CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF ENVIRONMENTAL SCIENCES

MASTER THESIS

Reflex bleeding as an anti-predation strategy in ladybirds (Coleoptera: Coccinellidae)

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

DIPLOMA THESIS ASSIGNMENT

Bishal Kumar Das

Nature Conservation

Thesis title

Reflex bleeding as an anti-predation strategy in ladybirds (Coleoptera)

Objective of thesis

The first aim of this diploma thesis will be to write a thorough literature review on the topic “reflex bleeding in insects”. Second part of the thesis will consist of following experiments: 1) investigation of reflex bleeding behaviour in various ladybird species; 2) investigation on of physiological costs of reflex bleeding.

Methodology

Literature review will be based on scientific papers searched in the Web of Knowledge database and relevant monographs (books). The review will be focused on reflex bleeding phenomenon in insects, with a chapter focused on this behaviour in ladybirds in more detail. Animals for the first experiment will be collected from the nature (various sites and habitat types) and transported to the laboratory. Then ladybirds will be reared for several days (provided by food and water) to standardize their physiological condition. In both experiments, we will simulate predator attack using standardized procedure to obtain hemolymph from ladybirds. Volume of hemolymph obtained during reflex bleeding will be measured. Differences between ladybird species and between sexes within species will be analyzed. The second experiment will be realized in cooperation with Dana Židlická. The experiment will be conducted using Harmonia axyridis as model species. Animals will be exposed to stressful (without food) and non-stressful conditions (food and water provided in surplus). Half of animals will be repeatedly forced to reflex bleeding (twice a week). At the end of the experiment, immune system (hemocyte concentration) of control animals and animals forced to reflex bleeding will be compared and modifying effect of stress (hunger) will be analyzed.

The proposed extent of the thesis

Ca. 50 pages + appendices as needed

Keywords

defense behaviour, insects, inter-species differences, ladybirds, reflex bleeding

Recommended information on sources

de Jong PW, Holloway GJ, Brakefield PM, de Vos H (1991): Chemical defence in ladybird beetles (Coccinellidae). II. Amount of reflex fluid, the alkaloid adaline and individual variation in defence in 2-spot ladybirds (Adalia bipunctata). Chemoecology 2: 15-19.

Hodek I, Honěk A, van Emden HF (2012): Ecology and Behaviour of the Ladybird Beetles (Coccinellidae). Chichester, UK: Willey-Blackwell.

Novgorodova TA (2015): Role of social and individual experience in interaction of the meadow ant Formica pratensis (Hymenoptera: Formicidae) with ladybird imagines and hoverfly larvae. Insect Science 22: 440-450.

Oliver TH, Jones I, Cook JM (2008): Avoidance responses of an aphidophagous ladybird, Adalia bipunctata, to aphid-tending ants. Ecological Entomology 33: 523-528.

Sato S, Kushibuchi K, Yasuda H (2009): Effect of reflex bleeding of a predatory ladybird beetle, Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae), as a means of avoiding intraguild predation on and its cost. Applied Entomology and Zoology 44: 203-206.

Suzuki N, Ide T (2008): The foraging behaviors of larvae of the ladybird beetle, Coccinella septempunctata L., (Coleoptera: Coccinellidae) towards ant-tended and non-ant-tended aphids. Ecological Research 23: 371–378.

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The Diploma Thesis Supervisor

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Prague on 13. 04. 2018

Declaration:

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

Prague, Date - 18/04/2018

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Bishal Kumar Das

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Abstract

Reflex bleeding or auto-haemorrhaging is one of the many defence mechanism against predator found in insects and in some reptiles. This behaviour is reported widely in several insect taxa, including ladybirds. However, there is a lack of interspecific comparative studies as well as intraspecific comparisons of reflex bleeding reflex behaviour in insects. In this study, reflex bleeding behaviour among several ladybird species was compared by measuring reflex blood volume released while auto-haemorrhaging. Intraspecific effects of sex, live weight and body size on the relative amount of reflex blood were also investigated. The physiological costs associated with the reflex bleeding behaviour were addressed by comparing haemocyte concentration per microliter of haemolymph in control individuals and these forced to repeated reflex bleeding. The significant difference was observed in the reflex bleeding behaviour among the studied ladybird species. Effects of sex and live weight on relative amount of reflex blood released were insignificant, but effect of structural body size was found to be significantly positive. This suggests the efficiency of reflex bleeding behaviour could be higher in species with large individuals. The loss in haemocytes due to repeated reflex bleeding illustrates possible physiological costs of reflex bleeding and probably trade-off associated with reflex bleeding behaviour exists, i.e. ladybirds have to select between actual defence against predators and future efficiency of their immune system.

Keywords: Reflex bleeding, auto-haemorrhaging, ladybird, physiological costs, coccinellidae, interspecific comparison, *Anatis ocellata*, *Calvia quatuordecimguttata*, *Ceratomigella undecimnotata*, *Coccinella magnifica*, *Coccinella quinquepunctata*, *Coccinella septempunctata, Coccinula quatuordecimpustulata, Exochomus quadripustulatus, Harmonia axyridis, Propylea quatuordecimpunctata*

Abstrakt

Reflexní krvácení je jedním z mnoha obranných mechanismů využívaných hmyzem proti predátorům. Toto chování je široce rozšířeno u několika skupin hmyzu, včetně slunéček. Existuje však jen omezené množství studií porovnávajících toto chování mezi různými druhy dané skupiny či mezi jedinci uvnitř vybraného druhu. Navíc téměř chybí informace o možné nákladnosti používání takové obrany proti predátorům. V této studii porovnávám ochotu reflexně krvácet pro několik druhů slunéček pomocí dráždění jedinců entomologickým špendlíkem, což má simulovat útok predátora. Zkoumám i vnitrodruhové rozdíly, konkrétně vliv pohlaví, živé hmotnosti a velikosti těla na relativní množství hemolymfy vykrvácené během reflexního krvácení. Fyziologické náklady spojené s reflexním krvácením zkoumám pomocí porovnání koncentrací hemocytů v hemolymfě kontrolních slunéček s jedinci, kteří byli opakovaně nuceni reflexně krvácet. Během studie jsem odhalil významné rozdíly v ochotě reflexně krvácet mezi druhy. Vliv pohlaví a živé hmotnosti jedince na relativní objem vykrvácené hemolymfy nebyl průkazný, zato však strukturní velikost těla průkazně pozitivně korelovala s objemem vykrvácené hemolymfy. Je proto možné, že účinnost reflexního krvácení je vyšší u druhů s větší velikostí těla. Dlouhodobá ztráta hemocytů v důsledku opakovaného reflexního naznačuje existenci značných fyziologických nákladů spojených s reflexním krvácením a tudíž naznačuje existenci trade-off mezi aktuálním obranným chováním slunéček a budoucím fungováním jejich imunitního systému.

Klíčová slova: Reflex bleeding, auto-haemorrhaging, ladybird, physiological costs, coccinellidae, interspecific comparison, *Anatis ocellata, Calvia quatuordecimguttata, Ceratomigella undecimnotata, Coccinella magnifica, Coccinella quinquepunctata, Coccinella septempunctata, Coccinula quatuordecimpustulata, Exochomus quadripustulatus, Harmonia axyridis, Propylea quatuordecimpunctata*

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1. Introduction

Reflex bleeding is an effective defensive behaviour that is widely distributed among insects, as reflex blood consists of toxic alkaloids and provide physical defence due to high coagulation and sticky nature (Hodek *et al*, 2012; Zvereva and Kozlov, 2016). When insects are attacked by the predator, ejection of haemolymph occurs mainly through tibia-femoral articulation in adults or exuding through the intersegmental membranes in larvae (Happ and Eisner, 1961; Bateman and Fleming, 2009). In Plecoptera, the expulsion of haemolymph to some distance of few inches occurs as a reflex bleeding mechanism (Benfield, 1974). Lampyridae reflex bleed from protonal and elytral margins, and also from region around the antennal sockets (Fu *et al.*, 2006). Reflex bleeding is also one of the defence mechanism in some vertebrates, like horn lizards and snakes (Sherbrooke and Middendorf III, 2001; Iiftime and Iftime, 2014). In family Coccinellidae reflex bleeding is a very prominent defence mechanism, occurring in many ladybird species (Hodek *et al.*, 2012).

Reflex bleeding is an effective defence against various predators. e.g. ants, carnivorous beetles, spiders, lizards, small mammals and birds (Blum and Sannasi, 1974; Matsuda, 1982; Nachappa *et al.*, 2006). Reflex bleeding is also reported to provide effective protection against intraguild predation in ladybirds (Sato *et al.*, 2009). Mechanism of reflex bleeding and its occurrence in the insects is well investigated. But, there is general lack of the studies addressing interspecific differences in relation to reflex bleeding. Comparative studies are much required for the deep understanding of effectiveness and efficiency of reflex bleeding behaviour varying between the several species. In the present work, such study was performed by comparing the volume of haemolymph released by the ladybirds and intraspecific effects of sex, size and body weight on relative reflex blood is also analysed. As a second part of the study, physiological costs are also investigated by comparing control and individuals undergone several reflex bleeding sessions. As haemolymph consisting of haemocytes, alkaloid, protein etc., making the reflex bleeding a highly energetically costing defence mechanism. Possible difference between species can shed a light on the species with greater anti-predation defence.

2. Purpose and aims of study

The thesis is consisting of two major parts.

First part covers literature review about reflex bleeding behaviour in the animal kingdom with ladybirds being at the focus. The source of information mainly focused on scientific research article and books published on reflex bleeding behaviour.

The second part is consisting of following experiments: 1) Investigation of reflex bleeding behaviour in several ladybird species and analysing the effects of sex, size and body weight on the amount of haemolymph devoted towards reflex bleeding; 2) determination of physiological cost existing due to the reflex bleeding behaviour in ladybird beetles.

Haemolymph volume obtained from different ladybird species reflects their strength of defensive mechanism. Factors like size, body weight and sex may be affecting the reflex bleeding in ladybirds. It is hypothesised that species with a larger body size will be able to perform reflex bleeding behaviour more effectively than the species having smaller body size. The loss in haemocytes due to reflex bleeding can indicate the trade-off bear by the ladybirds, which can possibly affect the immunity and loss of valuable substances.

3. Review of literature

3.1 Reflex Bleeding

Ejection of fluid (blood or mixture of blood with glandular secretion) as a means of defence from the body of the animal in response to any kind of threat or molestation is considered as reflex bleeding or auto-haemorrhaging. Lacordaire (1838) being one of the first investigators to mention the phenomenon of ejection of liquid from different parts of the body of certain insects. Emittion of milky and fetid fluid was observed in *Dytiscus* sp. and *Gyrinus* sp., from articulation between the head, thorax and abdomen. Similarly, *Meloe* sp. are found to emit a yellowish-orange liquid from the articulations of the legs. The odour of the fluid is not disagreeable. But, an analogous liquid consisting of the different and strong odour was observed by him in Coccinellidae and Chrysomelidae. This emitting of liquid isn't termed as reflex bleeding or auto-haemorrhaging in his work. An investigation by Cuenot (1894), describes the behaviour of emitting defensive fluid in genera *Timarcha, Adimonia, Coccinella* and *Meloe* by using the term "reflex bleeding" for the very first time. Upon touching, all these beetles fall to the ground, feign death and perform reflex bleeding. The reflex discharge is yellowish or reddish in colour in this groups of species. The discharged fluid from *Cocccinella* has a strong and very unpleasant odour, and that of *Timarcha* is odourless, but very persistent and astringent taste. In the early 1900s, Charles Hollande termed the phenomenon of emitting defensive fluid as "auto-haemorrhage" (Stocks, 2008). Part of the terminological problem is due to simply the vast diversity of behaviours and physiologies present in the insects. As in some species, like in chrysomelids, it may involve only glanduliferous products, a mixture of glandular products and haemolymph, or a genuine release of haemolymph through a deliberately made tear in the cuticle (Stocks, 2008). A summarized investigation of reflex bleeding in Coleoptera and Orthoptera is described by Cuenot (1896). *Timarcha, Galeruca, Megalopus*, coccinellids and meloids among the Coleoptera, and *Eugaster* and *Ephippiger* among the Orthoptera perform the reflex bleeding behaviour. Many kinds of literature state the importance of reflex bleeding as an anti-predator strategy or a defence mechanism against natural enemies. It serves both, as physical (Blum and Sannasi, 1974) and chemical (Smedley *et al.*, 2017) defence (consists deterrent and unpalatable/toxic substances) against predators and natural enemies in invertebrates, but also found to be a considerable defence strategy in some vertebrates. According to McIndoo (1916), it also aids some beetles in recognizing the different individuals and sexes of the same species.

3.1.1 *Reflex bleeding in insects*

Reflex bleeding behaviour in invertebrates is highly studied in insects. Auto-haemorrhaging or reflex bleeding is predominantly found in order Coleoptera, Hemiptera, Plecoptera and Orthoptera. In Lepidoptera, larvae or caterpillar of *Heliconius himera* of family Nymphalidae, is known to perform reflex bleeding. But this kind of reflex discharge cannot be considered as true reflex bleeding, as it is mediated by a baculovirus. Viral genes encoding chitinases and proteases that are transcribed by the host – causing the integument to rupture. When infected larvae are provided with mechanical stimulation, it exudes fluid through the spines on the back of the larvae. This phenomenon is not observed in healthy larvae of *Heliconius himera* (Hay-Roe *et al.*, 2003). In Plecoptera, stoneflies *Pteronarcys proteus* and *Peltoperla maria* are known to perform reflex bleeding at the coxal and tibio-femoral joints (Benfield, 1974). In *Pteronarcys proteus*, the auto-haemorrhaging behaviour is usually accompanied by a popping or explosion sound. Peck (2000) reported bleeding in froghoppers (Hemiptera: *Prosapia* sp.) from the pre-tarsal pad of the legs. This bleeding behaviour is restricted to adults, nymphs of *Prosapia* sp. do not show reflex bleeding. As mentioned above, grasshoppers like *Eugaster* and *Ephippiger* among the Orthoptera are also known to perform reflex bleeding (Cuenot, 1896). Also, another orthopteran of family Tettigoniidae (*Acanthoplus discoidalis*) is reported to perform reflex bleeding (Bateman and Fleming, 2009). About 86% of *Acanthoplus discoidalis* individuals in the study responded to the stimulation by auto-haemorrhaging with nearly 20 mg of haemolymph, whereas average body mass of males is 5.6 ± 0.8 g and of females is 7.0 ± 0.6 g. The reflex bleeding behaviour in Coleopterans is a very much widely observed phenomenon.

As this behaviour is reported in Chrysomelidae, Coccinellidae, Erotylidae, Lampyridae and Meloidae (Blum and Sannasi, 1974; Carrel and Eisner, 1974; Drilling and Dettner, 2010). Reflex bleeding is found in all the 3 instars of cucumber beetle, *Diabrotica unidecimpunctata* and *Diabrotica baltcata* at intersegmental membranes of head and abdomen (Wallace and Blum, 1971). Adults and pupae are devoid of reflex bleeding behaviour. Larvae of *Gallerucida, nigromaculata* is also known to perform reflex bleeding from the sides of the thoracic or abdominal segments (Matsuda, 1982). A mixture of haemolymph and exocrine glandular secretion is also observed in larvae of the chrysomelid beetle *Agelastica alni* (L.) (Galerucinae, Sermylini). Most of the studies in Chrysomelidae regarding reflex bleeding suggests an absence of this behaviour in adults. Also, the reflex bleeding may or may not be accompanied by endocrine secretion in Chrysomelidae. In Erotylidae, the reflex bleeding consists of both glandular secretions and reflex blood and is observed from the abdominal tip of *Tritoma bipustulata* (Drilling and Dettner, 2010). Adults of Lampryridae, *Pyrocoelia pectoralis* and *Photinus pyralis* show reflex bleeding from protonal and elytral margin (Blum and Sannasi, 1974; Fu *et al.*, 2006), whereas Meloidae show very localized reflex bleeding from the articulation of the legs (Carrel and Eisner, 1974).

3.1.2 *Reflex bleeding in Coccinellidae*

Reflex bleeding or auto-haemorrhaging is a very prominent feature of ladybirds. Many of the studies state its existence in both adults and larvae. Due to this usual characteristic of ladybirds, McIndoo (1916) mentioned reflex bleeding as the ejection of liquid from the tibia-femoral articulation. As almost all ladybirds known to perform reflex bleeding in a similar way. Many species like, *Adalia bipunctata*, *Coccinella septumpuctata*, *Coccinella magnifica, Diomus thoracicus, Epilachna borealis, Epi1achna varivestis* and *Harmonia axyridis* are thoroughly studied in respect to their reflex bleeding behaviour (McIndoo, 1916; Happ and Eisner, 1961; de Jong *et al.*, 1991; Holloway *et al.*, 1991; Grill and Moore, 1998; Sloggett and Majerus, 2003; Roux *et al.*, 2017). Mainly in ladybirds auto-haemorrhaging serves as defensive arsenal against ants. Preying on ant-tended aphid leads the ladybirds towards ant’s aggressiveness, from which reflex bleeding protect ladybirds and minimize the deadly attack of ants on them (Sloggett and Majerus, 2003; Roux *et al.*, 2017). Apart from usual reflex bleeding from tibia-femoral articulation, when threatened or attacked by the predator, larvae of *Epi1achna varivestis* emits haemolymph from the spines covering their body, which breaks due to its brittle structure (Happ and Eisner, 1961). Holloway et al. (1991) found females to emit more reflex fluid than males due to their larger size. Reflex bleeding also depends on ladybird diet and continuous availability of their food, as for *Adalia bipunctata* 3 days of ad libitum food supply can make it able enough for normal reflex bleeding (de Jong *et al.*, 1991).

3.1.3 *Reflex bleeding in vertebrates*

Although the phenomenon of reflex bleeding or auto-haemorrhaging is quite evident in invertebrates, some vertebrates, especially some snakes and lizards bear reflex bleeding as an anti-predator strategy in their defence arsenal. Members of subfamily Phrynosomatinae of family Iguanidae are explored mainly among the lizards with respect to reflex bleeding. Even in Phrynosomatinae, this behaviour is reported in only two genera, *Phrynosoma* (Sherbrooke and Middendorf III, 2001) and *Sceloporus* (Sherbrooke, 2000). Middendorf III and Sherbrooke (1992) reported auto-haemorrhaging for the first time in vertebrates in lizard *Phrynosoma cornutum*. *Phrynosoma cornutum* reported squirting blood by increasing blood pressure and rupturing vessels around the eyelids. Similarly, *Sceloporus Jarrovvi* is reported to expel blood from the vascular system due to increased pressure in the sinus orbitalis and ruptures of one or more capillaries (Sherbrooke, 2000). A comparative study is conducted by Sherbrooke and Middendorf III (2001) to identify the presence of reflex bleeding behaviour in genus *Phrynosoma*. The reflex bleeding or blood squirting response rate against dogs for species tested are: *P. mcallii* (0%), *P. modestum* (0%) *P. platyrhinos* (0%), versus *P. cornutum* (70-100%), *P. hernandesi* (50%), *P. solare* (60%; three of five lizards in dog trials). *P. ditmarsi* and *P. asio* are also known to perform reflex bleeding (Hodges, 2004). The squirting of blood helps the lizard to defend itself against – dogs, kit fox (*Vulpes macrotis*), greater roadrunners (*Geococcyx californianus*)

(Sherbrooke, 1990) or southern grasshopper mice (*Onychomys torridus*) (Sherbrooke, 1991). Aesculapian Snake *Zamenis longissimu* is known to perform reflex bleeding from lips and nostrils, which is always followed by death feigning behaviour. The snake completely relaxes its muscular tonus (Iiftime and Iftime, 2014). Emission of blood does not appear to weaken the specimen. Smith et al. (1993) summarized the presence of auto-haemorrhaging in *Tropidophis haetianus*, *Rhinocheilus lecontei*, *Heterodon platyrhinos*, and *Nerodia erythrogaste*. Though, reflex bleeding in snakes is not explored as much as lizards, but considerable enough to recognize its existence among them. But, there is a general lack of study in vertebrates, in association with reflex bleeding behaviour.

3.2 Bleeding behaviour

The bleeding behaviour of many animal species while performing reflex bleeding or auto-haemorrhaging varies to a greater extent. It may be a generalized behaviour for some groups of species or it can be species specific. Most of the invertebrates are known to emit reflex fluid; from the articulations of the leg in adults and from the intersegmental membranes in larvae. Whereas, vertebrates are known to squirt their blood from their nostrils, mouth or eyelids by building pressure and rupturing capillaries or vessels (Middendorf III, Sherbrooke and Braun, 2001; Iiftime and Iftime, 2014).

It is a very well-known fact among the entomologists when disturbed or threatened, beetles of family Coccinellidae cover themselves by folding their legs and antennae under elytra and exude haemolymph from the articulations of tibia and femur (McIndoo, 1916). The haemolymph that exudes out is usually yellow-reddish in colour and with high coagulating ability. It’s usually viscous in nature. Many beetles also feign death after they are exhausted and devoid of reflex blood. Normally beetles eject small droplets of reflex blood from tibia-femoral articulation in nature, when slightly irritated. Response to the stimulation is generally received by the tactile hairs. As soon as these tactile hairs receive the sensation, there is contraction of muscles folding the tibia against the femur and forcing the blood into the chamber containing the gland cells. At once the gland cells undergoes a high blood pressure which immediately stimulates them. The haemolymph or reflex discharge usually passes through hypodermal pores inside and around (proximal end of the tibia and the distal end of the femur) the articular membrane (figure.1). Pores at the proximal end of the tibia and distal end of the femur are generally connected with the reservoir, but the pores at articular membranes do not consist of any reservoir. Lutz (1895) asserts that in the Coccinellidae, blood coming from the distal end of the femur issues through a slit on either side of the articular membrane which surrounds the chitinous rods (Selane) to which the extensors of the tibia are attached. The blood exudes by a forced contraction of the abdomen and by the flexors of the tibia, and it is a voluntary act. McIndoo (1916) also described that a hind leg was able to eject as many as six drops of defensive fluid with only a few seconds of gap between each successive drop. A middle or front leg was not able to eject quite so many drops. The exact above-mentioned location of liquid is confirmed by observing under a microscope. *Epilachna borealis* is used in the study by McIndoo (1916) to observe the reflex bleeding behaviour, but this reflex bleeding mechanism is typical for many ladybird beetles with some slight variations. Happ and Eisner (1961) also confirmed the same kind of reflex bleeding behaviour in adults of *Epilachna varivestis*. They reported reflex bleeding behaviour in *Epilanchna varivestis* larvae from the intersegmental membrane of the body. The body of the larvae is covered with hollow brittle spines, that breaks upon attack by the predators or natural enemies. The breaking of these spines ejects defence fluid on the back of the larval body. This might not be a voluntary reflex bleeding behaviour, but many other ladybird larvae like *Coccinella magnifica* and *Harmonia axyridis* show intersegmental ejection of defence fluid (Grill and Moore, 1998; Sloggett and Majerus, 2003). Larvae of *Diomus thoracicus* bear special structures to perform reflex bleeding. When irritated or attacked by predators (e.g. ants), transparent droplet appears at the tip of the two thoracic tubercles as well as on the surface of the two abdominal humps accompanied by an anterior-posterior bending movement (figure. 2). They also respond by flattening themselves, which makes them difficult to be grabbed by ants (Roux *et al.*, 2017). Lateral bulges and flat ventral faces make it difficult for the ants to have a hold on the larvae. The reflex fluid emitted by *Diomus thoracicus* coagulates quickly, entangling the ant’s mouthparts. In spite of having a special structure for reflex bleeding, an association of thoracic tubercles and abdominal humps with glandular structure is absent.

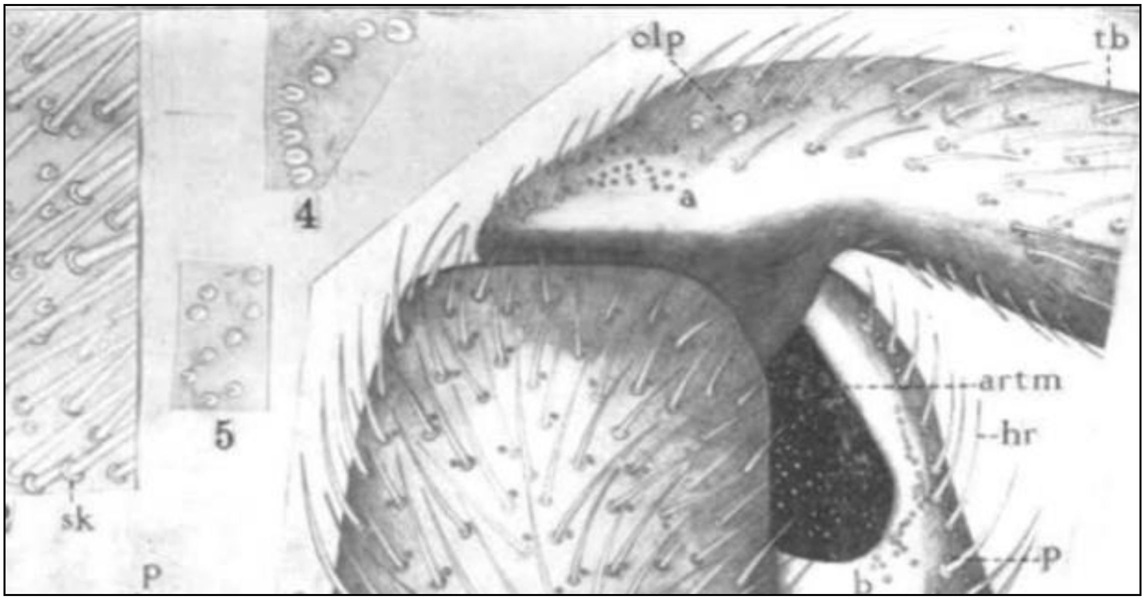

Figure 1. Representing hypodermal pores inside and around the articular membrane between tibia and femur of *Epilachna borealis*. “artm” = articular membrane; “tb” = tibia; “hr” = hairs; “p” pores; “olp” = olfactory pores (McIndoo, 1916).

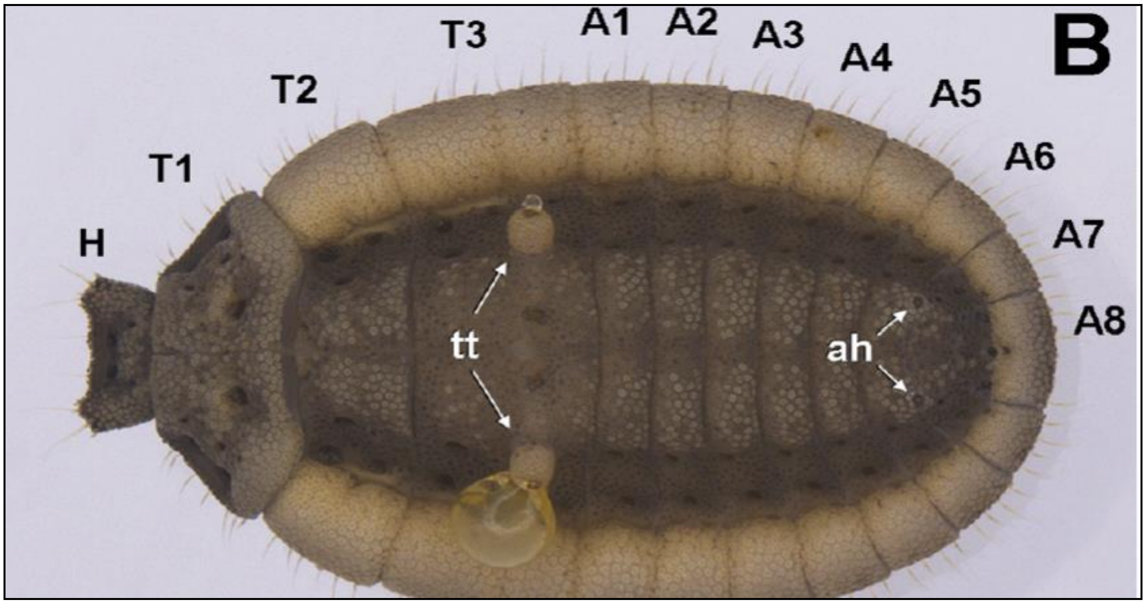

Figure 2. In vivo dorsal view of larva of *Diomus thoracicus* after gentle prodding, causing the emission of fresh droplets by the thoracic tubercles. Whereas "tt" representing thoracic tubercles and "ah" representing abdominal hump. H = head, T = Thorax and A = abdomen (Roux et al., 2017).

Like Coccinellidae, blister beetles (Meloidae) also perform much localized reflex bleeding, especially from legs (Carrel and Eisner, 1974). Each individual leg can selectively bleed. This is an adaptive specialization against small predators rather than large one. When beetles are attacked by ants they show localized bleeding until the number of predators is high enough. It performs generalized bleeding from all the legs when overwhelmed completely by the predators. Also feigned death until ants dragged it up to a distance and abandoned, by thinking of it as a corpse. *Pteronarcys proteus* (Plecoptera: Pteronarcyidae) is also known to perform similarly localized auto-haemorrhaging (Benfield, 1974). Droplets were observed to carry up to 10 inches from the animal and their expulsion is accompanied by a very audible and rather startling "popping" sound. Benfield (1974) also observed the same reflex bleeding behaviour in another species of stoneflies, *Peltoperla maria*, but without popping sound. Unlike ladybird and blister beetles, fireflies (Lampyridae) show reflex bleeding from elytral and pronotal margins and the region around the antenna1 sockets (Blum and Sannasi, 1974; Fu *et al.*, 2006). Blood is discharged at points closest to the source of tactile stimulation and, unless the insect is subjected to repeated stimuli, simultaneous bleeding from several sites does not occur. Phase-contrast microscopy of the pronotal and elytral margins demonstrates that these regions contain a series of irregular depressions which constitute the reflex bleeding sites. An examination of the depressions by scanning electron microscopy reveals that they are relatively shallow and under laid by a continuous layer of uninterrupted cuticle. Reflex bleeding results when this relatively weak membrane of cuticle ruptures as a result of increased hydrostatic pressure (Blum and Sannasi, 1974). Fu et al. (2006) observed reflex discharge also from the anus in both male and female firefly adults several times. Larvae of many insect taxa show common intersegmental reflex bleeding behaviour. Some Chrysomelidae larvae show a slight variation in the position of reflex discharge. *Diabrotica* larvae auto-haemorrhage from intersegmental membranes between the head and the 1st segment and between the penultimate and last abdominal segments (Wallace and Blum, 1971), whereas *Gallerucida, nigromaculata* auto-haemorrhage from either side of the thoracic and abdominal segment (Matsuda, 1982). Chrysomelids may or may not be consist of defensive glands. Presence of glands makes more of a glandular secretion than reflex bleeding (Wallace and Blum, 1971; Matsuda, 1982). Due to the presence of such complexity in insects Izquierdo (1896) categorized liquids discharged by insects as a means of defence into three groups: (1) those from organs which are furnished with glands. Such organs are found in all the families of insects and their exits may be found in the thorax, abdomen, at the anus or in the last portion of the intestine; (2) liquids which are discharged from the tibia-femoral articulations; and (3) liquids that are discharged from the mouth. Such variation in reflex bleeding behaviour among species may be because of their evolution towards specific predator or group of predators.

3.3 Nature of reflex fluid

Many studies questioned and investigated the true nature of reflex blood. Whether it is true blood or glandular secretion. Two important questions are mentioned by McIndoo (1916) in relation to nature of reflex blood: "*If the reflex blood is true blood or haemolymph, then how it's able to exude out of the vascular system of the body*?" And, "*if it's glandular secretion, then how it's possible to release such amount of secretion in short period of time*?" Porta (1903) mentioned that the discharge of the secretion from *Coccinella*, *Timarcha* and *Meloe* spp. is caused by a reflex bleeding phenomenon brought about by any stimulation. The liquid is secreted by a glandular follicle in the reticulum formed from the fibres of connective tissue, situated in the wall of the middle intestine. The liquid has an acrid reaction on predators, and it is perhaps only a bile secretion. Porta (1903) provided three reasons why this liquid is not blood: (1) It is unacceptable that insects should constantly pass such an important fluid; (2) after a prolonged excitation the liquid ceases to exude; and (3) it has an acrid reaction while we know that blood in all animals has an alkaline reaction. He fails to explain how this secretion reaches the exterior from where it is produced. Berlese (1909) mentions that the discharged fluid from *Meloe* spp. is a combination of both blood and hypodermal secretion. In his work, he also mentions unicellular glands lying just beneath the hypodermis on both sides of the articulation on the leg.

McIndoo (1916) studied the cross-sections of the extreme proximal end of the tibia and distal end of the femur in *Epilachna borealis*. The lumen in the extreme proximal end of the tibia and extreme distal end of the femur is divided into two almost equal chambers extending across the leg. One chamber containing the nerve (n) and trachea (tr) lie only a few scattered gland cells and with a small amount of blood in it. The other chamber is full of blood and it is the one that contains all the gland cells (glc) belonging to the two groups of pores found on the tibia (figure.3). No muscles are seen in the tibia at this place. Liquid from reflex bleeding is compared with the blood in the wings (which are free from hypodermal glands). It is found to be more viscid and slowly dissolving in the water than the haemolymph from wings. All these evidences confirms a combination of both glandular secretion and haemolymph discharge in *Epilachna borealis*. Study on *Agelastica alni* larvae reported the presence of exocrine glandular cells which are connected to cuticle sacs located close to the spiracle at the abdominal segments 1–8. The fluid discharged from these tubercle openings contains haemolymph and most probably also glandular secretion (Bunnige and Hilker, 1999). In contrary to McIndoo (1916), there are many studies stating that reflex fluid is similar to blood or haemolymph. Cellular features were found to be same as haemolymph in some reflex blood (Happ and Eisner, 1961; Benfield, 1974). Many demonstrated by amino acid analysis that discharged fluid from reflex bleeding and blood collected had identical compositions (Blum and Sannasi, 1974; Matsuda, 1982). Cells in the reflex blood of *Adalia bipunctata* and *Coccinella septempunctata* ladybirds are consistently seen at similar levels in all reflex blood samples examined (Karystinou, Thomas and Roy, 2004). Blood samples taken by puncturing and reflex bleeding in adults of *Harmonia axyridis*, *Coccinella septempunctata* and *Ceratomegilla undecimnotata*, mentioned no significant difference in haemocyte number (Knapp *et al.*, 2018). Both the sampling methods suggest that the content of reflex blood is the same as haemolymph. The fluids discharged from different parts of the body, like the pronotal and elytral margins, and around the antennal sockets as well as the anus, were rich in the same kinds of haemocytes seen in samples of the haemolymph in *Pyrocoelia pectoralis* (Fu *et al.*, 2006). True nature of reflex blood can vary from species to species, with or without glandular secretion, but always haemolymph is found to be contributing to a greater extent. Presence of haemocytes demonstrated by many studies make it quite evident that reflex blood is actually consisting of blood or haemolymph, but sometimes with slight variations.

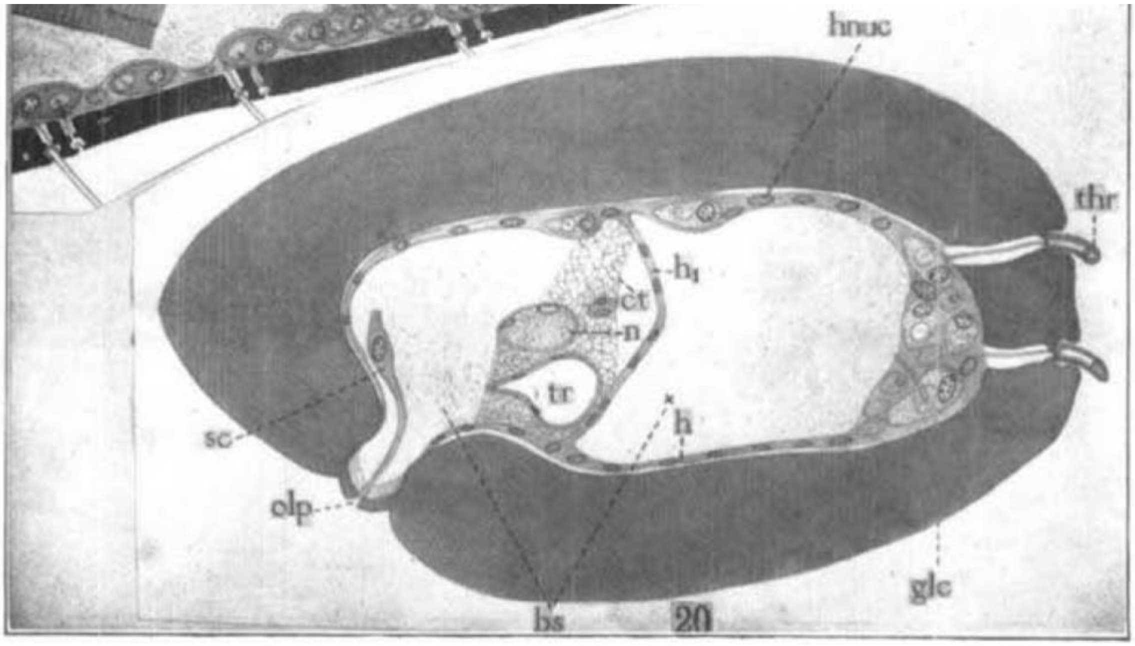

Figure 3. Cross -section of lumen in the extreme proximal end of the tibia of *Epilachna borealis*. “thr” = tactile hair; “h” = hypodermis; “h_1” = membrane diving lumen of the leg into two chambers; “tr” = trachea; “n” = nerve; “glc” = gland cell with reserviour; “ct” = connective tissue; “sc” = sense cell; “olp” = olfactory pore; “hnuc” = hypodermal nucleus (McIndoo, 1916)

3.4 Deterrents in reflex blood

Reflex fluid is mostly known to be irritant or deterrent to the predators. This deterrent nature of reflex blood is always due to the presence of some chemicals in the discharged fluid. These chemicals help the animals possessing reflex bleeding behaviour by making it unpalatable and toxic to the predators (de Jong *et al.*, 1991; Holloway *et al.*, 1991). Upon attack, persistent and distasteful nature of the haemolymph and irritating chemicals make the predator withdraw from the predatory act on the particular animal species. This toxic nature is sometimes accompanied by aposematic colouration, stating the toxicity or unpalatability of the species. Very much effective for the predators, that depends on visual recognition. Gas chromatography analysis of reflex blood from *Coccinella septempuntata* and *Adalia bipunctata* reported the presence of deterrent called coccinelline in *Coccinella septempuntata* and adaline in *Adalia bipunctata* (de

Jong *et al.*, 1991; Holloway *et al.*, 1991). All these compounds are synthesized inside the body of the ladybirds (Pasteels *et al.*, 1973). Coccinelline compound same as in reflex blood is detected in body parts of the ladybird, making it distasteful to predators, irrespective of whether it has just reflex bled or not. The concentration in the reflex blood, though, is found to be around 10 times that of any of the other body parts. It is also in high concentration during overwintering period despite ladybird's inability to reflex bleed. Females are found to consisting of more of this kind of alkaloids than males in their reflex blood (Holloway *et al.*, 1991). Similar to *Coccinella septempuntata* and *Adalia bipunctata,* possession of alkaloids is also observed in *Coccinella magnifica*. The chief alkaloid in *Coccinella magnifica* is hippodamine, which is more repellent than coccinelline of *Coccinella septempunctata* (Sloggett and Majerus, 2003). Like other alkaloids, harmonine is very prominent in *Harmonia axyridis* (Haulotte *et al*., 2012).

The biosynthesis of coccinelline (1), adaline (2), and harmonine (3) from fatty acids precursors in *Coccinella septempunctata*, *Adalia bipunctata*, and *Harmonia axyridis*, respectively, are now clearly established. Fatty acid precursor undergoing straightforward oxidation and amination reactions. Stearic acid is the precursor of coccinelline and harmonine, whereas myristic acid is at the origin of the carbon skeleton of Adaline (Haulotte *et al*., 2012). Larvae of sawfly species *Athalia rosae* are able to take up glucosinolates from their host plants and concentrate them effectively in the haemolymph, which is very different from the internal synthesis of the compounds in ladybirds (Müller *et al.*, 2001). This is somewhat mechanism with less energy requirement than biosynthesis of compounds. The dominant glucosinolate in leaves of *Sinapis alba* was sinalbin, which was found in a concentration of 0.1–0.3 mol/g fresh weight. Sinalbin was found in fed larvae of *A. rosae* in about eightfold higher concentration per gram fresh weight compared to leaf material. Sinigrin was detected in *Brassica nigra* leaves, and glucobarbarin in *Barbarea stricta* leaves. Larvae switched at second instar to either of these species, also sequestered sinigrin and glucobarbarin, respectively. Although, the efficiency is not similar to sinalbin. In *A. rosae*, the importance of glucosinolates in reflex bleeding as a defensive compound remains to be confirmed. Most of these compounds are volatile in nature, some can be non-volatile. The compounds present in the reflex blood is not always simple. In *Tritoma bipustulata* the main component in reflex blood samples was identified as indole. The following compounds were identified, and their structures were confirmed by injection of authentic standards: phenol, p-cresol, 3-methylindole, 2-phenyl pyridine, lauric acid, palmitic acid and pentacosane. The chemical composition of the reflex blood and the haemolymph did not differ between the sexes (Drilling and Dettner, 2010). Nuclear magnetic resonance and mass spectroscopy in *Aulacoscelis appendiculata* reflex blood identified two azoxyglycoside compounds, cycasin and macrozamin, in the reflex bleeding. These compounds are taken directly from the gut to the haemolymph through an active transport mechanism. Adults obtain azoxyglycoside by in taking cycad leaves (Prado *et al.*, 2011). Generally, animals possessing reflex bleeding behaviour are equipped with a chemical deterrent that can be very effective against the predators. These chemicals can be species, genus or family specific. Presence of such chemical in haemolymph is either by sequestration or by biosynthesis of compounds.

3.5 Cost of reflex bleeding

As from above, it is clear that haemolymph plays an important role in reflex bleeding. Such kind of discharge can really cost the organism in terms of energy. *Epilachna varivestis* is able to produce an average of six drops of blood or haemolymph from each leg (Happ and Eisner, 1961), which is a considerable amount for a small organism. Wallace and Blum (1971) described 3-13 % of loss of total body weight in the reflex bleeding in larvae of *Diabrotica*. In captured *Decaptoma lunata* the weight loss due to reflex bleeding averaged 16.5 ± 1.6 mg (Nicolson, 1994). Body size varied considerably from 90 – 460 mg. By taking blood loss as a function of body mass, with a mean value of 7.4 % body mass lost during defensive bleeding. The insects could be induced to bleed repeatedly but decreasing volumes of haemolymph were produced with successive bleedings. In *Decaptoma lunata* the initial blood loss was 17.7 ± 3.8 mg, followed by losses of 11.8 ± 1.7 mg and 4.1 ± 1.1 mg. Organisms that expel a large amount of body fluid are at greater risk of dehydration, as well as nutrient loss, and possibly increased predation risk due to reduced effectiveness of the bleeding response with repeated use (Grill and Moore, 1998). Diet quality and repeated reflex bleeding can affect the larvae to take more time to develop, grow smaller and develop a pale coloration. Cercopidae (froghoppers) species produced less exudate after 6 h without food (1.05 ± 0.14 ml, mean ± SE) when compared individuals with food (2.01± 0.28ml). Assaulted froghoppers with access to food regained the full capacity to bleed (1.47± 0.25ml) within that same period (Peck, 2000). The reflex bleeding is very much improved when the organism is well fed (Holloway *et al.*, 1991). Nicolson (1994) offered 43 *Decaptoma lunata* with water after reflex bleeding, only three rejected it. The volume of water drunk after bleeding depended on the mass of haemolymph lost. The meloids did not take advantage of contact with water to replace the water deficit completely: on average, 60 % of the mass loss due to bleeding was replaced. If replenishment of haemolymph volume does not diminish defensive potential, then meloids are in a better position to recover from encounters with predators than some insects with glandular defences. Refilling of defensive glands can take two weeks or longer in dytiscid or tenebrionid beetles or in cockroaches. *Adalia bipunctata* are able to exude large quantities of reflex blood as proportions of their body weights after the ladybirds are fed properly. Rate of depletion of the reserves in *Adalia bipunctata* is observed to be much faster than the *Coccinella septempunctata*. As *Coccinella septempunctata* are 3 times larger than *Adalia bipunctata*. Size makes a big difference when it comes to reflex bleeding. The larger size of organisms or individuals enables animals to have a larger reserve for longer reflex bleeding behaviour. Reflex bleeding also involves loss of important chemical substances or alkaloids. In many insect species, the important chemicals are produced by biosynthesis in the body, which is energy consuming metabolic action. Upon continuous reflex bleeding the alkaloid concentration will drop. This drop is more significant in males than females (Holloway *et al.*, 1991). The synthesis of this alkaloid in haemolymph really takes time as the biosynthetic pathway is rate limiting (de Jong *et al.*, 1991). Along with alkaloids, there is also a high risk of a decrease in haemocyte concentration in the haemolymph of many insects. The decrease in haemolymph concentration can affect the immunity of the particular individual, also making it vulnerable to parasitism (Zvereva and Kozlov, 2016).

3.6 Effectiveness of reflex bleeding behaviour

Presence of reflex bleeding or auto-haemorrhaging behaviour in insects and reptiles dominantly serves as a type of defence mechanism against natural enemies or anti-predator strategy. Reflex bleeding may also serve for other purposes like inter or intraspecific chemical signalling or communication (McIndoo, 1916), Effectiveness of reflex bleeding in firefly *Photinus pyralis* was tested against fire ants, *Solenopsis invicta*, *Pogonomyrmex badius*, and *Crematogaster atkinsoni* (Blum and Sannasi, 1974). The reflex fluid is discharged by the firefly adult from elytral and pronotal margins upon attack by fire ants. Discharged fluid covered the mandibles and often spread, entangling ant's leg. Cessation of the attack on the firefly occurs due to this behaviour and ants begin intensive cleansing activities. Fireflies which had auto-haemorrhaged to exhaustion, due to previous tactile stimulation, were easily overrun by the ants. This clearly indicates the effectiveness and importance of reflex bleeding in fireflies against fire ants. In the same study, *P. pyralis* were also subjected to be attacked by green anole lizard *Anolis carolinensis* and leopard frog *Rana pipiens*. Green anole lizards rejected the firefly by spitting them out with discomfort immediately after the encounter and wiped their snouts. On other hand leopard frogs ate the fireflies without any discomfort. Ineffectiveness of defence strategy of *P. pyralis* against leopard frogs may be due to predator's feeding method, by providing nearly no reaction time to perform any defensive or anti-predator act. Also, sometimes this defence mechanism may be specific for some predators and may not act effective for others, as frogs are very well known voracious feeder with generalized feeding habit. Similar type of study also conducted with two lined spittlebug *Prosapia bicincta* with wide range of potential generalist predators found in turf grass: big-eyed bugs *Geocoris uliginosus* and *Geocoris punctipes*; red fire ant, *Solenopsis invicta*; wolf spiders (Lycosa sp.); carabid beetles *Harpalaus pensylvanicus* and *Calosoma sayi*; and tiger beetles *Megacephala carolina carolina* (Nachappa *et al.*, 2006). Extensive grooming of mouthparts and antennae of predators is observed when they came in contact with the haemolymph of the adult spittlebug. Ladybird *Epilachna varvestis* and leaf beetle *Gallerucida nigromaculata* are also known to show mechanical defence via reflex bleeding against ants (Happ and Eisner, 1961; Matsuda, 1982). Matsuda (1982) reported lack of deterrence of reflex fluid in *Gallerucida nigromaculata*, mentioning the inability of the reflex blood as chemical defence. Lack of chemical defence in reflex bleeding may exist in some species, but along with its mechanical defence many studies demonstrated the existence of the chemical defence through reflex bleeding behaviour. Blister beetles are well known for causing blisters on human skin, when irritated by human hand. This is mainly due a chemical substance released via reflex bleeding, called as cantharidin (Carrel and Eisner, 1974). It acts as powerful systemic poison, 0.5 mg/ kg is reportedly lethal for humans. The deterrence of cantharidin is tested against an ant species *Formica exsectoides*. Acceptance of glucose solution isn't followed by ants even in the presence of cantharidin in concentration 0.77 x 10^{-5} M. A second bioassay is carried out by observing mouth response of carabid beetle *Calosoma prominen.* Demonstrated cleansing activity when concentrations of cantharidin ranging from 0.66 x 10^{-1} to 0.66 x 10^{-5} provided with help of a brush to mandible of the beetle. Cleansing activity was evident every time, even at lowest concentration. Many ladybirds e.g. *Coccinella septempuntata*, *Coccinella magnifica*, *Adalia bipunctata* and *Harmonia axyridis* are also known for effectiveness of their reflex blood against ants (de Jong *et al.*, 1991; Holloway *et al.*, 1991; Grill and Moore, 1998; Sloggett and Majerus, 2003). But, chemical part of defence of the reflexing bleeding is also a protection against vertebrate predators like birds or small mammals. High coagulation and sticky nature of reflex bleeding serves well for invertebrate predators as a physical defence. The alkaloids, coccinelline in *Coccinella septempuntata* and adaline in *Adalia bipunctata* are known to be distasteful and toxic to some bird predators (Marples, Brakefield and Cowie, 1989). In order for a predator, such as bird, to experience the effects of alkaloids, and overwintering ladybird may have to be mutilated or even killed. However, winter and early spring ladybirds are often found together in hibernating groups (Majerus & Kearns 1989) or feeding gregariously on early spring aphids (as were the ladybirds collected for the current study). De Jong *et al.* (1991) described that unpleasant experience with one ladybird species can protect other ladybird species. As it is mentioned that unpleasant experience of the birds with *Coccinella septempunctata* will defend *Adalia bipunctata* for being preyed on by birds. Although adaline is found to be less effective than coccineline, it still has a considerable deterrence. The possibility of Batesian mimicry of

Coccinella septempunctata by *Adalia* due to similar aposematic colouration as mentioned by De Jong *et al.* (1991) requires more study to understand this kind of relationship. Aposematic colouration plays an important role in anti-predator strategy, by serving as a warning signal. More colouration with patterns indicates the toxicity of the chemical substance in the haemolymph (Peck, 2000). The main alkaloid in *Coccinella magnifica* is hippodamine, which is known to be even more repellent than coccinelline of *Coccinella septempunctata*. This suggests that presence of chemicals/alkaloids contributing towards the effectiveness of reflex bleeding and it will vary with the varying alkaloids.

Not only just colouration, there are other behavioural mechanisms available that are employed by the insects to increase the efficiency of reflex bleeding behaviour. Froghoppers and spittlebugs are known to jump and fly after reflex bleeding attempts against predators (Peck, 2000; Nachappa *et al.*, 2006). As in nature few seconds achieved by distracting the predator with reflex bleeding is enough to escape from the predators. Upon attack by the ants, the *Diabrotica* larvae immediately became turgid and inflated in the region of abdominal segments I-V. While performing the reflex bleeding, the larvae began twisting and spinning, often elevating and moving the head in a serpentine fashion. This process helps to spread the secretion in all the directions, it also becomes a less accessible target and helps the larvae to avoid being glued to its own secretion. The reflex bleeding didn’t show any action against Mouse. No obvious distasteful or repellent properties for vertebrates (Wallace and Blum, 1971). Behavioural characteristics like, preferring untended or non-tended aphids for their feeding activity and carrying the aphids from the edge of aphid colonies if they are tended by ants by minimising the time of interaction with ants, reducing the chance of ant attacks on *Coccinella magnifica* larvae as compared to *Coccinella septempunctata* larvae. As *Coccinella septempunctata* move very fast around the ants and feed on site. Under ant attack, *Coccinella* septempunctata larvae usually produced copious quantities of reflex blood (83% of attacked larvae). In contrast *Coccinella magnifica* larvae reflex bled to deter ants visibly on two occasions (25%) and the quantity produced was visibly far less than that of *Coccinella septempunctata* larvae (Sloggett and Majerus, 2003). Stonefly *Pteronarcys proteus* is known to make “popping” sound along with reflex bleeding.

Which is effective against carnivorous myrmicine ant *Aphaenogaster*, but also deterred mouse *Peromyscus leucopus* (Benfield, 1974). Even after the mouse was deterred, it consumed the stoneflies, which may not occur in nature, as the study is conducted in a closed environment. *Peltoperla maria* performed a serpentine motion to avoid ant attack and eventually performed reflex bleeding. Stonefly *Peltoperla maria* show an effective reflex bleeding against myrmicine ant but failed to defend itself against mouse as a predator. Stridulating behaviour is also coupled with reflex bleeding in *Acanthoplus discoidalis* (Bateman and Fleming, 2009). An effective form of defence against bearded dragon lizards *Pogona vitticeps* (Agamidae), as a lizard, immediately dropped the cricket and retreated, extruding its tongue and wiping its mouth on the cage floor and with its forefeet. *Acathoplus discoidalis* haemolymph applied to *Gryllus bimaculatus* nymphs (which have no such chemical defence) successfully saved them from predation by striped skinks *Trachylepis punctatissima* (Scincidae). Apart from attack by predators, insects like ladybirds are subjected to intra-guild predation. Sato et al. (2009) demonstrated in the work that reflex blood is very much effective as a defence mechanism for intra-guild predation. Larvae of *Coccinella septumpunctata* cannibalized over 80% of the eggs when painted with water, this fell to 20% when haemolymph of *Harmonia axyridis* was present on the eggs. The squirting of blood or reflex bleeding helps the lizard to defend itself against, dogs, kit fox (*Vulpes macrotis*), greater roadrunners (*Geococcyx californianus*) (Sherbrooke, 1990) or southern grasshopper mice (*Onychomys torridus*) (Sherbrooke, 1991). As squirting movement may scare some predators or irritate due to spray fall of the reflex blood.

All the above indicates ants to be the most general predator mainly of all the insects possessing the reflex bleeding strategy. Insectivores beetle, spiders, lizards and birds can also be considerable predators for which reflex bleeding is an effective strategy. Along with some behavioural acts like stridulating motion, making “popping” sound and certain movements, reflex bleeding seems to be more effective. As popping sound and stridulating movement seemed to be working on predators like lizards and mouse. Presence of alkaloid also found to be enhancing the effectiveness of reflex fluid by making it deterrent for predators. It is also quite effective against birds. Integration of aposematic colouration along with alkaloid make the prey species look less palatable, even if it doesn’t perform reflex bleeding. Also, quite an effective strategy for the predators depending on visual cues.

4. Methodology

The whole study is divided into two experiments: -

1. Comparing reflex bleeding behaviour among several ladybird species and analysing intraspecific effects of sex, size and body weight on the amount of haemolymph devoted towards reflex bleeding.
2. Determination of physiological cost existing due to the reflex bleeding behaviour in ladybird beetles.

4.1 Collection of ladybird beetles

For the first part of the study, a total of 206 individuals of different ladybird species were sampled. But in the final dataset, some of the species were excluded due to the relatively low number of individuals sampled. Among these sampled individuals, some were excluded from the final dataset because of high coagulation of haemolymph while processing the sample. The final data set is consisting of 10 species from 8 genera.

Following are the included species and number of individuals (n) in the final dataset: (i) *Anatis ocellata* (n = 5), (ii) *Calvia quatuordecimguttata* (n = 7), (iii) *Ceratomigella undecimnotata* (n = 6), (iv) *Coccinella magnifica* (n = 6), (v) *Coccinella quinquepunctata* (n = 44), (vi) *Coccinella septempunctata* (n = 36), (vii) *Coccinula quatuordecimpustulata* (n = 8), (viii) *Exochomus quadripustulatus* (n = 12), (ix) *Harmonia axyridis* (n = 39), (x) *Propylea quatuordecimpunctata*, (n = 22). All species included in the study are aphidophagous ladybird beetles. The ladybird samples were collected from Prague and Central Bohemian Region, Czech Republic, during the month of September and October 2017. The ladybirds were collected using entomological net and aspirator. The entomological net was used with sweeping and beating methods.

Then the ladybirds were sorted out from the other insects inside the net, using the aspirator. The aspirator was also used directly to catch ladybirds sitting on grasses and shrubs. All the collected samples were stored in a small glass jar, as soon as they were captured from the wild, with a perforated lid to provide aeration.

Similarly, 80 individuals of *Harmonia axyridis* were collected from the nature using the aforementioned technique to determine the physiological cost bear by the ladybird beetles during reflex bleeding.

4.2 Rearing of ladybird beetles inside the laboratory

The ladybirds were taken out of the glass jar and maintained in Petri dishes (diameter 9 cm) in the laboratory. For the species, which are comparatively large in size, like *Anatis ocellata*, *Coccinella magnifica*, *Coccinella septempunctata* and *Harmonia axyridis*, maximum of five individuals were kept per petri dish. For the remaining species, maximum of 10 individuals were kept in each petri dish. This is done in order to ensure sufficient space and resources for each individual beetle. Each petri dish contained a piece of paper – folded in a zig-zag manner to provide more surface area and substratum. Also, small round ball of wet tissue paper was used as a source of water, and eggs of *Ephestia kuehniella* were provided to the beetles as a food source. Both water and food were provided ad libitum. The average temperature inside the room was 22 to 25 °C. Beetles were maintained in a similar condition in the laboratory for at least one week before processing them for the experiment.

4.3 Processing of ladybirds for the collection of haemolymph

Each individual ladybird beetle is weighed by using Sartorius analytical balance to a precision of 10^{-5} g before processing it for the collection of haemolymph. Also, individual body size measurements consisting of elytron length was measured by using digital callipers to a precision of 0.01 mm. All the 206 ladybird beetles selected for the first part of the experiment were glued to two-sided adhesive tape on their back and stimulated with the help of an entomological pin, near to tibia-femoral joints of each leg. Ejected haemolymph by reflex bleeding was collected with the help of glass micro-capillary tube (Hirschmann, Germany) and the amount sampled was measured using a Vernier calliper with a precision of 0.01 mm. 15 mm of micro-capillary tube corresponds to the volume of 1 μl. The collected haemolymph was diluted in anticoagulant buffer (62 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, and 26 mM citric acid) (Knapp *et al.*, 2018) and used immediately for counting haemocytes present in it. After counting the haemocytes, all the beetles were frozen at -25 degrees Celsius. The number of haemocytes was counted by using a Bürker chamber viewed under a Carl Zeiss Primo Star microscope (Germany). In the end, sex of all the ladybird beetles was determined based on presence and absence of copulatory organ by dissecting them from the frozen individuals.

4.5 Processing of ladybirds for the determination of physiological cost due to reflex bleeding behaviour

A total of 80 individuals were included, but data for 42 *Harmonia axyridis* individuals were analysed for this experiment, due to mortality and undesirable amount of haemolymph required of the analyses. 10 beetles (Males = 6 and Females = 4) from the total were stressed by starvation (no food, only water). From the stressed individuals, 3 beetles (Males = 3 and Females = 0) were stimulated to reflex bleed twice per week for two and half week period (Total bleeding = 5 times). Rest of the (Males = 3 and Females = 4) stressed beetles were stimulated for reflex bleeding only at the beginning and end of the experiment. Thirty-two beetles (Males = 17 and Females = 15) from the total individuals were provided with ad libitum food and water (not stressed). From these non-stressed individuals, 15 beetles (Males = 7 and Females = 8) were stimulated to reflex bleed. Rest of the 17 (Males = 10 and Females = 7) non stressed beetles were stimulated for reflex bleeding, also beginning and end of the experiment. Stimulation of reflex bleeding and collection of haemolymph is performed same as it is mentioned above in the previous section. The concentration of haemocytes for all experimental beetles was also counted twice, one at the beginning and one at the end of the experiment by using the Bürker chamber viewed under a Carl Zeiss Primo Star microscope.

4.6 Data Analyses

As mentioned above the analyses were performed on the final dataset by using R 3.4.3. A single (vector) variable was created for reflex bleeding behaviour (rbv) observed in the sampled individuals by integrating two variables, reflex bleeding performed by the individual (RB) and reflex bleeding not performed by the individual (RB2) by using "cbind" function. To analyse the willingness to perform reflex bleeding in different ladybird species, a generalized linear model with a binomial distribution of errors was applied to the dataset. Another variable created for relative volume of reflex blood (RBrel) devoted towards reflex bleeding was calculated as the amount of reflex blood (RB_vol) divided by the total weight (Wlive) of the sampled individual. The difference in the relative volume of reflex blood is checked between the species by using analysis of variance (ANOVA) and F-test. To verify the effect of Size (elytron length), live weight and sex on reflex bleeding behaviour, the generalised linear mixed models (GLMMs) in the "glmmPQL" function with a binomial distribution of errors was implemented in nlme package. Similarly, the effect of Size (elytron length), live weight and sex on the relative volume of reflex blood was analysed, but with the Gaussian distribution of errors. For analysing the effect of Size (elytron length), live weight and sex, only following species were taken into account: *Coccinella quinquepunctata*, *Coccinella septempunctata, Exochomus quadripustulatus, Harmonia axyridis* and *Propylea quatuordecimpunctata*. This was performed due to a small sample size of other species in the final dataset. By employing generalised linear mixed models (GLMMs) with Gaussian distribution of errors in the "glmmPQL" function in the "nlme" package, the effect of Size on the relative volume of reflex blood is also analysed.

For analysing the haemocyte number in the different species ladybirds, only individuals that provided haemolymph successfully were considered. Also, individuals with total haemolymph volume 0.2 μl or more were included. Generalized linear model with a quasipoisson distribution of errors was utilized of the analysis. Tukey's honestly significant difference tests were utilized to test the significant differences between the species using the ‘glht’ function in the ‘multcomp’ package for all the mentioned analysis.

To analyse the physiological cost due to reflex bleeding behaviour, the effect of treatment (with or without food) and reflex bleeding was tested against the difference in the number of hemocytes present in the haemolymph at the start of the experiment and at the end, by using analysis of variance (ANOVA).

5. Results

5.1 *Experiment 1*

The willingness to show reflex bleeding behaviour in ladybird beetles was tested between the different ladybird species. Reflex bleeding behaviour is found to be significantly varying between the different ladybird species ($F = 7.51$, $p < 0.001$). All individuals of *Anatis ocellata*, *Ceratomigella undecimnotata* and *Coccinella magnifica* showed reflex bleeding behaviour, whereas *Coccinula quatuordecimpustulata* individuals were devoid of this behaviour. Individuals of *Harmonia axyridis* and *Coccinella septempunctata* also showed strong willingness towards reflex bleeding behaviour (Figure 4).

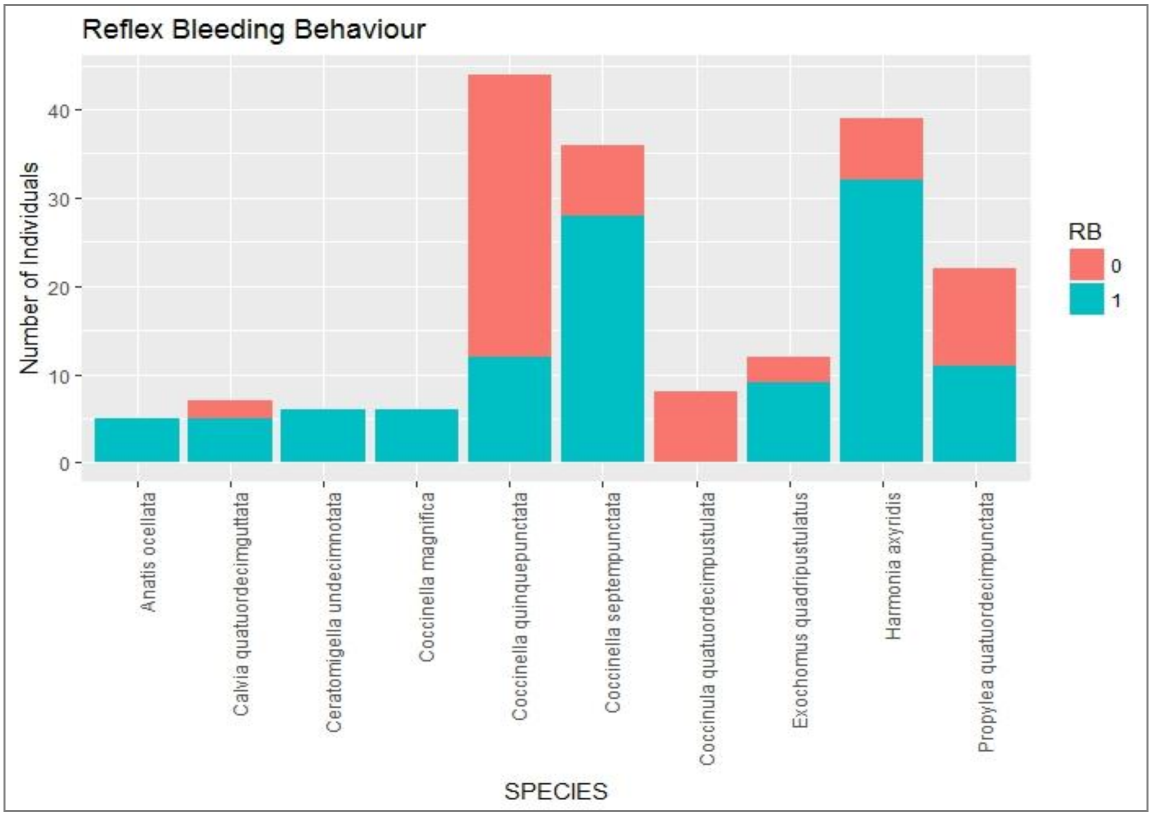

Figure 4. The reflex bleeding behaviour observed in the different species of ladybird beetles. Individuals performing reflex bleeding are shown in blue, whereas red is indicating the individuals that didn’t perform reflex.

The relative amount of reflex blood devoted towards reflex bleeding is also found to be differing between the species (F = 8.54, P < 0.001). *Harmonia axyridis* is found to be devoting a higher proportion of haemolymph towards reflex bleeding among all the species studies in the experiment (Figure 5). *Anatis ocellata, Coccinella septempunctata, Ceratomigella undecimnotata* and *Coccinella magnifica* also showed significantly higher proportion of the relative amount of haemolymph devoted to reflex bleeding compared to rest of the species, except *Harmonia axyridis* (Figure 2).

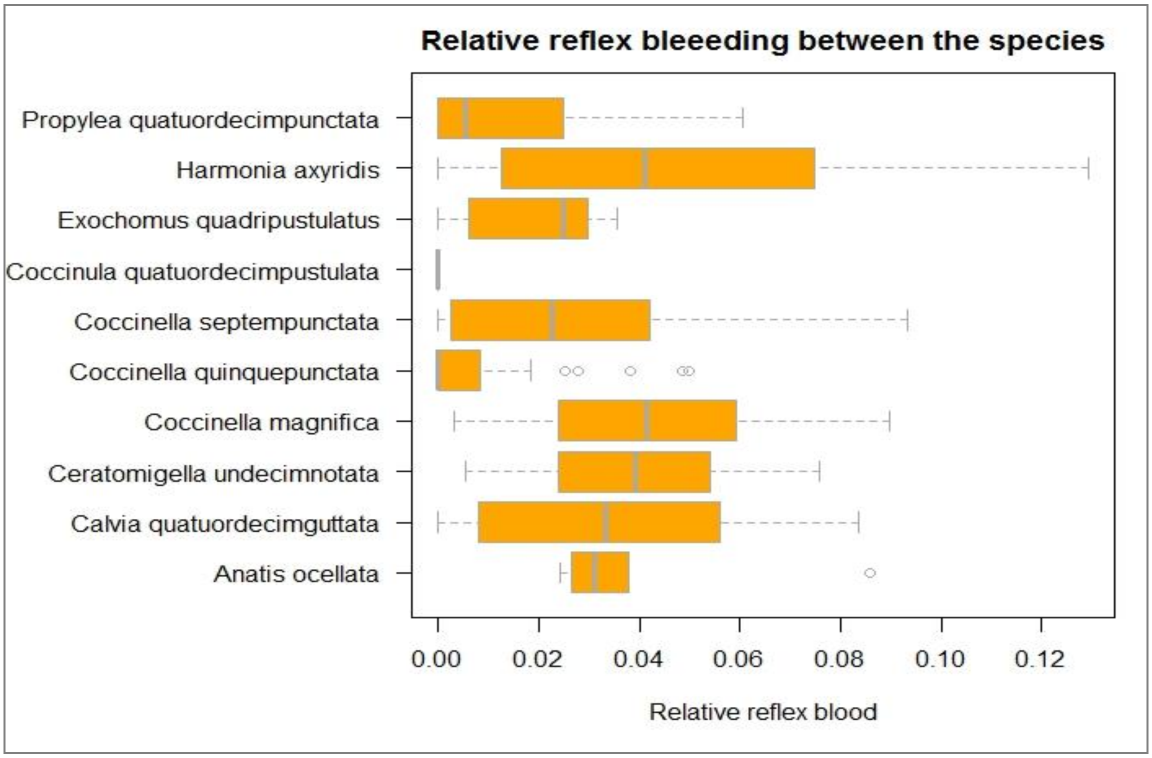

Figure 5. Showing relative reflex bleeding observed between different species of ladybird beetles.

Size, live weight and sex were found to have no significant effect on willingness towards showing reflex bleeding behaviour when provided with stimulation (GLMM, P > 0.05 in all cases). The amount of relative reflex blood devoted towards reflex bleeding is significantly affected by the size of the individuals (GLMM, F = 5.49, P = 0.020), whereas live weight and sex had no effect (GLMM, P > 0.05 both). When the only effect of size is tested against the amount of relative reflex blood, the effect was found to be highly significant (GLMM, F = 10.94, P = 0.0011).

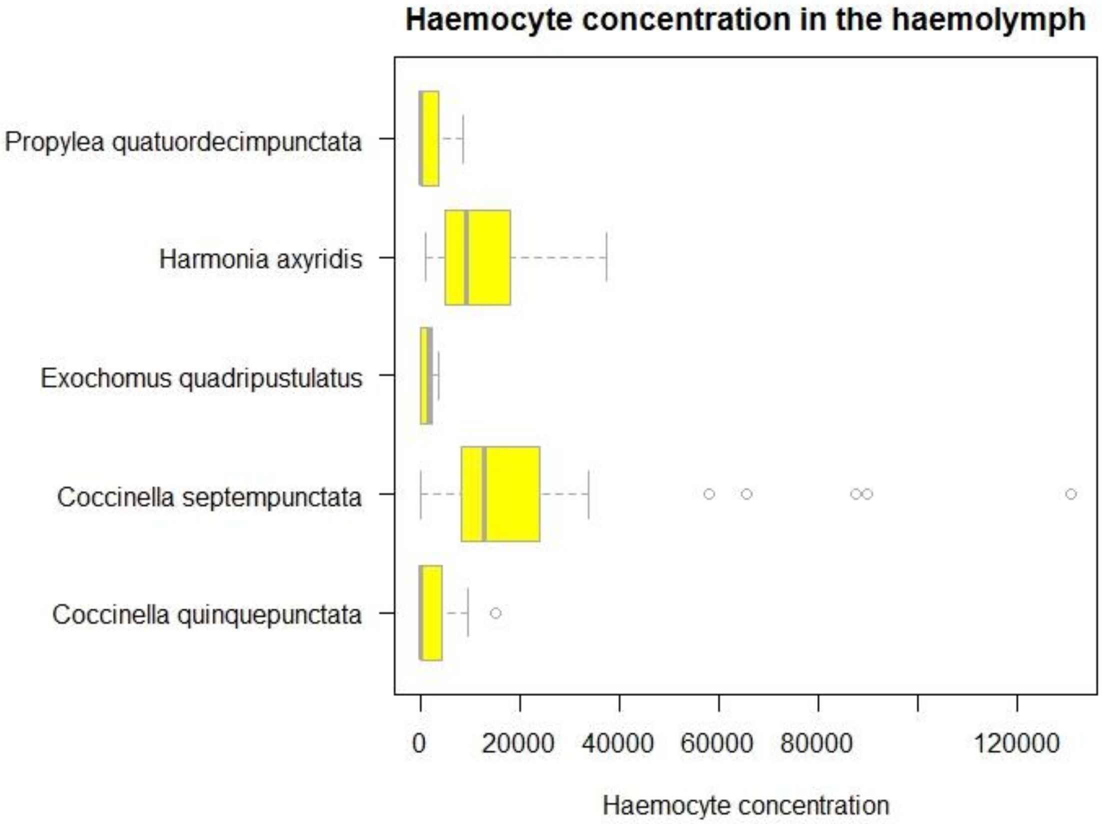

Figure 6. Displaying the concentration of haemocytes in the haemolymph among the different ladybird species.

Significantly varying concentrations (per microliter) of haemocytes were observed in the haemolymph from different species of the ladybird beetles (GLM, F = 25.57, P < 0.001). A number of haemocytes are found to be highest in the haemolymph from *Coccinella septempunctata* (Figure 6), then followed by *Harmonia axyridis*.

5.2 *Experiment 2*

The physiological cost associated with reflex bleeding behaviour is observed to be very much evident. The effect of reflex bleeding behaviour on the difference between the numbers of haemocyte before and after the experiment is marginally significant (F = 4.14, P = 0.049). Effect of treatment (with or without food) on the difference between the concentration of haemocytes before and after the experiment is found to be also significant (F = 6.21, P = 0.017). Whether individuals starved or fed, the difference in the haemocyte concentration is quite higher for the individuals that performed reflex bleeding, as compared to the control individuals (Figure 7). No sex-specific effect was observed (F = 0.07, P = 0.788). Also, no effect of the interaction between treatment and reflex bleeding was found (F = 0.03, P = 0.872).

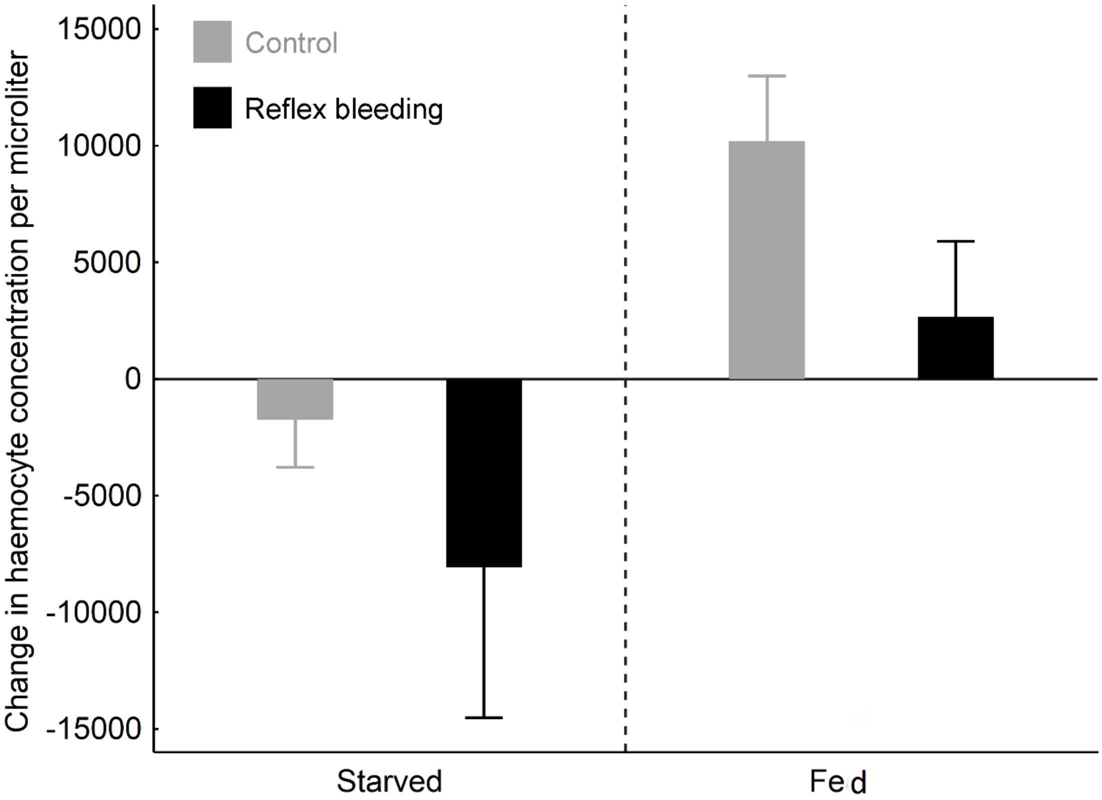

Figure 7. Change in haemocyte concentration in *Harmonia axyridis* haemolymph due to repeated reflex bleeding and starvation stress.

6. Discussion

Presence and mechanism of reflex bleeding are explained for insects by many studies. Especially, the occurrence of reflex bleeding behaviour and the mechanism of reflex bleeding in ladybirds are well addressed. Investigations by McIndoo (1916) provides a very clear picture of how the reflex blood is ejected from the tibia-femoral articulations with exact point locations responsible for the ejection of reflex blood. Most of the studies on reflex bleeding behaviour in ladybirds suggest a similar type of bleeding behaviour, whether it's larvae or adult (McIndoo, 1916; Happ and Eisner, 1961; de Jong *et al.*, 1991; Knapp *et al.*, 2018). There can be a slight variation in some species due to special structures like spines in the larvae of *Epi1achna varivestis* and bulges on the abdomen in *Diomus thoracicus* responsible for the reflex bleeding. According to many literatures, the general similarity in reflex bleeding is evident, but possible similarities or dissimilarities associated with the amount of reflex blood that is devoted towards reflex bleeding and the willingness of a particular species – that how readily the species is able to show reflex bleeding behaviour when provided with stimulation is not well explored. The present study reveals the occurrence of interspecific variation in reflex bleeding behaviour among the several species of lady beetles. All individuals of *Anatis ocellata*, *Ceratomigella undecimnotata* and *Coccinella magnifica* showed reflex bleeding behaviour. Whereas no individual of *Coccinula quatuordecimpustulata* reacted towards stimulation with reflex bleeding behaviour. Individuals of both *Harmonia axyridis* and *Coccinella septempunctata* showed higher willingness towards reflex bleeding behaviour when provided with stimulation in comparison to rest of the species, except *Anatis ocellata*, *Ceratomigella undecimnotata* and *Coccinella magnifica*. This difference in reflex bleeding behaviour between *Anatis ocellata*, *Ceratomigella undecimnotata* and *Coccinella magnifica*, and *Harmonia axyridis* and *Coccinella septempunctata* may be due to the low sample size of *Anatis ocellata*, *Ceratomigella undecimnotata* and *Coccinella magnifica* as compared to *Harmonia axyridis* and *Coccinella septempunctata*. The willingness of reflex bleeding in *Exochomus quadripustulatus* and *Calvia quatuordecimguttata* is close to that of *Harmonia axyridis* and *Coccinella* *septempunctata*. In *Propylea quatuordecimpunctata* half of the total individuals is found to show reflex bleeding. Despite of having similar ecological role as *Coccinella septempunctata* (Hodek, Emden and Honěk, 2012), *Coccinella quinquepunctata* individuals showed very low willingness towards reflex bleeding behaviour.

The interspecific variation of reflex bleeding behaviour in ladybird species may be existing due to their behaviour, feeding habits or ecological role. Ladybirds feeding on ant-tended aphids have higher chances of having aggressive interaction with ants. Having better reflex bleeding behaviour serves important defence strategy for such ladybird species (de Jong *et al.*, 1991; Holloway *et al.*, 1991; Grill and Moore, 1998; Sloggett and Majerus, 2003). But in case of some ladybirds having herbivorous diet might not be requiring such defence strategy (Hodek, Emden and Honěk, 2012). Also, in case of myrmecophilous ladybirds (e.g. *Coccinella magnifica*) the use of reflex bleeding behaviour might not be the primary defence strategy. Some behavioural characteristics in ladybirds – as reported in *Coccinella magnifica* larvae – preferring untended or non-tended aphids as their food source and carrying the aphids from the edge of aphid colonies if they are tended by ants by minimising interaction with ants, might not be readily showing the reflex bleeding behaviour (Sloggett and Majerus, 2003). As suggested by Sloggett and Majerus (2003), such behaviour in *Coccinella magnifica* may be persisting due to evolving towards myrmecophily, the expression of certain behaviours of *C. magnifica*, such as carrying aphids, have been enhanced and whereas the expression of reflex bleeding have been reduced. In the present study, interestingly *Coccinella magnifica* found to be showing 100% willingness towards reflex bleeding. Being myrmecophilous as adult and possession of special behavioural characteristics in larvae are found not be reducing the willingness to show reflex bleeding behaviour as defence strategy according to the findings of the present study, but this may be resulting due to less number of individuals present in the study. Individuals of aphidophagous ladybird beetle *Coccinula quatuordecimpustulata* found not be reflex bleeding can possibly due to feeding on untended aphids, but further investigation is required for clarity of the particular issue.

From the results, a significant interspecific difference in the relative amount of reflex blood devoted towards reflex bleeding is found to be existing among the ladybird species present in the study. *Harmonia axyridis* is found to be contributing highest amount of relative reflex blood as compared to other species present in the study. The relative amount of reflex bleeding is found to be slightly less in *Coccinella septempunctata* as compared to *Anatis ocellata*, *Calvia quatuordecimguttata, Ceratomigella undecimnotata* and *Coccinella magnifica* (figure 4.). As discussed earlier, the results for the species *Anatis ocellata*, *Calvia quatuordecimguttata, Ceratomigella undecimnotata* and *Coccinella magnifica* may be getting affected due to their low sample size. As in case of *Harmonia axyridis* and *Coccinella septempunctata* the sample size is relatively higher as compared to rest of the species.

Analysis of intraspecific effects of sex, size and body weight, reveals that, the effect of size is much greater on the relative amount of reflex blood devoted towards reflex bleeding. No significant effect of sex and body weight is observed on relative amount of reflex blood. Also, no effect of sex, size and body weight is found on willingness towards reflex bleeding in the ladybird species. Size can be one reason why some species readily showing reflex bleeding with comparatively higher amount of relative reflex blood, when compared to worldwide dominant species like *Coccinella septempunctata* or *Harmonia axyridis* (Slipinski, 2007; Hodek, Emden and Honěk, 2012). May be the similar reason lies for the low amount of relative reflex blood found in the species with smaller body size. Holloway *et al.* (1991) in their study described that the species with larger body size tend to be consisting of larger reserve, hence making then able to devote larger amount of relative reflex blood. This also reduces the chance of the species to get exhausted due to repeated reflex bleeding, as exhausted individuals are likely to be overrun by the predators (Blum and Sannasi, 1974).

Reflex bleeding being the most efficient defence mechanism in ladybirds (Hodek, Emden and Honěk, 2012), comes with a trade-off. Evidences proving that the haemolymph being the major constituent of reflex blood makes it valuable substance to lose in the process of reflex bleeding. The presence of glandular substance, cellular components (e.g. haemocytes) and proteins in reflex blood, is widely reported

(McIndoo, 1916; Happ and Eisner, 1961; Blum and Sannasi, 1974). Grill and Moore (1998) described that expelling a large amount of body fluid can put the ladybird beetle at greater risk of dehydration, as well as nutrient loss, and may increase the possibility of predation risk. Along with haemolymph, loss of essential alkaloids is associated with reflex bleeding (Holloway *et al.*, 1991). Same situation stand for the glandular secretion associated with reflex bleeding (Nicolson, 1994). As biosynthesis of glandular substances and alkaloid are rate limiting (de Jong *et al.*, 1991; Nicolson, 1994). Similar physiological cost associated with reflex bleeding is addressed in this study by analysing the effect of reflex bleeding on haemocyte concentration. Reflex bleeding is found to have a significant effect on concentration of haemocytes present in the haemolymph (per microlitre). Individuals that went through the sessions of reflex bleeding are found to show a drop in the haemocyte concentration, and the effect is more dominant when they are devoid of food. Such drop in the haemocyte concentration may affect the immunity of the particular individual, also increasing the possibilities of parasitism (Zvereva and Kozlov, 2016). Individuals of smaller body size are at the greater risk of losing haemocytes as compared to individuals with larger body size, as rate of depletion of reserves is faster in the individuals with smaller body size (Holloway *et al.*, 1991).

7. Conclusion

Willingness towards reflex bleeding is very species specific, some readily show this behaviour, and some do not. Species consisting individuals with larger body size can contribute more relative amount of reflex blood towards reflex bleeding as compared to the individuals/species with smaller body size. Having the ability to devote more haemolymph towards reflex bleeding enhances the effectiveness of reflex bleeding as defence strategy. Having large reserve also reduces the rate of loss. The study did not find any effect of sex or live weight on relative amount of reflex blood, but body size is somewhat linked to the sex and weight. Females are generally of larger body size than males in insects. Also, individual with larger body size are able to accumulate resources as compared to individual with smaller body size, hence gaining more live weight (Holloway *et al.*, 1991; Nicolson, 1994). Having comparatively large body size may be is playing an important role in worldwide dominance of some species like *Coccinella septempunctata* and *Harmonia axyridis* over other ladybird, by providing better defence strategy. But such phenomenon cannot be addressed with a single factor, as reflex bleeding comes with trade-offs. Multiple reflex bleeding sessions can result in depletion of haemocytes, alkaloids and proteins along with haemolymph. In resource limited environment reflex bleeding can pose great danger on physiological condition and development of the individual. Peck (2000) states that the reflex bleeding is very much affected by the diet availability. Individual with continuous supply of food can perform reflex bleeding for longer period. The chemical part of defence of reflex bleeding is also affected even if animal is in constant supply of food and water. As biosynthesis of alkaloids and glandular products are rate limiting.

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