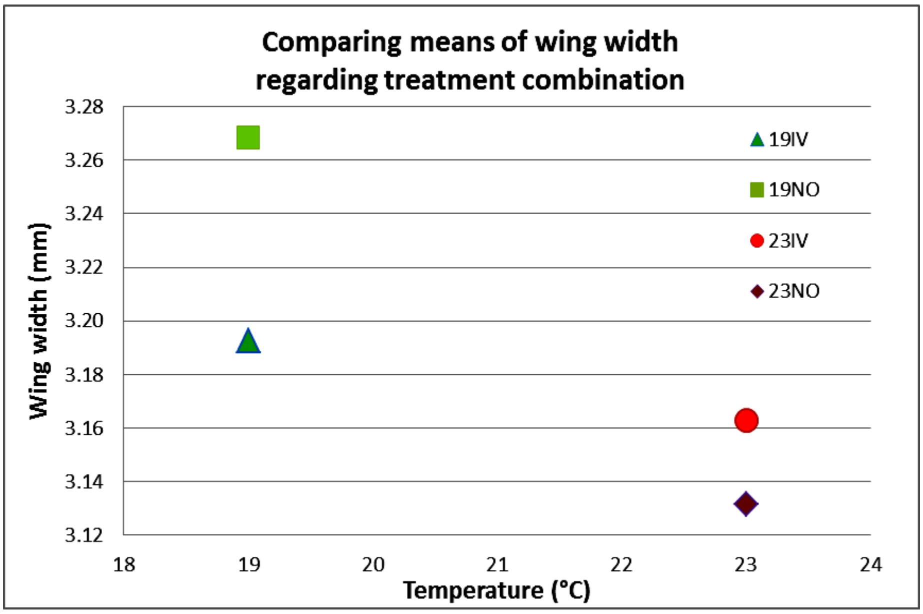
Appendix 1 Table with dissected parts of the individuals and information about the used type of treatment. (Photo: K. Beňová)

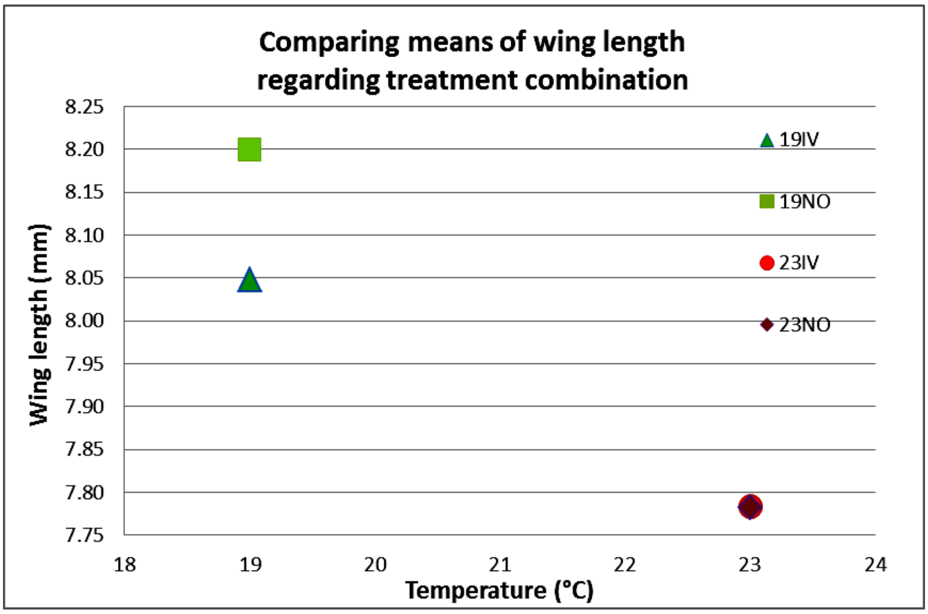


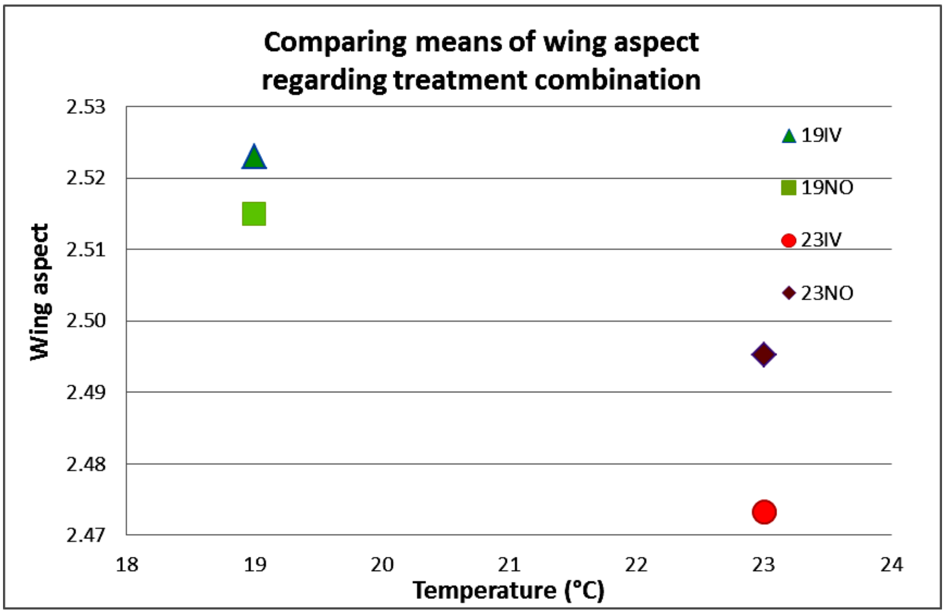
Alica Date started: 2011 07 29		Page: 34			Name:				<	>	<	>	<	>					
Label (ID, generation, date, temperature, treatm.)					Sex	Wet mass1	LW	RW	LHL	RHL	LML	RML	LFL	RFL	Wet mass2	Notes	Dry mass1	Dry mass2	Lipids
16576	F4	2011-07-29	19	IVA	M	0,0044	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0022	[illegible]			
15399	F4	2011 07 29	19	NoB	M	0,0202	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0139				
25932	F4	2011 07 29	23	NoA	M	0,0121	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0072				
15511	F4	2011 07 29	19	NoB	M	0,0219	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0147				
19523	F4	2011 07 29	19	IVA	M	0,0229	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0168				
186 84	F4	2011 07 29	19	IVA	M	0,0139	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0092				
15239	F4	2011 07 29	19	IVA	M	0,0044	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0028				
21026	F4	2011 07 29	23	NoA	M	0,0171	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0106				
21907	F4	2011 07 29	23	IVA	M	0,0106	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0071				
17444	F4	2011 07 29	19	IVA	M	0,0073	LW	RW	LHL	RHL	LML	RML	LFL	RFL	0,0050				

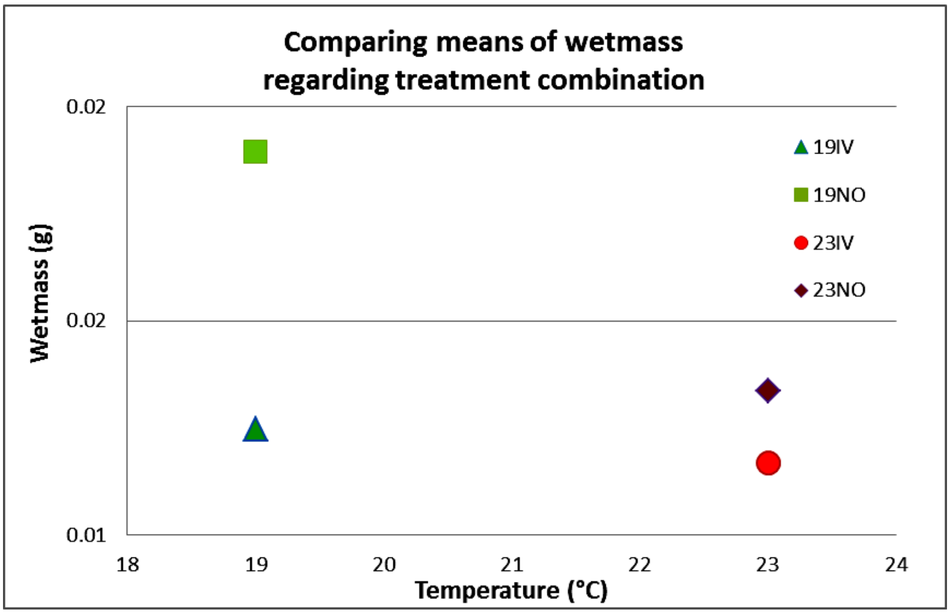
Appendix 2 Mating of *S. stercoraria* in polypropylene tube during field work. (Photo: A. T. Laugen)

Appendix 3 Four graphs comparing means of the four traits used in the study.






Comparing means of wing aspect regarding treatment combination
Wing aspect
2.53
2.52
2.51
2.50
2.49
2.48
2.47
18
19
20
21
22
23
24
Temperature (°C)
19IV
19NO
23IV
23NO


Comparing means of wetmass regarding treatment combination
Wetmass (g)
0.02
0.02
0.01
18
19
20
21
22
23
24
Temperature (°C)
19IV
19NO
23IV
23NO