Feeding Live or Dried Black Soldier Fly Larvae (*Hermetia illucens*) to laying hens – Effects on Immune Traits, Egg Quality, Feather Condition, Body and Organ Weights

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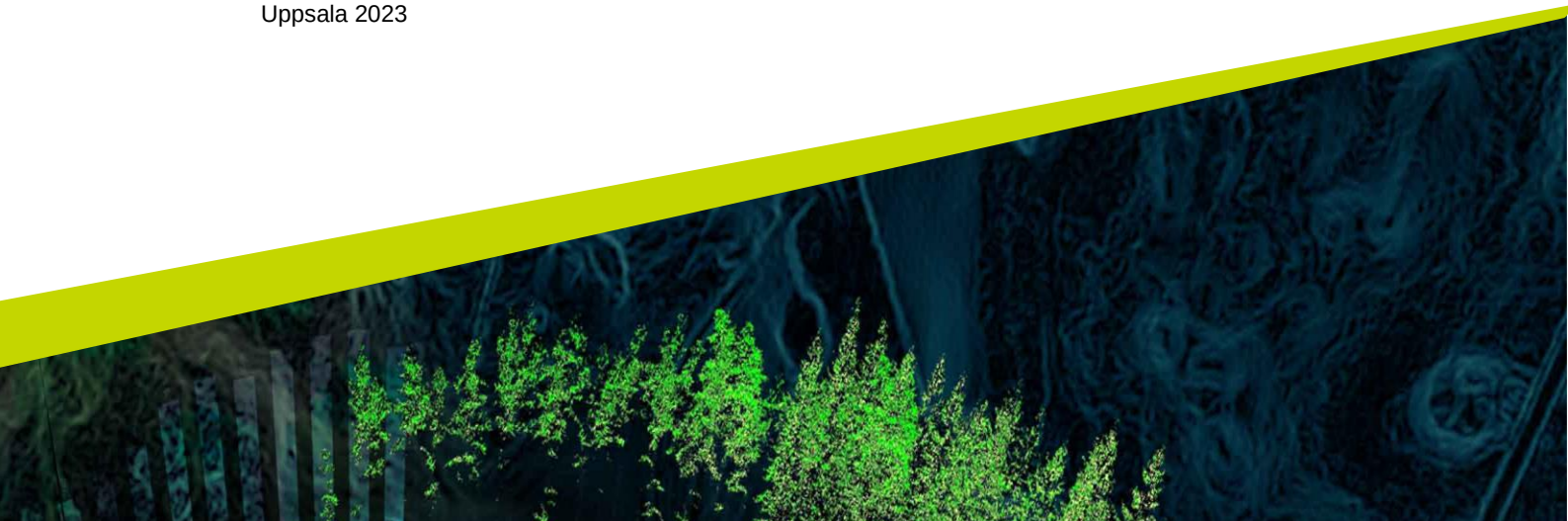

Feeding Live or Dried Black Soldier Fly Larvae (Hermetia illucens) to laying hens – Effects on Immune Traits, Egg Quality, Feather Condition, Body and Organ Weights

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Abstract

Due to increasing prices and potential scarcity of soybean meal (**SBM**), there is growing demand for alternative and sustainable protein sources in poultry production. Black soldier fly larvae (**BSFL**) have proven to be a suitable alternative based on nutritional value and sustainable production. Feeding live larvae to laying hens can stimulate natural foraging behaviour and improve animal welfare, but requires careful management to standardize nutrient composition of the larvae. Dried larvae, on the other hand, have a longer shelf life and are easier to handle. Various studies have analysed the effects of live BSFL and BSFL meal feeding in laying hens, but there is a gap regarding the effects of dried whole larvae feeding, and there is a scarcity of literature regarding the impact of BSFL feeding on immune parameters. This study aims to evaluate the effect of live and dried black soldier fly larvae feeding to laying hens.

For this purpose, 360 Bovans Robust White hens were randomly allocated at 18 weeks of age to three diet groups: control, live larvae, and dried larvae. The control group received a standard soybean meal based diet, while the live and dried larvae groups received 20% replacement of daily dry matter intake as live or dried larvae, respectively, along with a balanced pelleted feed. Data was collected from 30 to 50 weeks of age. Immune parameters recorded were white blood cell counts and specific antibody responses to infectious bronchitis virus (**IBV**) booster vaccination. The white blood cell populations enumerated were heterophils, monocytes, lymphocytes, thrombocytes, and some subpopulations of lymphocytes, including MHC II+ lymphocytes (B cells), CD4+CD25- (T helper cells), CD4+CD25+ (activated helper and/or regulatory T cells), and CD4-CD25+ (non-CD4 activated T cells). In addition, the expression levels of MHC II and chicken mannose receptor MRC1L-B on monocytes was measured. Internal and external parameters of egg quality and feather condition score were evaluated. At the end of the experiment, hen body weight and gastrointestinal tract (**GIT**) organs were weighed.

The control group diet was unintentionally manufactured with low sodium content, which is known to cause reduced body weight, cannibalism, and feather pecking. This makes it difficult to interpret the results. Nonetheless, comparisons between dried and live larvae are still relevant. Hens in both larvae groups had darker yolk colour with age ($p<0.0001$) and higher yolk weight ($p=0.05$). The feather condition of the control group on the neck, back, cloaca, and tail was poorer than both larvae groups, and control hens had more rear body skin injuries ($p<0.0001$). The control group hens were significantly lighter in weight than the larvae group hens ($p=0.005$). However, there was no significant difference between the treatments in organ weights (i.e. proventriculus, liver, gizzard, and fat pad). Additionally, there were no significant differences between groups in leukocyte counts, expression of surface markers, and antibody response to IBV vaccination.

Moreover, egg quality parameters, such as egg weight, eggshell breaking strength, eggshell thickness, eggshell weight, and albumen thickness, were similar in all groups ($p>0.05$).

The findings of this study are in agreement with previous research on BSFL meal feeding, demonstrating no difference in leukocyte counts and antibody responses to IBV vaccination, suggesting that the form of larvae feeding may not have an impact on the immune response. The results indicate that the low sodium diet of the control group had an impact on the integument condition and body weight. Egg quality and organ weight were also unaffected with treatment, indicating that substituting 20% of daily dry matter intake with live or dried larvae can serve as a sustainable alternative feed ingredient. Dried larvae, with their consistent nutritive value and easy management, appear to be the more practical option. Further research is needed to validate the findings with a proper control group.

Keywords: Laying hens, black soldier fly larvae, leukocyte count, antibody response, egg quality, feather condition, organ weight

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Abbreviations

BSF	Black Soldier Fly
BSFL	Black Soldier Fly Larvae
CD	Cluster of Differentiation
D	Day
IBV	Infectious Bronchitis Virus
ELISA	Enzyme-Linked Immunosorbent Assay
SBM	Soybean Meal
W	Week

Introduction

The demand for poultry products is rising, leading to a surge in demand for feed ingredients (Iji et al., 2017). Soybean meal (SBM) is the primary source of protein in poultry feed production (Hsiao et al., 2006). However, due to increasing demand, the prices of soybean meal are soaring, and there will be scarcity as well (Iji et al., 2017). Therefore, there is a pressing need for alternative, sustainable protein sources for poultry production. Black soldier fly larvae have gained popularity in recent years due to their sustainable production (Baiano, 2020) and minimal land and water requirement (Siddiqui et al., 2022).

Black solider fly larvae (BSFL) have been successfully used as a component of a balanced diet to replace part of the protein in the diet since the nutritive value of BSFL can be higher than conventional protein sources depending on the substrate used (Siddiqui et al., 2022). Furthermore, BSFL has been used as an environmental enrichment by providing them whole and live to attract hens (Veldkamp & Van Niekerk, 2019; Star et al., 2020). Alternatively, BSFL can be provided as dried meal included in a pelleted feed (Maurer et al., 2016; Marono et al., 2017; Secci et al., 2020; Liu et al., 2021; Patterson et al., 2021).

Providing live larvae can act as an attractive environmental enrichment, as the larvae activity can stimulate natural foraging behaviour and improve animal welfare. However, BSFL are difficult to manage and need to be kept at lower temperatures to slow down the process of turning into pupae (Tahamtani et al., 2021) since their nutritional value change with the different stage of larvae development (Liu et al., 2021). The effects of both live larvae and larvae meal feeding on laying hens have been studied, but there is a scarcity of knowledge about the effects of whole dried BSFL feeding to laying hens. Providing dried BSFL feeding could prove to be a more practical option for replacing protein in the diet and provide an environmental enrichment compared to feeding laying hens with live BSFL.

The effects of BSFL feeding on immune parameters in laying hens remain largely unknown. The immune response can be influenced by the chitin content of the exoskeleton of the larvae (Elieh Ali Komi et al., 2018) or by enhancing the birds' affective state (Campo et al., 2005). While a few studies have examined the effects of BSFL on some immune parameters, the findings are mixed. For example, Lee et al. (2018) found that broiler chickens fed with BSFL meal exhibited a positive response, showing better survivability upon induced infection. Tippayadara et al. (2021) did not find any significant effect of BSFL meal feeding on the various populations of leukocytes in fish. De Souza Vilela et al. (2021) reported decreased leukocyte counts in young broiler chickens fed a diet supplemented with BSFL

meal. However, the impact of whole live and dried BSFL feeding on laying hens' immune parameters remains unknown.

Most of the studies that have investigated the effect of BSFL feeding on integument condition in laying hens have focused on the provision of live larvae in a feeding utensil or mixing larvae meal with feed (Maurer et al., 2016; Veldkamp & Van Niekerk, 2019; Star et al., 2020). However, there is a knowledge gap regarding the effect of scattering BSFL in the litter, which could potentially stimulate foraging behaviour and lead to improved feather condition. Similarly, there is a gap in the knowledge regarding the effect of dried BSFL feeding on egg quality and gastrointestinal organ weight.

To address these knowledge gaps, this study investigated the effects of replacing 20% of daily dry matter intake with live or dried BSFL in the diet of laying hens. The experiment evaluated the impact of this dietary intervention from 30 to 50 weeks of age on leukocyte counts, antibody responses to infectious bronchitis virus (IBV) booster vaccination, egg quality, feather condition, hen body weight, and organ weight. Blood sampling was started at 30 weeks of age and carried out at 4-week intervals for a total of 5 times to assess leukocyte counts with flow cytometry and antibody responses with ELISA methodology, and egg quality parameters including external and internal parameters were measured at 40 and 49 weeks of age. The Integument condition was scored at 40 and 50 weeks of age on a scale of 1 to 4 with 1 being worst and 4 being best. At the end of the study, the birds were euthanized and gastrointestinal tract (GIT) organs and fat pad were weighed.

Objectives and Hypothesis

The aim of this study was to assess the effect of including BSFL in the diet of laying hens on leukocyte count, antibody response, egg quality, feather condition, and organ weight. The hypothesis of this study was that replacing 20% of the daily dry matter intake with either live or dried BSFL, when compared to a control group fed SBM-based pelleted feed, can modulate leukocyte counts and antibody responses to IBV booster vaccination, in addition to improved egg quality and feather condition in the hens. We also expect that dried BSFL will have a similar effect on egg quality, feather condition, immune parameters, and organ weight in laying hens as live BSFL, as they have a similar nutrient composition except for moisture content and mobility.

Literature Review

BSFL are gaining popularity as an alternative source of protein in poultry production. Larvae feeding can affect the production and health parameters in various ways. The potential impact BSFL feeding on the avian immune response, egg quality, and feather condition was assessed using pertinent scientific literature.

Black Soldier Fly Larvae: A Sustainable and Nutritious Alternative Protein Source for Poultry Feeding

In poultry production, soybean meal is the primary source of protein (Hsiao et al., 2006). Demand for feed ingredients has increased due to the rapid growth of the poultry sector, resulting in higher prices. The price of conventional feedstuff is expected to increase further in the future, and there can also be some shortage (Iji et al., 2017). Therefore, it is necessary to find alternative protein sources that can either partially or totally replace the traditional source of protein. There is continued effort to find sustainable alternative protein sources, and Black Soldier fly larvae (BSFL) can be a good option. (Siddiqui et al., 2022).

Additionally, waste production is also increasing with the increase in human population. Over the last decade, BSFL have become popular as a way of converting organic waste to highly quality feed components for poultry. (Baiano, 2020). BSFL are polyphagous and have the capability to convert a waste range of organic waste into good quality protein. Black Soldier flies do not serve as disease carriers or cause pest issues for humans, as they primarily depend on body fat as an energy source stored during their larval stage. (Sheppard et al., 2002). Adult flies exhibit avoidance behaviour towards humans and human food. The BSFL are receiving significant interest due to its minimal resource requirements for small-scale production. Compared to other poultry protein sources, such as SBM, larvae production requires significantly less water and land. (Siddiqui et al., 2022).

Many studies have identified a positive impact of BSFL feeding on health and production parameters in poultry (Bovera et al., 2018; Secci et al., 2018; Veldkamp & Van Niekerk, 2019). Larvae can be fed to laying hens as a meal, whole dried or alive. Live larvae provide nutritional and environmental enrichment to laying hens, mimicking their natural insect consuming behaviour. As the nutritional value of the

live larvae varies with age, maintaining a lower temperature than the ambient condition during storage is crucial. The lower temperatures help minimize nutritional changes. The most notable difference is observed in the fat content, which decreases from 28% in the larval stage to 8% in the pupa stage (Liu et al., 2017). To overcome storage challenges and mitigate potential nutritional changes associated with live larvae feeding, utilizing dried larvae presents a practical solution. Dried larvae offer advantages in terms of ease of handling and storage. Moreover, the drying process significantly prolongs the shelf life of the larvae. In terms of the nutritional composition of BSFL, the protein content varies between 35% and 57%, depending on the substrate utilized and the age of the larvae. (Veldkamp et al., 2012).

Effects of Larvae Feeding on Chicken Immune Response

Feed ingredients that stimulate the immune response, such as amino acids, can lead to improved production performance and better response to vaccines in poultry (Faluyi et al., 2015). The feeding of BSFL has the potential to influence the immune response in two ways. Firstly, it can have a direct effect due to the presence of chitin, which may stimulate immune cells and promote their functions, such as the production of immunoglobulins. Secondly, it may have an indirect effect by improving the welfare and affective state of chickens, which can subsequently enhance immune function. The chicken immune system is similar to the mammalian immune system and comprises innate and adaptive immunity (Kaiser & Balic, 2015). Before discussing the effects of larvae feeding on chicken immune parameters, an overview of the circulating chicken immune cells is presented.

Innate Immune Cells

The innate immune system initiates the first and most rapid response when any foreign particle invades the body (Göbel & Smith, 2022). This system has more abundant cells than the adaptive system and tackles the pathogen until the adaptive immune response is activated (Göbel & Smith, 2022). A brief description of innate immune cells is provided below.

Heterophils are the chicken homologue to mammalian neutrophils (Kaiser & Balic, 2015) and one primary function of these immune cells is phagocytosis (Sjaastad, 2016). A bacterial infection or exposure to bacterial compounds such as lipopolysaccharide can cause a dramatic increase in the number of heterophils in the circulation (Juul-Madsen et al., 2014).

Monocytes are immature macrophages circulating in the blood (Tizard, 2013). When monocytes enter the tissue, their size increases, converting them to macrophages (Sjaastad, 2016). They are crucial fighters in long-term infections due to phagocytosis (Juul-Madsen et al., 2008). Macrophages bear several receptors on their surface which facilitate them in their functioning. During disease conditions such as coccidiosis, the number of MRC1L-B+ macrophages increases rapidly (Sutton et al., 2022).

Thrombocytes (blood platelets) are the most abundant white blood cells. They can be 20-40 times more numerous than other leukocytes (Sjaastad, 2016), and only erythrocytes are more numerous than thrombocytes in the blood (Astill et al., 2022). The primary function of platelets is to stop bleeding by attaching to injured sites (Kaiser, 2010; Sjaastad, 2016). In addition, thrombocytes play a role in innate and adaptive immunity through phagocytosis and the release of cytokines (Astill et al., 2022).

The natural killer (NK) cells are part of the innate and adaptive immune systems (Juul-Madsen et al., 2008). NK cells destroy abnormal cells by identifying foreign proteins and phospholipids in the membrane of cancer cells or virus-infected cells (Sjaastad, 2016). The duration of the high number of NK cells can vary depending on the type of infection. For instance, in *Mycoplasoides gallisepticum* infection, the increase can be sudden but short-lived (Juul-Madsen et al., 2008). In contrast, in the case of infection with Marek's disease virus, the higher levels can be maintained for an extended period in genetically resistant chickens (ibid.).

Adaptive Immune cells

The adaptive immune system comprises lymphocytes, and lymphocyte products e.g., antibodies. There are two major types of lymphocytes: B-cells and T-cells (Sjaastad, 2016). The lymphocytes develop into memory cells after encountering a foreign particle, resulting in a rapid response when the body is exposed to the same foreign material (Tizard, 2013).

T-cells control cellular immunity (Szenberg & Warner, 1962) which is more complex than the antibody-dependent immune response (Day & Schultz, 2014). The most important aspect of T cells is antigen recognition (Viertlboeck & Göbel, 2008). T –cells are categorised into four major types: T helper (Th) cells, cytotoxic T cells, regulatory T cells, and memory T cells (Sjaastad, 2016). The Th cells regulate the cellular immune response by producing cytokines (Tizard, 2013). The cytotoxic T cells are particularly important in fighting intracellular viruses, bacteria, parasites, and cancer cells (Sjaastad, 2016). T-cells increase in response to disease

(Viertlboeck & Göbel, 2008), vaccination (Nii et al., 2014), and stress (Maxwell, 1993).

B-cells produce antibodies that combat antigens (Pastoret et al., 1998). When naive B-cells encounter a new antigen, it takes several days for the B cells to produce antibodies. During the first exposure, sometimes the animal can die before a proper immune response is initiated (Sjaastad, 2016). Time taken for the identification of antigens and proliferation of cells causes the delay in response (Tizard, 2013). Also, during the first exposure, the antibodies are produced to a limited extent and are short-lasting. However, during the second exposure, the response to an antigen is rapid, intense, and longer-lasting (Sjaastad, 2016).

Antibodies, also known as immunoglobulins (Ig), are produced by B-cells and found in the blood and lymph (Litman et al., 1993). Natural antibodies are found in non-immunized chickens (Lammers et al., 2004), whereas immunization with a non-replicating antigen can result in increased levels of antibodies when a booster dose is given at the peak of primary immunization (Nagase et al., 1983). However, if the booster dose is given during the decline phase of the primary vaccine response, it does not result in significantly higher titer values (Härtle et al., 2022). After immunization, peak titers are typically obtained within seven days (Kreukniet et al., 1992). Titers against various antigens can persist for varying durations. For instance, titers against IBV can persist for up to ten weeks (Darbyshire & Peters, 1985).

Factors Modulating the Chicken Immune System

It is well-established that dietary feed ingredients and the animal's physiological state can regulate the immune response (Dalgaard et al., 2022). Both of these factors are discussed briefly below.

Physiological States

Any factor relevant to housing or animal environment that causes stress to the animal can modulate the immune response (Dohms & Metz, 1991). The factors that can influence the animal's physiological state can be temperature, floor system, stocking density, light intensity and type, geographical location, and environment enrichment (Dalgaard et al., 2022). For instance, rearing chickens on slatted floor results in an elevated heterophil-lymphocyte ratio and lower antibody titers than birds on the litter system (El-Lethey et al., 2003). Higher stocking density is considered compromised welfare, resulting in decreased avian immune organ size (Heckert et al., 2002). On the other hand, the provision of perches is linked with

improved welfare, resulting in a lower heterophil-monocyte ratio in laying hens due to less stress (Campo et al., 2005).

Dietary Effects on Immunity

The chicken immune response can be modulated with various dietary nutrients, a process known as nutritional modulation or nutritional immunology (Klasing, 1998). The primary goal of immunomodulation with nutrients is to reduce the use of antibiotics and improve the health and welfare of chickens (Korver, 2012). Amino acids and microminerals are the main nutrients that regulate the immune response (Dalgaard et al., 2022). Amino acids play a role in facilitating the synthesis of proteins essential for the immune response (Bortoluzzi et al., 2020). Among microminerals, zinc and copper are the central modulators of the immune response, as they play a role in the production of immune cells and their products (Percival, 1998; Wessels et al., 2017). In addition to that, the lipid content of feed ingredients can also modulate the immune response (Huo et al., 2019).

The Potential Immunomodulatory Effects of Black Soldier Fly Larvae Feeding on Poultry: A Review of Current Research

The exoskeleton of larvae contains certain substances, such as chitin, which is not produced in birds and mammals. Chitin can stimulate the immune response (Elieh Ali Komi et al., 2018) and can be identified in the gut by chitinases and chitinase-like proteins, which can initiate cytokine production (Lee et al., 2018). Cytokines further stimulate the immune system by recruiting macrophages and leukocytes (Deng et al., 2008). Mannose receptors, located on the surface of macrophages, also initiate an immune response against chitin. The immune response to chitin can vary based on the source, size, and shape of the chitin (Alvarez, 2014). In addition to chitin, the lipid content of BSFL can also modulate the T lymphocyte subpopulations (Huo et al., 2019).

A few studies have investigated the impact of BSFL feeding on the immune response of poultry. Lee et al. (2018) found that BSFL meal feeding in broiler chickens significantly enhanced the population of CD4+ T lymphocytes in the spleen, potentially improving the ability to combat nonspecific infections. The authors also concluded that broiler chickens fed with BSFL could combat the *Salmonella* challenge better. In contrast, in fish, Tippayadara et al. (2021) found no significant difference in the population of leukocytes, including neutrophils, monocytes, lymphocytes, and thrombocytes, when BSFL meal was included in the fish diet. Liu et al. (2021) observed that a 3% inclusion of BSFL meal in the diet of broiler chickens improved the plasma antioxidant status and led to a linear increase in antibodies against Newcastle disease virus. Mat et al. (2022) reported that the

inclusion of BSFL meal in broiler chicken diets did not affect the total leukocyte counts or numbers of granulocytes in blood but resulted in higher numbers of monocytes at 42 days of age.

Other than the findings of positive or no effect on leukocyte counts or antibody production, de Souza Vilela et al. (2021) identified in one study on broiler chickens that BSFL meal feeding significantly decreased the total leukocyte and lymphocyte counts in blood as wells as some intestinal lymphocyte populations at the age of 21 and 42 days with increased inclusion of BSFL.

Given the different results found and limited information currently available on the effect of BSFL feeding on immune parameters in laying hens, further research is warranted to clarify the impact of feeding live or dried whole BSFL to laying hens on these parameters.

The Positive Impact of BSFL Feeding on Immunity

The feeding of BSFL to laying hens has been shown to enhance their welfare by mimicking their natural diet in the wild (Koutsos et al., 2023). BSFL feeding can reduce stress in poultry and improve immunity, as stress causes immunodepression (Dohms & Metz, 1991). According to Jong and Guémené (2011), any factor that can increase or decrease stress can ultimately affect the immune response. Star et al. (2020) concluded that the provision of live BSFL can enhance the natural behavior of laying hens, which is linked to enhanced welfare and decreased stress. Therefore, by providing larvae as a source of environmental and nutritional enrichment, it can have a positive impact on the immune response.

Effects of BSFL Feeding on Egg Quality

Egg quality is influenced by various factors, with feed ingredients and age being the most common under normal circumstances (Sherwood, 1958). Egg quality parameters include egg weight, eggshell breaking strength, eggshell thickness, albumen weight and height, yolk colour, and yolk weight (Fig. 1).

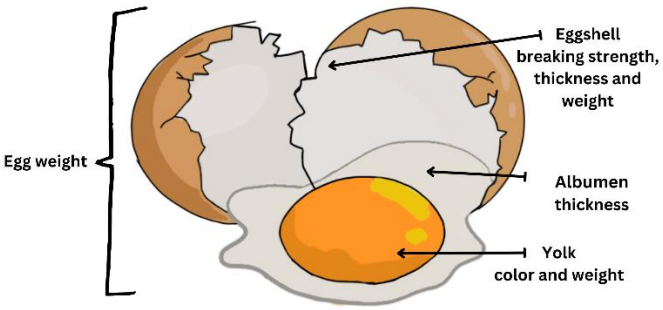

Figure 1: Egg quality parameters. Illustration by Muhammad Adnan Aslam

The use of BSFL as a replacement for SBM in laying hen diets has been the subject of several studies. Many of these studies have found no negative impact of BSFL feeding on egg production and quality. For instance, Tahamtani et al. (2021) found no effect of live BSFL feeding on egg production and quality parameters in hens aged 18-30 weeks compared to providing a standard pelleted diet. Similarly, Maurer et al. (2016) reported no significant difference in egg production between hens fed BSFL meal and those fed SBM, regardless of the replacement percentage, in hens aged 64-74 weeks. In a study involving older laying hens (67-78 weeks), Star et al. (2020) also observed no effect on egg production and quality with the provision of live BSFL. While Patterson et al. (2021) reported no significant impact of BSFL meal on egg production or other egg quality parameters, they did observe a change in yolk colour. Likewise, Liu et al. (2017) found no significant effect on egg quality parameters with BSFL meal as a partial replacement for SBM. Table 1 provides a summary of these findings.

Other studies on the effects of BSFL as a replacement for SBM in laying hen diets have reported mixed results. Chronologically, Bovera et al. (2018) found a positive influence on egg production with 25% replacement of SBM with BSFL. However, Marono et al. (2017) found that complete replacement of SBM with BSFL meal resulted in decreased egg production and egg weight. Secci et al. (2018) did not find any significant difference in egg weight, shell weight, and yolk weight but did observe a negative effect on albumen weight with 100% replacement with BSFL meal. In more recent studies, Bejaei and Cheng (2020) found a negative effect on egg weight, eggshell weight, and albumen weight with 50% or 100% replacement of SBM with dried BSFL in mashed feed. Ruhnke et al. (2018) reported that provision of dried larvae in a free-range system led to decreased egg weight, eggshell thickness, and eggshell weight. However, Secci et al. (2020) found a positive effect on egg weight, eggshell weight, and yolk weight with 25% or 50% replacement with BSFL meal.

Overall, these studies indicate that the effects of BSFL as a replacement for SBM on egg production and quality in laying hens are not consistent and may depend on various factors such as the percentage of replacement, the form of larvae, method of feeding the larvae and the production system.

Effects of BSFL Feeding on Feather Condition

The most significant factor affecting the feather condition of laying hens is abnormal behaviour, such as feather pecking, which can have severe welfare and economic consequences. Proper management practices can decrease the incidence of feather pecking, resulting in improved feather condition. For instance, providing

Table 1: An Overview of Studies Investigating the Impact of Replacing SBM with BSFL Meal on Egg Quality and Integument Condition in Laying Hens: A Literature Review

Studies with No Effect on Egg Production and Quality					
Parameter	Effect	Laying Hen Age	BSF Treatment	Distribution	Reference
Egg Production and Quality	No Effect	64-74w	Dried, 50% and 100% replacement	Mixed with feed	Maurer et al., 2016
		67-78w	Live with soy free feed	Specialised dispenser	Star et al., 2020
		18-30w	Live, 10%, 20%, and ad lib	Feeding bowls	Tahamtani et al., 2021
		45-53w	Larvae meal, 1, 3, or 5% replacement	Mixed with feed	Liu et al., 2021
		25-37w	Live Larvae, 10, 20, 30 g/kg	-	Lokaewmanee et al., 2023
Studies with Positive or Negative Effect on Egg Production or Quality					
Egg Production	Negative Effect	24-45w	Larvae meal, 100% replacement	Mixed with feed	Marono et al., 2017
	Positive Effect	16-36w	Larvae meal, 25% replacement	Mixed with feed	Bovera et al., 2018
Egg Weight	Negative Effect	18-35w	Dried and chopped, 50 and 100% Replacement	Mixed with Mash feed	Bejaei & Cheng, 2020
	Positive Effect	16-36w	Larvae Meal, 25 or 50% replacement	Mixed with feed	Secci et al., 2020
	Negative Effect	47-56w	Dried Larvae *Ad libitum*	Insect feeders in range	Ruhnke et al., 2018
	Negative Effect	24-45w	Larvae meal, 100% replacement	Mixed with feed	Marono et al., 2017
Eggshell Breaking Strength	Positive effect	28-43w	Larvae Meal, 100% replacement	Mixed with feed	Mwaniki et al., 2020
Eggshell thickness	Negative Effect	47-56w	Dried Larvae *Ad libitum*	Insect feeders in range	Ruhnke et al., 2018
	Negative Effect	16-36w	Larvae Meal, 50% replacement	Mixed with feed	Secci et al., 2020
Eggshell Weight	Negative Effect	18-35w	Dried and chopped, 50 and 100% Replacement	Mixed with Mash feed	Bejaei & Cheng, 2020
	Negative Effect	47-56w	Dried Larvae *Ad libitum*	Insect feeders in range	Ruhnke et al., 2018
	Positive Effect	16-36w	Larvae Meal, 25 or 50% replacement	Mixed with feed	Secci et al., 2020

Albumen Height	No Effect	43-55w	Larvae Meal 8, 16, 24%	Mixed with feed	Patterson et al., 2021
Yolk Colour	Lower score	47-56w	Dried Larvae *Ad libitum*	Insect feeders in range	Ruhnke et al., 2018
	Higher score	43-55w	Larvae Meal 8, 16, 24%	Mixed with feed	Patterson et al., 2021
	Higher score	28-43w	Larvae Meal, 100% replacement	Mixed with feed	Mwaniki et al., 2020
Yolk Weight	Positive Effect	16-36w	Larvae Meal, 25 or 50% replacement	Mixed with feed	Secci et al., 2020
			Integument Condition		
Feather Condition	No Effect	64-74w	Dried, 50% and 100% replacement	Mixed with feed	Maurer et al., 2016
	Positive Effect	0-35d*	Live, 10% replacement of FI	Feeding plate	Veldkamp & Van Niekerk, 2019
	Positive Effect	67-78w	Live with soy free feed	Specialised dispenser	Star et al., 2020
Skin Injures	No Effect	64-74w	Dried, 50% and 100% replacement	Mixed with feed	Maurer et al., 2016
	Positive Effect	0-35d*	Live, 10% replacement of FI	Feeding plate	Veldkamp & Van Niekerk, 2019

*Turkeys aged 0-35 days

environmental enrichment, such as litter, perches, and outdoor access, has been shown to reduce the incidence of feather pecking and skin injuries in laying hens (Xu et al., 2022). Litter material, for example, enhances foraging behaviour and decreases the incidence of feather pecking (Huber-eicher & Wechsler, 1998)

Providing larvae to laying hens can act as environmental enrichment, resulting in improved feather condition scoring. In one study by Star et al. (2020), non-beak-trimmed laying hens aged 67-78 weeks were provided with live larvae at a rate of 10% of daily feed intake, resulting in better feather condition scoring. Similarly, Veldkamp and Van Niekerk (2019) observed better feather condition scoring in turkeys aged 0-35 days with the provision of 10% live BSFL in feeding plates. However, in a study by Maurer et al. (2016), no effect on feather condition was observed when laying hens aged 64-74 weeks were fed a diet with a 50% and 100% replacement of soybean cake with partially defatted dried BSFL meal. The same authors also did not observe any difference in skin injuries with larvae feeding. In contrast, Veldkamp and Van Niekerk (2019) noted a lower incidence in skin injury in turkeys aged 0-35 days when live BSFL were provided as a 10% replacement of daily feed intake offered in feeding plates. These findings suggest that whole larvae could be acting as an enrichment reducing the risk of feather pecking.

The content of sulphur-containing amino acids, particularly methionine, plays a critical role in the feather development of chickens (Eriksson et al., 2007). It has been observed that BSFL meal has a higher methionine content than soybean meal (Bovera et al., 2018). Consequently, based on methionine content and bioavailability, larvae feeding can influence feather condition

BSFL feeding can initiate an immune response in laying hens due to the chitin in the exoskeleton. The activation of the immune response can influence feather condition. Parmentier et al. (2009) challenged laying hens with the model antigen human serum albumin and immunogenic lipopolysaccharide (LPS) and observed that immune challenge resulted in feather and comb damage. Therefore, an immune challenge can also initiate feather pecking in laying hens.

Materials and Methods

Animals and Housing

Animals

The experiment involved 360 Bovans Robust White hens, which were obtained from a commercial rearing farm (Närkesberg Hönseri AB, Åsbro, Sweden) at 18 weeks of age and kept at the Swedish Livestock Research Center, Lövsta, until 50 weeks of age. The hens were not subjected to beak trimming. The data collection for this study commenced at 30 weeks of age and continued until the end of the experiment. Prior to the experiment, all hens were vaccinated against infectious bronchitis (IB). The primary vaccination was given at 5 weeks of age with IBV strain 4/91 in drinking water. Subsequent booster vaccinations were administered at 10 and 12 weeks (Ma5 strain), 15 weeks (4/91 strain), and 30 weeks (Ma5 strain) of age, in drinking water. In addition, the hens were also vaccinated against Marek's disease via the subcutaneous route in the neck, coccidiosis (at week 1) through drinking water, and chicken encephalomyelitis (at 14 weeks of age) via the wing web. Hens were individually marked with colour coded plastic leg rings to facilitate identification. The health status of the experimental hens was monitored visually daily. In the event of injury due to feather, toe, or vent pecking, affected hens were excluded from further data collection.

Housing

The Swedish Board of Agriculture (Jordbruksverket) regional ethical committee approved all animal protocols in this study under Diarienummer 5.8.18-03402/2020.

Birds were housed in 18 identical pens (20 hens per pen, pen dimensions: 3m × 3.56m × 3.62m, H × W × L). Each pen included a slatted floor area (2.30m × 3.56m, W × L), perches, and a litter area (1.32m × 3.56m, W × L) with wood shavings. In addition, two colony nest boxes (46 cm × 115cm, W × L each), two round feeders, and one bell drinker were provided in each pen (Figure 2). Hens had ad libitum access to feed and water, and water was supplied through bell drinkers, which were cleaned every day. The feed was provided in automatic round feeders. Lighting was provided for 16 hours daily with an intensity of 10 lux. Ventilation was automated

with a Fancom system (Netherlands), and the temperature was maintained at 20 to 23 °C.

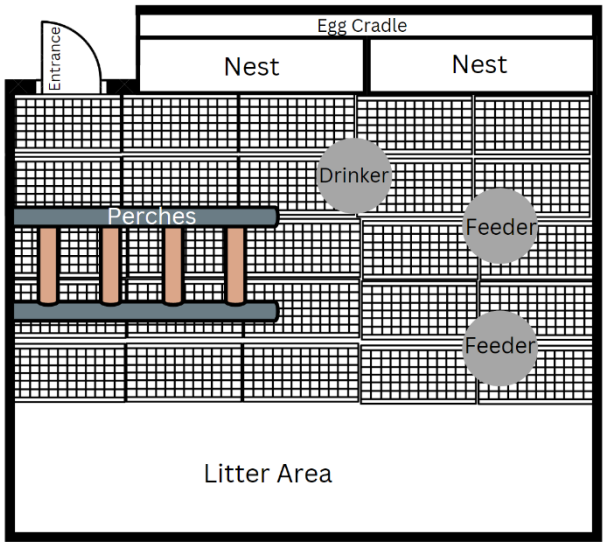

Figure 2: Schematic illustration of a pen

Experimental design

The experiment, which provided the hens for the current study, began with 360 Bovans Robust White hens. These hens were placed in groups of 20 hens per pen, resulting in a stocking density of fewer than 2 hens/m^2. This is significantly lower than the recommended stocking density of 9 hens/m^2 as outlined in Council Directive 1999/74/EC. The layout of the experimental pens inside the barn is shown in Figure 3.

The hens were randomly allocated to one of three dietary treatments: 1) control, 2) live larvae or 3) dried larvae).

Control	Live Larvae	Dry Larvae	Live Larvae	Dry Larvae	Live Larvae	Control	Control	Dry Larvae
Pen 10	Pen 11	Pen 12	Pen 13	Pen 14	Pen 15	Pen 16	Pen 17	Pen 18
Pen 9	Pen 8	Pen 7	Pen 6	Pen 5	Pen 4	Pen 3	Pen 2	Pen 1
Dry Larvae	Control	Dry Larvae	Live Larvae	Control	Live Larvae	Dry Larvae	Live Larvae	Control

Figure 3: Schematic representation of the poultry shed illustrating pen distribution and treatment group allocation. Pen 1 and pen 18 were facing the entrance door of the stable.

Dietary treatments

The hens were randomly allocated to one of three dietary treatments:

1. Control Group: With no black soldier fly larvae. Pelleted feed was provided ad libitum in the feeders and also approximately 60 grams of pellets were scattered daily in the litter area at 8:30 am

2. Live larvae Group: 20% of their expected daily dry matter intake was provided as live larvae (59.5g live larvae per hen/day). Larvae were provided daily by scattering in the litter area at 8:30 am.
3. Dried larvae Group: 20% of their expected daily dry matter intake was provided as dried larvae (21.8g dried larvae per hen/day). Larvae were provided daily by scattering in the litter area at 8:30 am.

Diet Design

The control group was provided with a soybean-based diet, formulated to meet the specific requirements of Bovans Robust White. In the treatment groups, based on the findings of Tahamtani et al. (2021), 20% dry matter of feed was replaced with live and dried larvae. The amount of replacement was determined by analysing the proximate composition of the larvae. The dry matter content of larvae was measured by drying at 103°C, and the ash content was calculated after burning at 600°C for three hours (Jennische & Larsson, 1990). The larvae were analysed for crude protein by the Kjeldahl method (NMKL, 2003) and crude fat was quantified by ether extract (European Commission, 1998). The crude fiber was measured as European Commission Directive 152/2009 EC (2009) described. The proximate composition of larvae is given in table 2. The amino acids profile was evaluated as described by Traksele et al. (2021). Based on the analysis, feeds for larvae groups were formulated to fulfil the daily nutritional requirements when 20% of daily dry matter intake was replaced with live and dried larvae.

The hens were housed in pens at 18 weeks of age and fed a respective diet according to their assigned groups (i.e. control, live larvae, or dried larvae) till 30 weeks of age when data collection for this experiment started. From 18 to 30 weeks, behavioural tests were conducted on the laying hens. The same diet was provided till the end of the experiment.

Table 2: Proximate analysis of larvae samples

Sample	Crude Protein (g/Kg DM)	Crude Fat (g/Kg DM)	Crude Fiber (g/Kg DM)	Ash (g/Kg DM)
Larvae	397.2	338.2	42.96	78.5
Dried Larvae	404.6	359.1	44.99	78.9

Black Soldier Fly Larvae Production

The larvae were produced at the larvae production unit located at the Department of Energy and Technology of the Swedish University of Agricultural Sciences (Uppsala, Sweden).

The larvae production was carried out in an environment-controlled facility between June 2022 and October 2022. The temperature was maintained at 23°C, and humidity at 65%. Racks were installed in the larvae production unit, with each

rack accommodating 11 boxes. Each box contained approximately 12,000 larvae (60cm x 40cm x 20cm). The larvae were fed with moistened pig's commercial feed (Svenska Foder, Sweden) from the Swedish Livestock Research Center, Lövsta, Sweden, with a feed-to-water ratio of 1:2 to produce larvae feed with 30% DM. The larvae were fed four times during the growth phase, twice a week. Harvesting occurred when 5% of the larvae entered the pupa stage to ensure a consistent chemical composition of the larvae.

To harvest the larvae, the top layer of substrate was removed manually. The rest of the larvae and substrate mixture was processed through a vibrating screen machine to separate the substrate from the larvae. Before putting the larvae in transportation boxes, the substrate was again removed, which larvae had brought on top with self-isolation from the substrate.

For the live larvae group, the larvae were transported to the Swedish Livestock Research Center Lövsta, where they were portioned into boxes according to the number of remaining hens in each pen (Fig. 4). Holes were made in the lid of the portioning boxes for air circulation. After portioning, the larvae were stored in a cold room at 15°C (for 1-4 days) to delay larvae turning into pupa as larvae where harvested and brought to farm twice per week.

Figure 4: Portioning of live larvae for live larvae group

For the dried larvae group, the larvae were initially euthanised by freezing in a freezer for 24 hours. Subsequently, they were dried in a forced air oven (MF 10/31, Weber) at a temperature of 60°C for a duration of 48 hours. Following the initial three-hour drying period, the larvae within each tray were thoroughly mixed and flipped to avoid any potential sticking together. Once the 48-hour duration elapsed, the larvae were collected from the oven, packed in a polythene bag, and stored at -20°C for future use. The dry larvae were portioned at Lövsta in transparent airtight plastic boxes to prevent the larvae from absorbing moisture from the environment.

Vaccination, Blood Collection and Processing

Blood samples were collected from two hens in each pen. The same hens, marked with leg rings, were used for blood collection each time. Twelve blood samples per group were collected from both the live and dried larvae groups. To induce antibody responses, hens were booster vaccinated with IBV Ma5 strain at 30 weeks of age. Blood samples were collected on five occasions at approximately 4 weeks intervals: two days before vaccination and 26, 48, 76, and 104 days after vaccination. All blood samples were collected from the jugular vein. After collection, blood samples

were divided into two parts: one was transferred to a blood collection tube with K_2-EDTA as an anticoagulant (EDTA vacutainer BD, USA), and the other to a test tube without any additive. EDTA-stabilized blood samples were used for leukocyte counts, as described below. Untreated blood samples were stored at room temperature overnight, centrifuged at 13,000 revolutions per minute for 10 minutes, and then the serum fractions were collected and stored at -20°C until analysis by ELISA.

Immunofluorescence Labelling for Flow Cytometric Analysis of Leukocyte Counts

For direct labelling of white blood cells, 25 µL of EDTA blood was mixed with 600 µL of FACS buffer (including 2mM EDTA as additive) in an Eppendorf tube. This diluted blood was vortexed on a mixer, and immediately after that, 50 µL of diluted blood was mixed with 50µL of antibody panel master mixes (table 3). To ensure the mixed sample is 100 µL, reverse pipetting was done. Single-colour controls and fluorescence minus one (FMO) controls were prepared. Samples were vortexed gently and incubated for twenty minutes at room temperature in the dark. Then, 275 µL of FACS buffer and 125 µL of 4% paraformaldehyde (PFA) were added to the sample, and reversed pipetting was done and vortexed again. The samples were then stored at 4 °C overnight. Prior to FACS, 75 µL of FACS-buffer and 25 µL of 123count eBeads counting beads (#011234-42, Invitrogen, ThermoFischer Scientific) were mixed and this 100 µL staining volume was added to each sample tube. Beads were vortexed for 15 seconds before pipetting. The concentration of beads was marked on each bead vial. For this experiment, it was 1009000 beads/ml. Samples were mixed well on a vortex mixer before running in FACSverse.

Flow cytometry was performed using a BD FACSVerse (BD Biosciences), equipped with 488 nm blue, 633 nm red and 405 nm violet lasers, and results were analysed using the FACSDiva (BD Biosciences) software. Single-stained compensation controls and FMO negative controls were included in the assay, and samples were recorded for 1 min. The gating strategy for leukocyte count is shown in appendix 1. The number of events counted in the bead gate was according to the manufacturer's recommendations (at least 1000). The bead concentration and total number of events were used to determine the volume of blood sample and to calculate absolute numbers of the leukocyte populations. Titrations of all antibodies were performed to determine optimal labelling conditions prior to the experiment. The following equation was used to calculate individual cell populations in each sample.

Absolute cell count/µL blood = (cells counted//(beads counted / total content of beads per tube))*300 (final blood dilution)

Table 3: Antibodies used for immunolabelling of 25µl whole blood in a no-wash absolute count assay

Abbreviation	*Clone*	*Specificity*	*Fluorochrome*
CD45-PerCP/Cy5.5	UM16-6†	Chicken CD 45, all isofroms	Peridinin chlorophyll-cyanine 5.5§
CD41/61-FITC	11C3†	Chicken CD41/CD61	Fluorescein¶
MHCII-PE	2G11ǂ	Chicken/Pigeon/Caiman MHC class II β-chain	R-phycoerythrin¶
Kul01-AF647	KUL01ǂ	Chicken Monocyte/Macrophage	Alexa fluor 647¶
CD4-pacific blue	CT-4ǂ	Chicken CD4	Pacific Blue¶
CD25-PECy5	AV142†	Chicken CD25, interlukin-2 receptor α-chain	R-phycoerythrin-cyanine 7§

† Purchased from Bio-Rad Antibodies

ǂ Purchased from SouthernBiotech

§ Fluorochrome conjugated using Lightning-Link conjugation kits (abcam) - according to the manufacturer's protocol

¶ Fluorochrome conjugated by manufacturer

ELISA

Antibodies to IBV were quantified by Enzyme-linked immunosorbent assay (ELISA) using a commercially available kit - IDEXX IBV Ab Test (IDEXX Laboratories, Maine, USA). The antibody test plate was first designed on paper with the same layout as of plate about how each sample will be placed on the plate. It made it easier to track the result of each sample. Serum from each blood sample was diluted 1:500. 500 µL commercial sample diluent (IDEXX Laboratories, Maine, USA) was poured in an Eppendorf tube with a pipette (Thermo Scientific, Finland), and each tube was marked according to sample number. Before dilution, the serum was shaken vigorously on a shaker (IKA MS 3 basic, staufen, Germany)

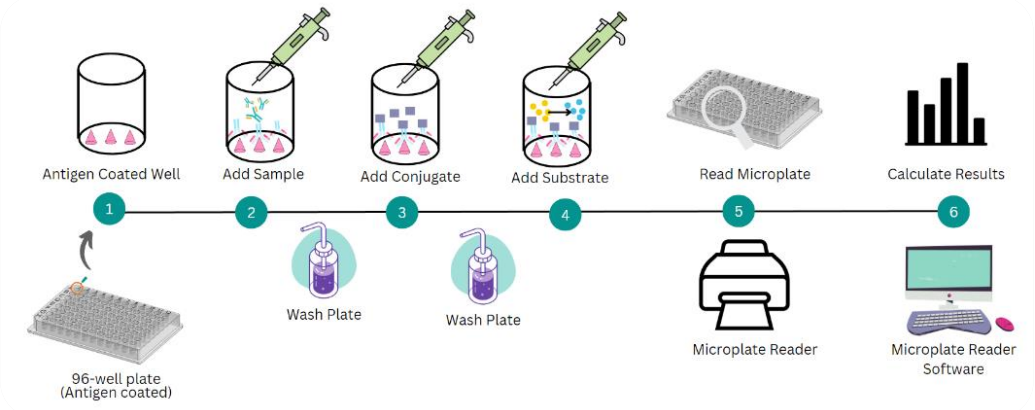

Figure 5: A workflow of enzyme-linked immunosorbent essay. Illustration by Muhammad Adnan Aslam, inspiration from moleculardevices.com

at a speed of 3000 RPM. A serum sample of 1 µL was taken with a micropipette (Thermo Scientific, Finland) to mix with diluent in an Eppendorf tube. Each sample was dispensed in duplicates in the antigen-coated plate. A 100 µL sample was dispensed in each well of the plate according to the positions drawn on the paper. Both positive and negative control, 100 µL each, were also dispensed in duplicate in undiluted form as provided by the manufacturer. After dispensing all control and test samples, they were allowed to incubate for 30 minutes at room temperature (18-26°C). After incubation time, the solution was removed from each well and each well was washed with approximately 350 µL of deionised water five times. The plate was taped on absorbent paper to remove all the residual deionised water. After that, 100 µL conjugate was dispensed in each well with a pipette and it was allowed again to incubate for 30 minutes at room temperature. All the wells of the plate were washed again with the same procedure. A 100 µL of TMB substrate, provided by the manufacturer, was dispensed in each well followed by incubation of 15 minutes at room temperature. Finally, 100 µL of stop solution, provided by the manufacturer, was dispensed in each well. Absorbance was measured at 650 nm in a microplate reader (Tecan, Switzerland). The validity of the test was measured based on the positive and negative control values. The workflow of ELISA is shown in figure 5.

Negative Control (NC) = NC 1 + NC 2 / 2
Positive Control (PC) = PC 1 + PC 2 / 2

Validity Criteria
Negative Control ≤ 0.150
Positive Control – Negative Control >0.075

Log10 titer was calculated by using following equations;
S/P = Sample Mean – Negative Control / Positive Control – Negative Control
Log10 Titer = 1.09 (log10 S/P) + 3.36

Egg Quality

Eggs were collected from individual pens. Egg quality was measured during week 40 and week 49. For egg quality, five fresh eggs from each pen, 30 eggs per treatment, were randomly collected on the day of the test and were marked with a permanent marker. The following parameters were measured:

Egg Weight

The weight of individual eggs was measured by using Mettler PE 160 laboratory scale. Egg weights were recorded with a two-decimal places accuracy.

External Quality

Eggshell Breaking Strength

Eggshell breaking strength was measured using an Egg Force Reader as shown in figure 6 (Orka Food Technology Ltd., West Bountiful, UT). Each egg was placed individually on the egg cradle with the pointed side of the egg downward. The probe of the Egg Force Reader exerted pressure on the egg until the egg cracked slightly. Values were recorded in kilograms-force exerted by the probe until the egg cracked.

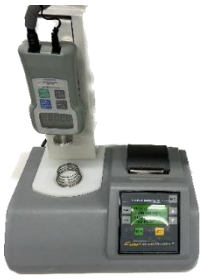

Figure 6: Egg Force reader used to measure eggshell-breaking strength

Eggshell Thickness

Eggshell thickness was measured after cracking the egg. The egg membrane was separated from the eggshell. Eggshell thickness was measured using a Mitutoyo Absolute digital micrometer (NO. 7360). Eggshell thickness was measured by placing the shell in the jaw of the micrometer, and three recordings were noted from three different points of the shell along the equator.

Eggshell Weight

To measure the shell weight, eggshells were dried along with the shell membrane at 103 °C for 16 hours. The eggshells were allowed to cool down overnight in the oven. The eggshells were weighed individually by using PE 160 laboratory scale.

Internal Quality

Albumen Height

Albumen height was measured after breaking the eggshell and pouring the egg contents on a levelled glass surface. The Ames S6428 micrometer (U.S.A) was placed on the glass surface with the measuring tip of the micrometer around 0.5cm away from the yolk. The measuring tip was lowered by revolving the knob until the tip touched the albumen. Albumen height was measured with 0.1mm accuracy.

Yolk Colour

The colour of the yolk was quantified by using a Roche Yolk Colour Fan (Switzerland). Yolk colour was measured on a scale of one to fifteen.

Yolk Weight

Yolk weight was measured on Mettler PE 160 laboratory scale after separating the yolk from the albumen. The yolk was separated from the albumen with the help of a yolk separator.

Integument Scoring

Integument scoring using the LayWel protocol (Tauson et al., 2005) was conducted in all hens at 40 and 50 weeks of age. Feather condition scoring was conducted as per LayWel protocol (Tauson et al., 2005) at six regions of each hen, including the neck, back, breast, tail, wings, and cloaca as show in figure 7. Hens were also scored for injuries on three regions of the body (comb, rear body, and footpad). The scoring system utilized a scale ranging from 1 to 4, where a score of 1 represented the worst integument condition, and 4 represented the best integument condition. (Table 4).

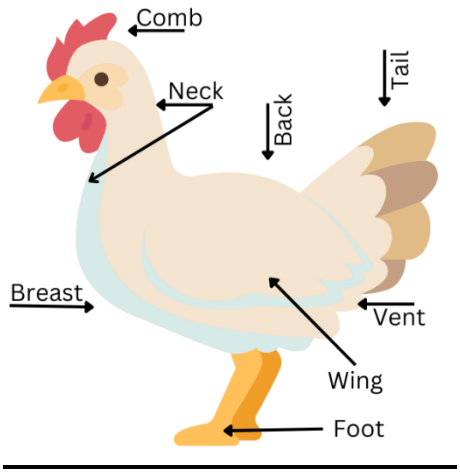

Figure 7: The body parts observed for feather and skin injury scoring

Table 4: Description of integument scoring used to assess neck, breast, cloaca/vent, back, wings, and tails of all hens of control and larvae group hens

Score	Description
4	No or minimal feather and skin damage (no bare skin visible, no pecking injury, no or slight feather damage, no inflamed bumble foot)
3	Minor feather wear or pecking and no bare skin visible. Minor scratches on the body and small dotted pecking injuries on the comb.
2	Skin is visible on half of the scoring area, for instance, a half-naked neck or breast. Bigger two or more pecking injuries on the comb and one skin wound <1 cm diameter.

1	Severe damage to the integument. For instance, intense feather pecking with almost total loss of feather from the scoring area, wound >2cm in diameter, aggressive pecking to the head area or inflamed bumble foot lesions, respectively.

Body and Organ Weight

At the end of the experiment, when the hens reached 50 weeks of age, they were euthanized via intravenous injection of pentobarbital. The gastrointestinal tract was then retrieved from the euthanized hens. The proventriculus and gizzard were emptied and weighed separately, and the weight of the liver and fat pad was also recorded.

Management of Feather Pecking

The outbreak of feather pecking was observed in the control group at 22 weeks of age. Due to the random allocation of treatments to pens, control pens were next to one of the other larvae treatments, allowing hens to see and occasionally pick larvae from the adjacent pens. This issue was addressed by placing opaque plastic between the control and larvae group pens. Initially, it was believed that the feather pecking and cannibalism were due to the control group hens' frustration with not getting access to larvae. To address this issue, pelleted feed was scattered in control group pens at the same time when larvae were provided to larvae groups. The intensity of feather pecking did not decrease and resulted in seriously injured hens. Considering the welfare of hens, two control group pens (pen 5 and pen 10) were offered larvae at 32 weeks of age, but this measure also did not solve the problem. Observing the high mortality, these pens were excluded from the experiment along with another control group pen 16.

Statistical Analysis

For immune parameters, data from two replicates of the control group were excluded from the analysis as larvae were offered to laying hens due to a severe outbreak of feather pecking. Data could not be obtained from another control replicate during the last sampling as that replicate was closed due to intense feather and toe pecking. The result of one blood sample during the first and second blood sampling was excluded due to blood clotting. The exclusion of the above-mentioned replicates did not make any statistical difference for integument and egg quality parameters, so those groups were included in the statistical analysis of these parameters.

Hen’s body weight was weighed as a group, and the collective pen weight was divided by the number of hens to get the average individual hen’s body weight. At the end of experiment, before weighing organs, hens were also weighed individually for the body weight.

Egg weight and Integument condition data were analysed using Restricted Maximum Likelihood (REML) analysis allowing for treatment, week number as fixed effects, with sample id nested within pen as random effect. First-order interactions were tested for inclusion. Other egg parameters were tested similarly, with the addition of egg weight as a random effect. The assumption of normality of the residuals was tested using the Shapiro-Wilk test. Data that did not pass this assumption was transformed to approximate normality. Tukey HSD adjustment for multiple comparisons was used in all post-hoc tests. The calculations of egg quality, integument condition, body weight, and organ weight were done using JMP 16 statistical analysis software (SAS Institute Inc., Cary, NC). Significance was determined at $P \leq 0.05$.

Descriptive analysis was performed on the flow cytometry and antibody titers data. The mean values and 95% confidence intervals (CI) were calculated for each type of leukocyte. Non-overlapping 95% CI at any given point indicated a significant difference between groups. All descriptive analysis calculations were conducted using MS Excel (2016).

RESULTS

Experimental Feed Analysis

At the end of the experiment, feed samples from both the control and larvae groups were analysed for nutrient composition, revealing that there had been an error during manufacturing of the feed resulting in feed for the control group having lower sodium content than the feed given to both larvae groups. The results of the feed analysis are presented in Table 5.

Table 5: Analysis of Control and Larvae Group Feed Samples

	Percentage in Milled Sample					Minerals g/kg Sample				Na
Sample Name	DM (103°C)	Ash	Crude Protein (N×6.25)	Crude Fat	Crude Fibre	Ca	K	Mg	Na	mg/kg
Control Group Feed	91.20	14.02	17.77	9.22	4.82	41.8	7.4	2.2	0.4	470
Larvae Group Feed	90.71	15.13	14.17	5.46	4.14	47.7	6.0	2.2	1.9	2000

Mortality and Excluded Hens

There was a severe outbreak of feather and toe pecking in the control group, resulting in the exclusion of 80 out of 120 hens from the experiment due to severe injuries and mortality. In the larvae groups, four hens were excluded from the live larvae group, while no hens were excluded from the dried larvae group. Percentage of excluded hens form each group is given in table 6.

Table 6: Number of Hens Excluded from each group due to mortality and feather pecking

Group	Number of Hens	Hens Excluded	Percentage
Control	120	80	67%
Live Larvae	120	4	3%
Dried Larvae	120	0	0%

Immune Parameters

Blood Leukocyte Counts

In the control group, 12 blood samples were collected in the first blood collection, 8 blood samples were collected in subsequent collections, and 6 blood samples were collected in the final blood collection. The number of samples decreased as two pens were excluded after the first blood collection due to severe feather pecking, and one control pen was closed before the last blood sampling due to the same problem. When any of the marked hens died, blood samples for

antibody measurement were discontinued from that replicate instead of marking a new hen.

There were no differences in the numbers of any of these cell populations between the control and larvae groups during the experimental period (Fig. 8).

The number of circulating heterophils decreased during the experimental period in the dried larvae group, while there was no specific trend in the control and live larvae group as the number of circulating heterophils fluctuated greatly (Fig. 8A). Near the end of the experiment, heterophils were higher in the control group than in the larvae groups, but the difference was not significant. The numbers of monocytes were slightly higher in blood samples taken from the live larvae group during the first 46 days of the experiment, and then it decreased (Fig. 8B). Like heterophils, the number of monocytes was slightly higher in the control group hens at the end of the experiment. The total number of lymphocytes was moderately higher in the larvae groups than in the control group at the start of the experiment. The levels of lymphocytes kept changing during the experimental period, but there was no clear trend, and there was no difference between groups during the entire experiment (Fig. 8C). At the end of the experiment, the number of lymphocytes was slightly higher in the larvae groups than in the control group. The number of circulating thrombocytes did not differ between groups, and over the duration of the experiment, the total number of thrombocytes decreased slightly in all groups (Fig. 8D).

The numbers of B cells (MHC II+ lymphocytes) increased slightly in all groups at the start of the experiment and then fluctuated inconsistently (Fig. 8E). There was no specific trend for the population of helper T cells (Fig. 8F). The helper and regulatory T cells decreased at the beginning of the experiment and then increased afterwards. The levels of these cells were marginally higher in the larvae group (Fig. 8G). A similar pattern was observed in the population of activated non-CD4 T cells (Fig. 8H).

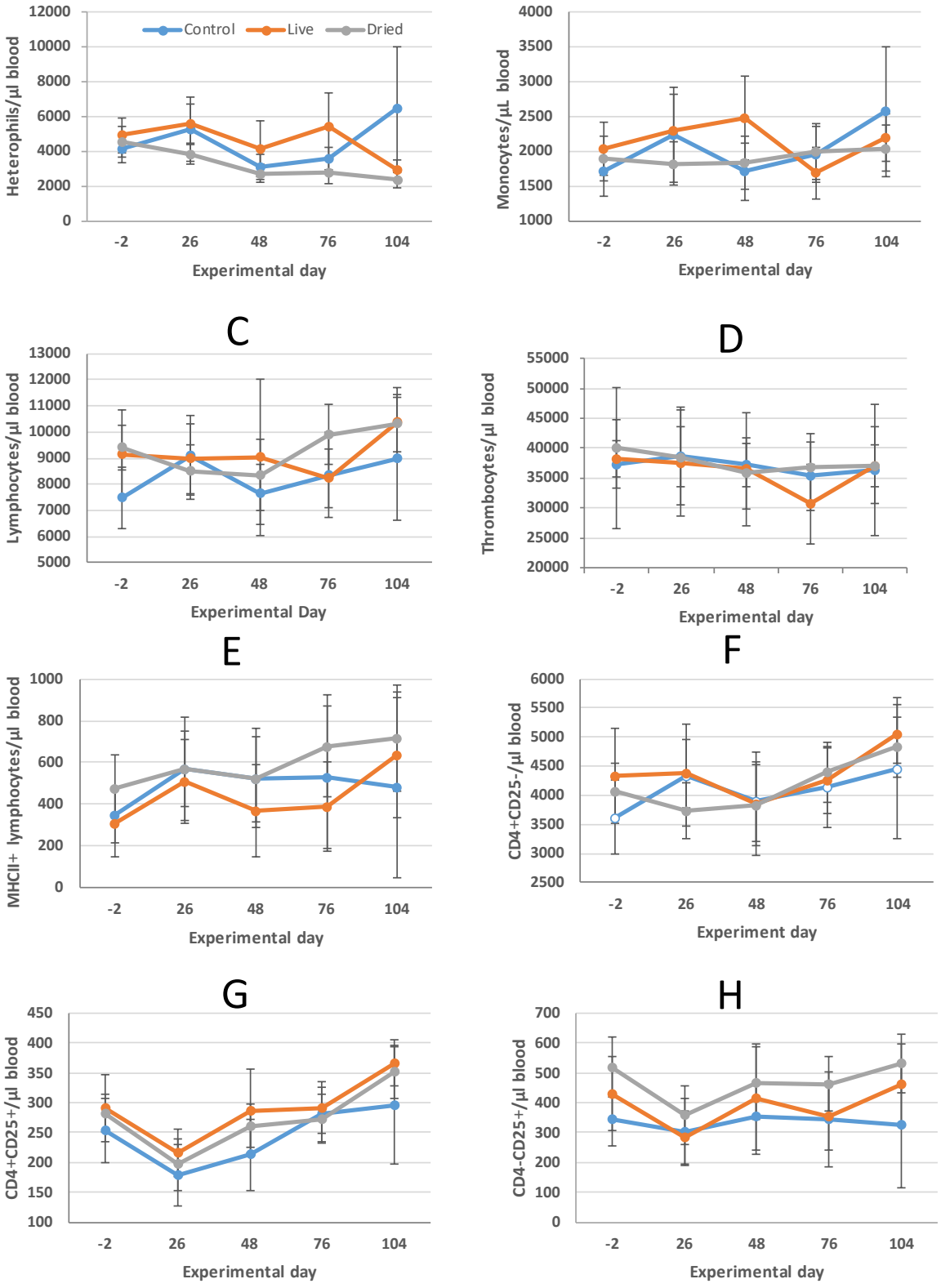

Figure 8: Total number of A) heterophils, B) monocytes, C) lymphocytes, D) thrombocytes E) B cells F) helper T cells G) activated helper and regulatory T cells H) activated non-CD4 T cells in blood samples of laying hens. Blood samples were taken on day -2, 26, 48, 76, and 104 of the experiment. Results are presented as group mean values ± 95% confidence interval (CI), and non-overlapping CI are considered a significant difference between groups. For live and dried group n=12/group. For the control group, on day -2 n=12; on day 26, 48, and 76 n=8; day 104, n=6

Expression of MHC II and Chicken Mannose Receptor MRC1L-B on Monocytes

There was no difference among groups in the expression of chicken mannose receptors MRC1L-B and MHC II on monocytes (Fig. 9). The values of MHC II were very similar in all groups. They resembled each other in how they varied during the whole experiment (Fig. 9B). The values for MHC II on monocytes dropped at the start of the experiment, followed by an increase for an extended period of time, and then reversed to nearly the same levels as at the start of the experiment.

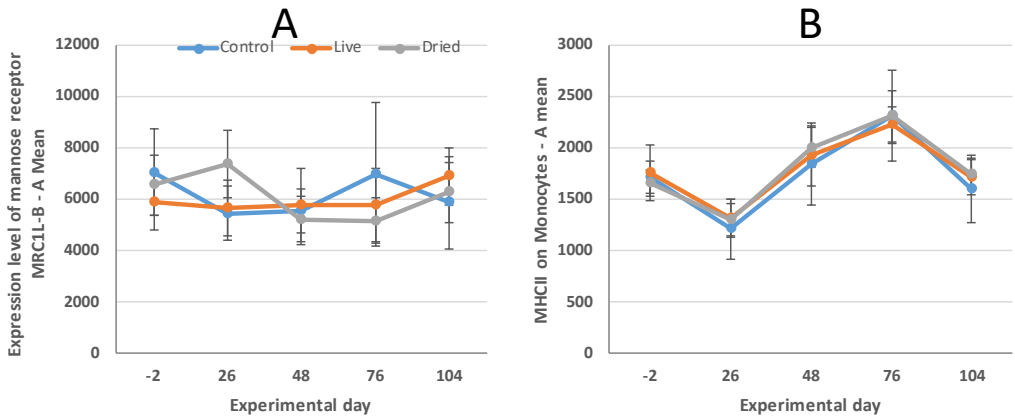

Figure 9: The expression of A) Mannose receptor MRC1L-B and B) MHC II on monocytes. The expression is presented as a mean value. The results are presented as group mean values ± 95% CI and non-overlapping CI represents statistically significant results. For live and dried group n=12/group. For the control group, on day -2 n=12; on day 26, 48, and 76 n=8; day 104, n=6

Antibody titers

Titer values for two samples were not obtained due to zero and negative S/P values. The control and larvae groups did not differ from each other in terms of antibody titers (Fig. 10A). The antibody titers were low before booster vaccination. The titers showed a slight increase after booster vaccination, but this increase was not significant and post-vaccination titers were also low. The mean titer values remained the same for each group throughout the experiment. The dried larvae group exhibited slightly higher titers than the live larvae group, while the control group had intermediate titers. The proportion of titer change within hens was calculated to reduce the influence of variation between individuals (Fig. 10B). There were no significant differences between the different treatments for the proportional alteration in titers to IBV after vaccination. The proportional alteration

in titers within hen after vaccination were moderately higher in the control group than in the larvae group, while both larvae groups exhibited very similar titer changes.

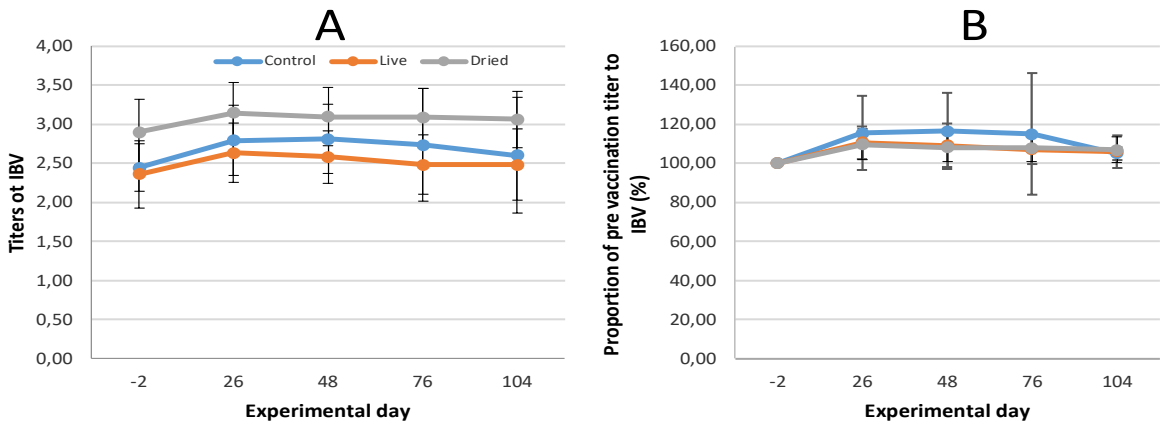

Figure 10: Titers(log10) to IBV B) proportion of vaccination titer to IBV (% before vaccination within hen). Blood samples were taken 2 days before IBV vaccination and then 26, 48, 76, and 104 days after vaccination. Log10 titers are presented as group means ± 95% CI. Significance is determined on the basis of non-overlapping CI. The group sizes for the experiment were as follows: dried group with n=12; live group on day -2 and 7 with n=12, and on day 26, 48, and 104 with n=11; control group on day -2, 26, and 48 with n=8, on day 76 with n=6, and on day 104 with n=4.

Body weight

There was a significant difference in body weight at 42 and 50 weeks of age with the control group having significantly lower weights at both ages ($p<0.0001$). There was an interaction between body weight and age with control and live groups increasing body weight from 42 to 50 weeks of age while the dried group remained the same (Fig. 11).

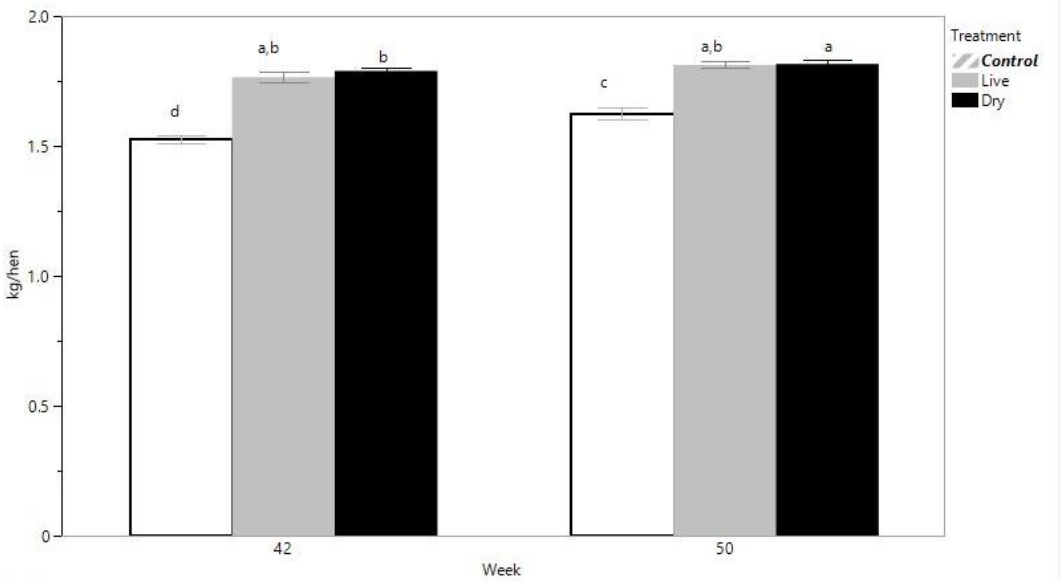

Figure 11: Hen body weight at week 42 and week 50, calculated as combined pen weight divided by number of hens. Different superscript letters indicate significant difference within age.

Egg Characteristics

The control group had a tendency for **yolk weight** to be lighter than larvae groups (LSM±SEM: control = 16.36 ± 0.18, Live = 17.16 ± 0.16, Dried = 17.09 ± 0.19, p=0.051). There was an increase in the yolk weight from week 40 to week 49 (16.66 ± 0.13 vs 17.15 ± 0.16, respectively. p=0.036). The yolk colour significantly became darker for the live and dried larvae group as their age increased. There were no differences in egg weight, eggshell-breaking strength, and albumen thickness among treatments and weeks of age, as shown in table 7.

There was no difference in shell weight and thickness among the control and larvae groups (p>0.5). However, shell weight increased from week 40 to week 49 (6.01 ± 0.05 vs 6.21 ± 0.05, respectively. p=0.22) and shell thickness decreased from week 40 to week 49 (0.40 ± 0.00 vs 0.39 ± 0.00, respectively. p=0.032).

Table 7: Egg quality parameters

	Week No					
	40			49		
	Control	**Live**	**Dried**	**Control**	**Live**	**Dried**
Egg weight (g)	(29) 61.59±0.82[a]	(30) 63.16 ± 0.79[a]	(30) 63.62 ± 0.68[a]	(22) 63.49 ± 0.87[a]	(30) 64.01 ± 0.66[a]	(30) 63.98 ± 0.89[a]
Breaking strength (kg)	(29) 4.48 ± 0.12[a]	(30) 4.51 ± 0.13[a]	(30) 4.43 ± 0.12[a]	(22) 4.31 ± 0.13[a]	(30) 4.51 ± 0.11[a]	(30) 4.55 ± 0.12[a]
Albumen thickness	(29) 9.49 ± 0.18[a]	(30) 9.66 ± 0.25[a]	(30) 9.76 ± 0.21[a]	(22) 9.90 ± 0.29[a]	(30) 9.94 ± 0.21[a]	(30) 9.91 ± 0.22[a]
Yolk colour	(29) 9.17 ± 0.30[b]	(30) 9.70 ± 0.19[b]	(30) 9.53 ± 0.22[b]	(22) 9.36 ± 0.28[b]	(30) 11.93 ± 0.20[a]	(30) 11.83 ± 0.16[a]
Yolk weight	(29) 16.20 ± 0.23[b]	(30) 16.86 ± 0.20[ab]	(30) 16.92 ± 0.22[ab]	(22) 16.56 ± 0.29[ab]	(30) 17.46 ± 0.23[a]	(30) 17.27 ± 0.30[a]
shell thickness (average)	(29) 0.40 ± 0.01[a]	(30) 0.40 ± 0.00[a]	(30) 0.40 ± 0.01[a]	(22) 0.40 ± 0.00[a]	(30) 0.39 ± 0.00[a]	(30) 0.39 ± 0.00[a]
Shell weight	(29) 5.89 ± 0.11[a]	(30) 6.10 ± 0.07[a]	(30) 6.04 ± 0.07[a]	(22) 6.22 ± 0.08[a]	(30) 6.17 ± 0.06[a]	(30) 6.23 ± 0.10[a]

Data are (n) mean ± SEM

Different superscripts indicate significant differences between treatments.

Integument Scoring

The experiment began with 360 hens at 18 weeks of age, but the control group began exhibiting feather, toe, and vent pecking at 22 weeks of age, resulting in the termination of 45 hens due to intensive feather pecking. Data collection began at 30 weeks of age with 315 hens, and the experiment ended with 276 hens at 50 weeks of age due to mortality resulting from feather pecking in the control group.

The control group had lower feather scores in the neck area compared to the live and dried larvae group (control=3.15 ± 0.07, live=3.68 ± 0.03, dried=3.56 ± 0.03, $p<0.0001$). However, no significant difference were observed between week 40 and week 50 (Table 8). There was no difference in the feather condition of the breast region among all groups and score significantly decreased from week 40 to week 50 (2.89 ± 0.027 vs 2.15 ± 0.029, respectively. $p<0.0001$). The control group had a significantly lower score on the cloaca region than the larvae groups ($p<0.0001$). The feather condition scoring around the cloaca also decreased substantially with age from week 40 to week 50 (3.30 ± 0.035 vs 3.15 ± 0.037, respectively. $p=0.001$). Feather condition of the back region was significantly affected by both treatment (Control=2.85 ± 0.06, Live=3.12±0.03, Dry 3.00±0.02, $p<0.0001$) and age (3.07±0.24 in week 40 vs 2.90±0.02 in week 50, $p<0.0001$). There was no significant difference in the feather condition of wings in all three groups, however, feather condition of wings improved from week 40 to week 50 (3.02±0.02 vs 3.09±0.02, respectively. $p=0.0005$). The feather condition of the tail was significantly affected with treatment as the control group had lower feather scores than both larvae groups ($p<0.0001$). Feather condition of the tail decreased from week 40 to week 50 (2.99±0.02 vs 2.86±0.02, $p<0.0001$).

The control group had a lower score for rear body skin damage as compared to larvae groups ($p<0.0001$) and there was no significant difference with age. The control group had a tendency for comb score to be higher than larvae groups (Control=2.77±0.07, Live=2.66±0.04, Dry=2.58±0.04, $p=0.0236$) and comb injury increased significantly from week 40 to week 50 (2.56±0.04 vs 2.79±0.04, respectively, $p=0.0001$). The foot lesion score decreased significantly in the control group from week 40 to week 50 (3.91±0.05 vs 3.33±0.17, respectively. $p < 0.0001$).

Table 8: Integument condition scoring of all the hens in each pen on week 40 and week 50

	Week No					
	40			50		
	Control	**Live**	**Dry**	**Control**	**Live**	**Dry**
Neck	(64) 3.25± 0.08^{c}	(117) 3.62 ± 0.04^{ab}	(120) 3.52 ± 0.05^{b}	(40) 3.00 ± 0.12^{c}	(116) 3.73 ± 0.04^{a}	(120) 3.61 ± 0.04^{ab}
Breast	(64) 2.84 ± 0.06^{a}	(117) 2.93 ± 0.04^{a}	(120) 2.90 ± 0.03^{a}	(40) 2.10 ± 0.08^{b}	(116) 2.12 ± 0.04^{b}	(120) 2.22 ± 0.05^{b}
cloaca/vent	(64) 2.33 ± 0.13^{b}	(117) 3.82 ± 0.04^{a}	(120) 3.82 ± 0.04^{a}	(40) 1.73 ± 0.14^{c}	(116) 3.80 ± 0.04^{a}	(120) 3.73 ± 0.05^{a}
Back	(64) 3.08 ± 0.05^{ab}	(117) 3.16 ± 0.03^{a}	(120) 3.03 ± 0.02^{ab}	(40) 2.48 ± 0.10^{c}	(116) 3.08 ± 0.05^{ab}	(120) 2.98 ± 0.04^{b}
Wings	(64) 3.05 ± 0.03^{ab}	(117) 3.03 ± 0.02^{b}	(120) 3.00 ± 0.00^{b}	(40) 3.03 ± 0.07^{ab}	(116) 3.15 ± 0.03^{a}	(120) 3.07 ± 0.02^{ab}
Tail	(64) 3.00 ± 0.02^{a}	(117) 3.05 ± 0.02^{a}	(120) 3.00 ± 0.00^{a}	(40) 2.38 ± 0.09^{b}	(116) 3.03 ± 0.03^{a}	(120) 2.98 ± 0.02^{a}
Pecking skin damage rear body	(64) 3.42 ± 0.10^{b}	(117) 3.98 ± 0.01^{a}	(120) 3.94 ± 0.02^{a}	(40) 3.13 ± 0.13^{c}	(116) 3.97 ± 0.02^{a}	(120) 3.93 ± 0.02^{a}
Pecking skin damage comb	(64) 2.67 ± 0.08^{ab}	(117) 2.54 ± 0.05^{b}	(120) 2.48 ± 0.05^{b}	(40) 2.93 ± 0.12^{a}	(116) 2.78 ± 0.06^{a}	(120) 2.68 ± 0.06^{ab}
Foot lesions	(64) 3.91 ± 0.05^{a}	(117) 3.97 ± 0.02^{a}	(120) 3.90 ± 0.03^{a}	(40) 3.33 ± 0.17^{b}	(116) 3.82 ± 0.04^{a}	(120) 3.85 ± 0.04^{a}

Data are (n) mean±SEM

Different superscripts indicate significant differences between treatments

Organ weight

The control hens from which we took tissue samples were significantly lighter ($p=0.005$) than the larvae group hens. However, there was no significant difference in the weight of the proventriculus, gizzard, and liver among the treatment groups ($p>0.5$). The larvae groups did have a slightly higher average weight of fat pad than the control group, but the difference was not significant (Table 9).

Table 9: Individual hen body weight and organ weight of all groups at 50 weeks of age

	Treatment		
	Control	**Live**	**Dried**
Hen body weight (kg)	(10) 1.63 ± 0.05[b]	(12) 1.82 ± 0.05[a]	(12) 1.87 ± 0.04[a]
Proventriculus - Empty (g)	(10) 8.36 ± 0.40[a]	(12) 8.80 ± 0.36[a]	(12) 8.38 ± 0.36[a]
Gizzard - Empty (g)	(10) 35.61 ± 1.47[a]	(12) 29.24 ± 1.34[a]	(12) 28.91 ± 1.34[a]
Liver (g)	(10) 50.56 ± 2.09[a]	(12) 52.73 ± 1.77[a]	(12) 51.19 ± 1.81[a]
Fat Pad (g)	(10) 75.62 ± 6.76[a]	(12) 87.48 ± 5.55[a]	(12) 85.14 ± 5.76[a]

Data are (n) mean ± SEM

Different superscripts indicate significant differences between treatments.

Discussion

This study evaluated the effects of replacing 20% of the daily expected dry matter intake with live and dried black soldier fly larvae (BSFL) feed on feather condition, egg quality, leukocyte counts, antibody responses to IBV vaccination, body weights, and organ weights. The control group in this study displayed severe feather pecking and cannibalism, resulting in poor feather scoring. At the end of experiment, feed analysis revealed that the control group's diet was mistakenly deficient in sodium, which is well-documented as a cause of cannibalism (Hughes & Whitehead, 1979) and a contributing factor to feather pecking (Van Krimpen et al., 2005). This dietary deficiency caused stress in the control group and impacted their health.

Given the compromised health status of the control group hens, the results of this study are discussed with this limitation in mind. Despite this limitation, valuable insights were gained into the effects of BSFL feeding in laying hens.

Integument Condition

The study found that live and dried larvae groups had better feather condition scores than the control group. The feather condition on the control group's cloaca, back, and tail decreased significantly with age. The control group also had lower score for rear-body skin injuries and foot lesions. The primary reason for poor integument scoring in the control group was most likely the outbreak of severe feather pecking and cannibalism, likely caused by the low sodium diet (Hughes & Whitehead, 1979). There was no incidence of feather pecking in any pen of the live and dried larvae group.

There was no significant difference in the integument condition between the live and dried larvae group. It was observed that the live larvae group had better feather scores on the neck and back regions than the dried larvae group. The pecking on these regions is associated with aggression (Bilcik & Keeling, 1999). In one study, Veldkamp and Van Niekerk (2019) also found a positive effect on the feather condition of the back region in turkey poults fed with live larvae. An interesting finding was reported by Star et al. (2020) in older laying hens, where the live larvae group hens had poor feather condition at the start of the experiment (67 weeks) but ended up with better feather condition at the end of the experiment (78 weeks) compared to the control group hens. The enhanced feather condition due to larvae feeding can be attributed to directed pecking behaviour towards larvae. As feather

pecking and cannibalism are considered to be the outcome of redirected foraging behaviour (Sherwin et al., 1999), and this is the leading cause of feather damage (Bilcik & Keeling, 1999).

Feather condition in the breast region significantly decreased from week 40 to week 50 in all groups. There was no incidence of feather pecking in larvae groups, and the poor feather condition on the breast region indicates that it is not linked only to feather pecking. Feather condition of the breast region is among the areas most affected by age, and it is the first area to get denuded with age without the incidence of feather pecking (Bilcik & Keeling, 1999). Feather damage on the breast area can result from abrasion against the floor. Also, perches were provided to laying hens, which are strongly motivated to sit on perches (Olsson & Keeling, 2002). As gentle pecking is normal behaviour that can result in minor feather damage (Van Hierden, 2003), Bilcik and Keeling (1999) discussed that hens sitting on perches can be more prone to be pecked at the breast.

Literature is scarce on the separate feeding of dried BSFL to laying hens and its impact on feather condition. The present study is a valuable addition to the literature revealing the implications of dried BSFL along with live BSFL. As there was no significant difference among larvae groups in terms of integument condition, both live and dried larvae can be offered to hens based on the convenience of the production system.

Egg Quality

The results of this study indicate that larvae feeding did not significantly impact most of the egg quality parameters analysed, including egg weight, eggshell-breaking strength, eggshell thickness, and albumen thickness. This finding is consistent with previous studies that reported no significant effect of larvae feeding on these egg quality parameters (Maurer et al., 2016; Secci et al., 2020; Star et al., 2020; Liu et al., 2021; Tahamtani et al., 2021). However, it should be noted that feeding larvae did significantly affect yolk colour and weight and the likely reasons for these are discussed below.

The eggs from the larvae group had a darker yolk colour (with an orange tone and higher score) than the control group. This result agrees with previous studies that have reported an improvement in egg yolk colour from laying hens fed with BSFL meal as a replacement for SBM (Mwaniki et al., 2020; Secci et al., 2020; Patterson et al., 2021). It has been suggested that the higher content of the pigments β-carotene and lutein in BSFL meal could be responsible for the improved yolk colour observed in the present study and previous studies (Leeson & Summers, 2009; Secci et al., 2020). Specifically, Secci et al. (2020) found that the lutein content in the BSFL-based diet was higher than in the SBM-based diet, and this was reflected in the higher lutein content of the yolk in eggs from BSFL-fed laying hens.

While the β-carotene content in the BSFL-based diet was lower, a higher deposition of this pigment was still observed in the yolk, possibly due to intact deposition against the concentration gradient, as suggested by Moreno et al. (2016). The higher content of lutein in the eggs from BSFL-fed hens is particularly beneficial for human health, as it has been linked to the prevention of heart disease and stroke and improved cognitive ability in older people (Ribaya-Mercado & Blumberg, 2004; Johnson, 2012). Additionally, yolk colour is an important characteristic that consumers pay attention to, with orange yolk colour being more desirable (Berkhoff et al., 2020).

Yolk weight was found to be comparatively higher in the eggs from the larvae group. This result is similar to the findings reported by Secci et al. (2020). However, they concluded that the increase in yolk weight resulted from a decreased albumen proportion, as albumen is the most sensitive component in the egg contents to the new protein source. In contrast, most other studies, such as those conducted by Maurer et al. (2016) and Tahamtani et al. (2021), did not report any effect of BSFL feeding on yolk weight.

As previously reported, the control group's diet was unintentionally manufactured low in sodium. Nesbeth et al. (1976) have reported cessation of egg production in laying hens within three weeks in response to the complete elimination of salt. Although egg production data for the present study is unavailable, no halt in the control group was observed.

This study and previous research suggest that feeding larvae does not negatively impact egg quality parameters in laying hens. Further research is needed for nutrient analysis and sensory studies of eggs from hens fed with BSFL.

Immune Parameters

There were no significant differences between the control group and treatment groups in any of the leukocyte counts quantified in this project. Due to the lack of a healthy control group, it is hard to say whether larvae feeding influenced the leukocyte populations or not. However, results agree with previously published studies by Mat et al. (2022) and Tippayadara et al. (2021), who also didn’t find any significant difference in the various subpopulations of leukocytes when BSFL meal was included in the diets of broilers and in aquaculture respectively.

Although feeding chickens with scattered live larvae are more beneficial for their welfare than feeding them with dried larvae (Ipema et al., 2022), the leukocyte populations did not differ significantly between the live and dried larvae groups. This suggests that the live larvae did not significantly impact the studied immune parameters. A large variation between individuals was recorded for most immune parameters in this study. This indicates that factors such as subclinical infections and stressors on the individual level may have had a greater impact on these traits than any putative general influence by the experimental treatments. Given that the low-sodium diet provided to the laying hens resulted in cannibalism and feather

pecking, which can lead to acute or chronic stress, it is crucial to consider stress a significant immune response modulator. (Dohms & Metz, 1991). Acute stress has been shown to result in significantly higher numbers of heterophils (Bedáňová et al., 2007), while chronic stress yields significantly lower numbers of lymphocytes (Bedáňová et al., 2003). However, considering the health parameters of the larvae group hens, such as body weight and integument condition, we can assume that they were healthy.

It was assumed that chitin could stimulate the leukocyte population, particularly heterophils, monocytes, and lymphocytes (Shanthi Mari et al., 2014), as it can be sensed as foreign particle (Lee et al., 2018), but no such effect was observed. One possible reason could be the size of chitin found in the exoskeleton of the larvae, as Small-sized chitin can go unnoticed, and the same is with larger size chitin (Alvarez, 2014). Before the first blood sampling, experimental hens consumed larvae for 12 weeks as part of a previous behavioural project. So, none of the variation in cell populations during early sampling could be attributed to the first encounter with larvae feeding.

Like the leukocyte counts, there was no significant difference among groups in the levels of antibody titers to IBV. Similar results were observed by Liu et al. (2021) in laying hens aged 45 weeks with different inclusion levels of BSFL; however, the authors reported a linear increase in antibodies to Newcastle disease virus (NDV) vaccination in response to higher inclusion levels of BSFL meal in the feed. Interestingly, the same study showed no difference in antibody levels among the treatment groups following vaccination against the avian influenza virus. Hussain et al. (2017) reported no difference in antibody titers to NDV in broiler chickens with various inclusion levels of meal worm (*Tenebrio molitor*). The titers to IBV in this study were low at 30 weeks of age before booster vaccination, and the same was the case after vaccination. Based on the results of Kreukniet et al. (1992), it is generally observed that peak antibody titers are typically obtained within seven days post-vaccination. Therefore, the low vaccination titer values observed in our study may be due to the sampling time. The first sampling was done two days before the booster vaccination at 30 weeks of age. The previous vaccination was done at 15 weeks of age. The long duration between the two vaccines can be a possible reason for the low titers before the booster vaccination. Similarly, post-booster vaccination, the first sampling was done after 26 days, resulting in low titer values. However, the timing of peak antibody titers can vary significantly between different antigens and studies, and therefore it is crucial to exercise caution while drawing firm conclusions about it, as it can be highly dependent on various factors (Fadly et al., 2009). Chronic stress also results in a lower response to vaccination (Mashaly et al., 2004). Further research is needed to verify the results of the present study.

This study's strength lies in using a modern and reliable technique, flow cytometry, for the leukocyte counts. Additionally, the extended sampling period

provides a better overview, and the sample size for the larvae groups was adequate. However, the sample size for the control group was insufficient. Another limitation of the study is the inability to observe the leukocyte response to the first exposure of larvae feeding.

In conclusion, the manufacturing mistake in the control group's diet was an unintended limitation of this study. The lack of a healthy control group made it difficult to draw accurate conclusions from the findings. The lack of significant differences in immune parameters between the BSFL fed and control groups have been reported in previous studies (Hussain et al., 2017; Liu et al., 2021; Tippayadara et al., 2021; Mat et al., 2022), which suggests that dried larvae, compared to live larvae, can be offered to laying hens with similar health and welfare outcomes as dried larvae are much easier to manage and to control nutrient content. Further research is needed to understand the impact of larvae feeding on immune response with a proper control group. Additionally, there is a need to analyse the leukocyte count in laying hens exposed to larvae at an early stage, as it would be interesting to see how the early exposure to chitin in the exoskeleton of larvae modulates the immune response. Immune parameters can also be studied with various inclusion levels of BSFL, as the study of (Liu et al., 2021) found the influence of inclusion levels on antibody levels.

Body Weight and Organ Weight

The results showed that the hens in the control group weighed significantly less than those in the larvae group. As previously mentioned, the pelleted feed given to the control group was deficient in sodium, which has been reported to result in reduced feed consumption and decreased body weight in laying hens (Nesbeth et al., 1976). In addition, low-sodium diets can lead to cannibalism and pecking, causing stress in laying hens (Cronin et al., 2011). Stress has also been linked to reduced feed intake and lower weight gain in hens (Mashaly et al., 2004).

Feeding larvae did not affect the weight of the proventriculus, liver, gizzard, and fat pad in the hens. Although the fat pad in the larvae group hens was slightly heavier than in the control group hens, this can be attributed to the larvae group hens having a higher body weight. Tahamtani et al. (2021) reported a heavier fat pad in laying hens fed with ad libitum live larvae and pelleted feed. Still, they observed no significant difference with a 20% feed replacement with live larvae which would explain why hens in the present study did not have excessive deposition of abdominal fat.

Overall, these results suggest that feeding larvae as 20% replacement of dry matter to laying hens can help to maintain standard body weight without affecting gastrointestinal tract organs.

Conclusion

The unintended low-sodium diet of the control group negatively affected body weight and integument condition and made it challenging to interpret results; however, the comparison between the live and dried larvae group is still valid. The study found that feeding 20% of daily dry matter intake as either live or dried black soldier fly larvae (BSFL) to laying hens results in a higher yolk colour with age and higher yolk weight. The larvae group hens had better integument condition than the control group, but the low sodium diet caused the poor integument condition of the control group. There was no difference in leukocyte counts and antibody response to IBV vaccination. Feeding larvae had no significant effect on the weight of the proventriculus, gizzard, liver, and fat pad. All other studied egg quality parameters were unchanged. These findings support the use of both live and dried BSFL as a sustainable alternative feed ingredient for poultry. The hypothesis that dried BSFL have a similar effect on egg quality, feather condition, immune parameters, and organ weight in laying hens as live BSFL was proven. Dried larvae can be a more practical option than live larvae, as they have similar effects on the studied parameters and are easier to manage and control the larvae's nutrient content. Further research is needed to analyse the effect of dried larvae feeding on hen's behaviour and welfare. It is crucial to validate the immune response to larvae feeding with a proper control group. It would also be interesting to analyse the immune response during the early stages of larval feeding. In addition, conducting sensory and nutrient analyses of eggs based on larval feeding is necessary to ensure consumer acceptance and to verify that taste is not negatively affected.

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Popular science summary

Scientists are seeking alternative and sustainable protein sources in poultry production due to increasing prices and the potential scarcity of soybean meal. Black soldier fly larvae (BSFL) have been proven to be a suitable alternative due to their nutritional value and sustainable production. Previous studies have analysed the effects of live BSFL and BSFL meal feeding in laying hens. Still, there is a gap regarding the effects of dried whole larvae feeding, and literature is scarce regarding the impact of BSFL feeding on immune parameters. This study evaluated the effect of live and dried black soldier fly larvae feeding on laying hens. The results showed that both live and dried larvae can be used as a 20% replacement of daily feed intake without any adverse effect on leukocyte count, antibody response, egg quality, feather condition, and gastrointestinal tract organs. BSFL feeding resulted in a darker yolk colour, which is highly preferred by consumers. This darker yolk colour is attributed to the higher accumulation of certain nutrients, which in turn contributes to its orange hue. These nutrients have notable health benefits, including improved heart health and enhanced cognitive abilities.

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Appendix 1

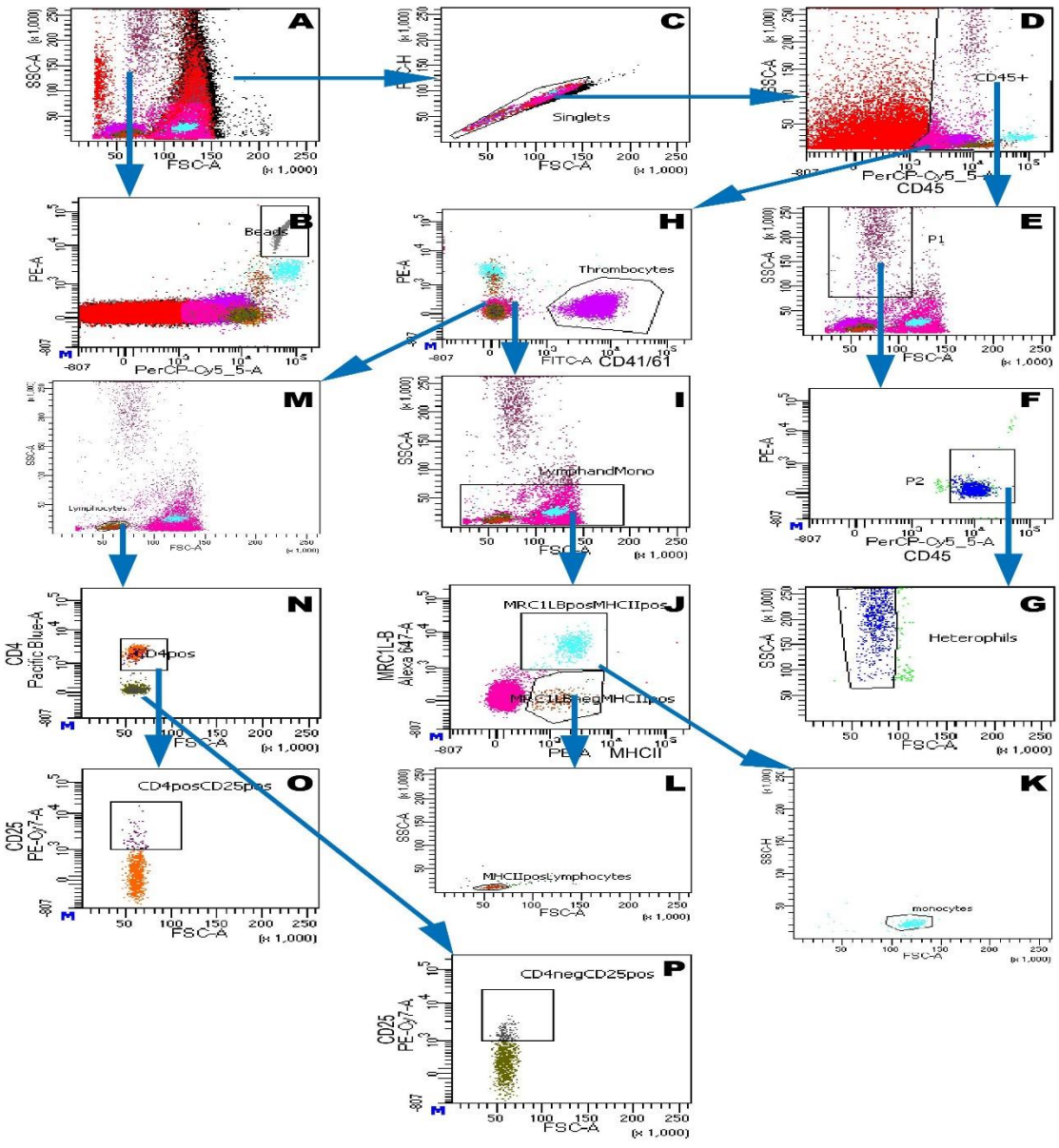

Figure 12: Gating strategy for flow cytometry: Identification of counting beads, heterophils, monocytes, thrombocytes, lymphocytes, MHCII+ lymphocytes, CD4+ lymphocytes, CD4+CD25+ and CD4-CD25+ lymphocytes through singlet gating, FSC/SSC characteristics and using CD45-PerCpCy5.5, CD41/61-Fitc, KUL01-AF647 (MRC1L-B), MHCII-PE, CD4-pacific blue and CD25-PECy7. From all

events in initial dot-plot in A) counting beads were identified as high fluorescent in B). From all events in A) gating through FSC-H vs FSC-A was performed in C) to identify singlets. From this gate high CD45 expressing events (leukocytes) and low SCC-A and medium to high CD45 expressing events (potential thrombocytes) were gated in D). From the CD45 gate in D) events were defined according to FSC and SSC characteristics in E) and high SSC events were defined in P1. P1 events were defined according to CD45 expression in F) and high CD45 expressing events were defined in P2. P2 events were defined according to FSC and SSC characteristics as heterophils in G). From the CD45 gate events were defined according to CD41/61 expression in H) and high CD41/61 events were defined as thrombocytes. Non-thrombocyte events from H) were defined according to FSC and SSC characteristics as "lymphocytes and monocytes" in I) and these were defined according to expression of MRC1L-B and MHCII in J. MRC1L-B+MHCII+ events from J were defined according to FSC and SSC characteristics as monocytes in K. MRC1L-B-MHCII+ events from J were defined according to FSC and SSC characteristics as MHCII+ lymphocytes in L.

Non-thrombocyte events defined in H) were defined according to FSC and SSC characteristics as lymphocytes in M). Lymphocyte events from M were defined according to CD4 expression in N and CD4+ events defined in N were defined according to CD25 expression in O. Non-CD4 events defined in N were defined according to CD25 expression in P

A representative blood sample from a hen on day -2 is shown. The antibody panel is described in Table 3.

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