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TESI DI LAUREA

The use of live insect larvae to improve sustainability and animal welfare in organic chicken production

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1. INTRODUCTION

It is known that worldwide meat consumption has drastically increased over the last decades, especially in developed countries. Indeed, recent studies show that animal-based proteins consumption increased on average from 61 g per capita per day in 1961 to 80 g per capita per day in 2011 (Sans et al.; 2015). This phenomenon is related to the rapid growth of global population, economic development of countries and urbanization (Godfray et al.; 2018). In fact, not only are countries getting richer (therefore increasing GDP and gaining access to foods that were once considered exclusive to the middle and upper class), but also meat is getting cheaper and quicker to produce (Sans et al.; 2015).

Meat is now easier to produce mainly due to:

1) genetic selection of the animals: these animals are able to produce more in less time due to higher adaptability, quicker development and better feed conversion ratio (FCR)

2) selection of feed: due to the high requirements of farmed animals, nowadays feeds present high nutritional values and are especially high in proteins

3) innovative farming systems: thanks to the constant research, we can now increase the welfare of farmed animals, therefore increasing productivity (Brameld et al.; 2016).

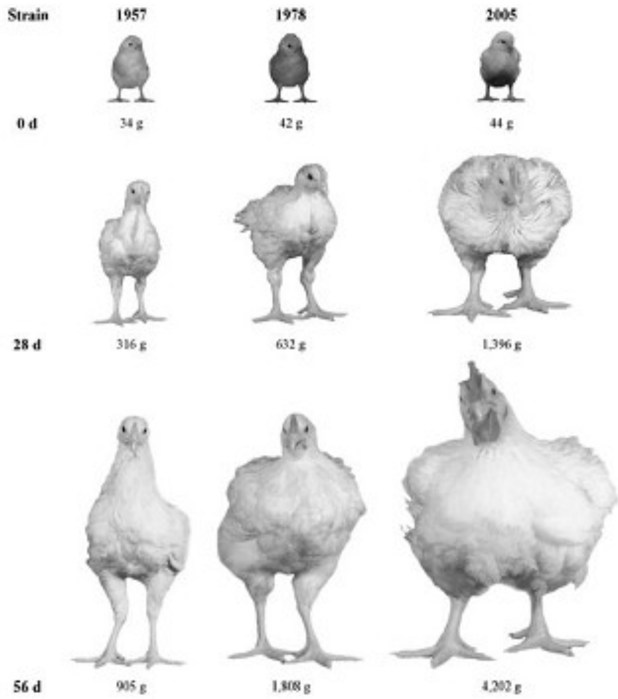
As stated before, meat consumption increased worldwide but some countries underwent strong economic transitions and are now consuming more meat than other countries. Among these, we can find Argentina, Australia, Brazil, Chile, China, New Zealand and U.S.A. The only exception to this trend is India, where most of the population prevalently consumes a vegetarian diet. This country doesn't show any important change in meat consumption over the last decades (www.ourworldindata.org). The type of meat consumed changes across each country. On average, poultry and pork are the most consumed worldwide (www.fao.org).

In 2018 FAO estimated that roughly 69 billion chickens were slaughtered for meat production. The countries with the highest poultry density are Brazil, China and U.S.A (www.fao.org). With this data overview, it is interesting to understand why poultry meat is largely consumed and why it is convenient to raise chickens for meat.

It is well known that chickens underwent an important selection within the past decades. The same genetic stock can grow globally, under any type of husbandry conditions. Through the genetic selection, the chickens' weight has dramatically increased, yet the FCR has decreased (Brameld et al.; 2016).

Chickens specifically reared for meat are called broilers. These animals underwent a strong selection in order to reach market weight at a very young age as, broilers are slaughtered at 43 days of age, on average (Bianchi et al.; 2007). In 1985, broilers at 35 days of age required 3.22 kg of feed to reach a weight of 1.4 kg and had a FCR of 2.3. In 2010, broilers only required 3.66 kg of feed in order to reach a body weight of 2.44 kg at 35 days, with a FCR equal to 1.5 (Siegel et al.; 2014). In other words, modern broilers are able to produce more meat while consuming less feed.

This development obviously comes with health implications since artificial selection led to several health and welfare problems.



Picture 1: visual representation of the evolution of
broilers over the years.
Source: www.mercyforanimals.org

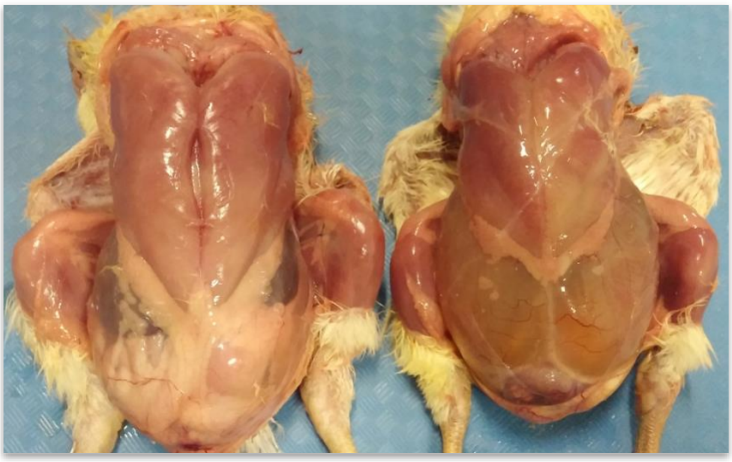
Broiler diseases may depend on their genetics and physiology. Moreover, also the farming condition can affect diseases development. Among the several diseases that affect broilers we can find:

1) Cardiovascular dysfunctions: broilers are selected to abnormally develop their breasts and thighs. The organs, on the other hand, do not grow proportionally to the targeted muscles. This incongruous ratio between energy-supplying and energy-consuming organs leads to various metabolic disorders, such as ascites and "sudden death syndrome" (Baghbanzadeh et al.; 2008).

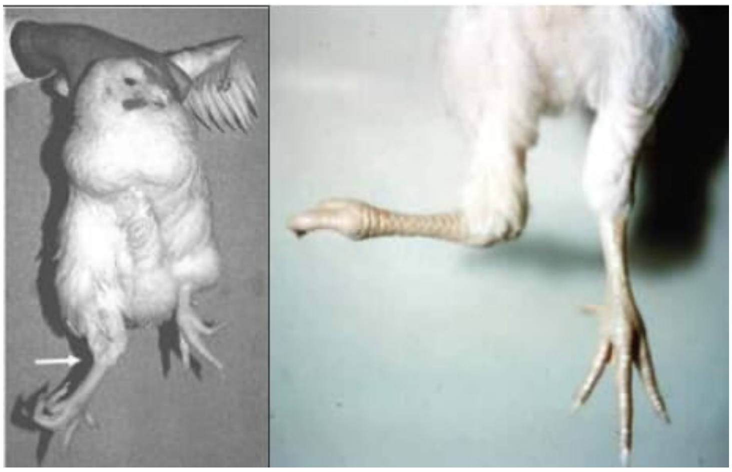
   a) Ascites (picture 2) is characterized by myocardial hypertrophy and dilatation, abnormal liver function, pulmonary insufficiency, and hypoxemia (Luger et al.; 2003)
   b) SDS ("sudden death syndrome") mainly affects fast-growing chickens. Suddenly the broiler, even though it appears to be healthy, flaps its wings, falls to the side and dies. This all happens under a minute (Newberry et al.; 1987). In Europe this syndrome usually affects 3% of birds (Turner et al.; 2014).

2) Skeletal dysfunctions (picture 3): varus and valgus deformities, osteodystrophy, dyschondroplasia and femoral head necrosis are common in broilers. These dysfunctions lead to a severe lameness in the chickens, inducing them to spend more time lying on the ground and sleeping. If the broilers spend too much time lying down, under the abnormal weight of their bodies, not only can they suffocate, but also, they will develop integument lesions (Juliani; 1998).

3) Integument lesions: these birds are often subjected to dermatitis (e.g.: hock burn, footpad lesions), hyperkeratosis and necrosis of the epidermis (picture 4). This is not only due to the poor blood circulation, but also due to the prolonged contact with the ammonia in the litter (Greene et al.; 1985).



Picture 2: ascites on the right.
Source: www.semanticscholar.org



Picture 3: skeletal disorders in broilers.
Source: www.msdvetmanual.com

Picture 4: footpad dermatitis.
Source: www.joneshamiltonag.com

Another issue related to the production of poultry meat is its important environmental impact. Feeding poultry requires a huge quantity of feed and these animals annually excrete important amounts of nitrogen and phosphorus to the environment, which conditions the production sustainability of this chain (Andretta et al., 2021). Nevertheless, poultry production has been found to be relatively environmentally friendly compared to other livestock productions, such as that of cattle. (Leinonen et al., 2016). The environmental impact of poultry production can roughly be divided into feed production and transportation, housing emissions and manure emissions.

One of the main critical aspects related to poultry production is the amount of feed necessary to grow these animals. These feeds are particularly high in proteins, in order to satisfy the high requirements of chickens. Poultry feeds are made of cereals and their by-products (e.g.: corn, wheat, corn gluten meal), vegetable protein meals (such as soybean meal), oils, vitamins and minerals. The most important protein source in poultry feed is soybean, usually given as a meal. This ingredient is high in proteins, low in fibers and high in lysine and tryptophan (even if deficient in methionine). Soybean meal is relatively inexpensive compared to other protein sources, such as corn gluten meal. The main issue related to soybean is that it has a strong impact on the environment, mainly due to the fact that in the past couple of decades some areas around the world (like South America and South Asia) have been converted from natural forests to soya crops (Kastens et al.; 2017). Then this ingredient must be transported to the feed mills around the world (mainly Europe, America and Asia). The loss of ecosystem carbon storage as a consequence of such conversion was added to the carbon dioxide emissions, therefore to the global warming potential arising from this system (Leinonen et al; 2016).

For what concerns the housing emissions, recent studies show an important difference in terms of emissions based on the type of housing system. Three systems were taken into consideration: standard (indoor), free range and organic. Studies show that less intensive poultry systems had higher environmental impacts compared to the more intensive ones (Leinonen et al; 2016) in such way: organic systems have higher contributions in terms of eutrophication potential and acidification potentials (due to the emissions of NH_3 and N_2O), but extensive poultry production can reduce the use of fossil fuels, fertilizers and has lower housing emissions (Leinonen et al.; 2016). Although organic systems show less manure in the litter, it still has an environmental impact. On average, a single broiler excretes 0,6kg of N and 0,1kg of P each year. The amount of N found in the uric acid, expresses as kg/year, is equal to 0,5 (Rotz; 2004). Usually, poultry manure is used as a fertilizer, although it must be used with caution due to the high concentration of N, P and K. If used incorrectly, it could severely damage the crops and it could lead to the excessive eutrophication and acidification of the soil (Leinonen et al.; 2016).

Despite what preceded, how could we possibly reduce the environmental impact of poultry meat production? Scientists all over the world are trying to find new farming strategies in order to produce high quality meat with a lower environmental impact. Genetic selections, as stated before, has improved the FCR of animals (chickens can now produce more while eating less feed, at a faster rate), but the main ingredients in feed cannot be totally substituted now. The main challenge nowadays is to find an appropriate substitute for soybean meal, which is known to be the least environmental-friendly ingredient.

The purpose of the project POULTRYNSECT is to test the effects of live insect larvae on slow and medium-growing organic chickens to allow sustainable meat production and to improve animal welfare. Insect larvae are reared on organic food by-products and are used as feed ingredient and environmental enrichment for chickens.

Picture 5: POULTRYNSECT logo.
Source: www.poultrynsect.eu

2. ORGANIC CHICKEN FARMING

GENERAL ASPECTS AND LEGISLATION

In the European Union, the standards for organic production are defined by EC Regulation 834/2007 and EC Regulation 889/2008. As stated in the Council Regulation 834/2007, organic production is a system of farm management and food production that integrates optimal environmental practices, biodiversity, the conservation of natural resources and the application of high animal welfare standards. A farm, in order to be considered organic, has to align with specific standards. In the European Union, the standards for organic production are defined by EC Regulation 834/2007 and EC Regulation 889/2008.These regulations define the main aspects of organic poultry farming, both for layers and broilers, such as:

-Feed: all kinds of feeds and feed ingredients must be of organic origin and certified. Synthetic amino-acids, GMOs and animal by-products (fishmeal, bonemeal and slaughter by-products) are prohibited.

-Housing: organic farming system focuses on the animal physiological needs, ensuring free access to outdoor areas but what really differentiates organic farming systems from other extensive rearing systems is the animal density. In fact, the limits are imposed to reduce the amount of nitrogen at 170 kg/hectare/year (Castellini, 2004) and to ensure the welfare of chickens.

Animal cat.	**Indoor no. poultry/m^2**	**Outdoor no. poultry/m^2**	**Cm of perch/chicken**	**Nest (laying hens only)**
Laying hens	6	4	18	7 hens/nest
Broilers in fixed housing	10	4	-	-
Broilers in mobile housing	16	2.5	-	-

Table 1: directives on poultry husbandry according to CE Regulation 889/2008.

According to Art. 12 of EC Regulation 889/2008, at least one third of the floor area must be solid and covered with a litter material (e.g.: wood shavings). The access to outdoor areas must be ensured at any time for at least one third of the chickens' lives. Natural lighting must be guaranteed, but some small changes are allowed as birds must be provided with a maximum of 16 h of light per day. During autumn and winter, these hours of light can be supplemented with artificial lights, but 8 hours of continuous nocturnal rest must be ensured.

-Animal reproduction: the organic labeling can be ensured only if animals come from organic breeding facilities. For breeding purposes, natural methods must be used even though artificial insemination is allowed. Reproduction cannot be induced by hormonal treatment, unless as a veterinary therapeutic treatment for a single animal.

-Slaughter: CE Regulation 889/2008 establishes the minimum age at slaughter for different poultry strains. For what concerns organic chickens used for meat production, the minimum allowed age at slaughter is 81 days. For capons, the minimum age is 150 days.

-Veterinary practices: any preventative veterinary treatments are forbidden, such as the use of growth promoters. Vaccinations are allowed, as one of the purposes of the organic rearing system is to prevent diseases rather than curing them. In case of diseases, it is possible to administer homeopathic and phytotherapeutic remedies. In case of administration of allopathic remedies, the organic certification might be denied if the appropriate withdrawal period isn't correctly observed. Any kind of mutilation (e.g.: wings clipping) is forbidden, except for beak trimming. In some specific and rare cases, it might be allowed by the competent authority only for safety reasons or when they are intended to improve animal health. In fact, beak trimming is advised in some specific cases in order to reduce the incidence of cannibalism. Castration is allowed only to maintain the quality of traditional products, such as the case of capons.

3. CHICKEN WELFARE

3.1 WELFARE ASSESSMENT IN CHICKENS

In 1965, Professor R. Brambell, on commission of the UK government, led an investigation on the welfare of intensively farmed animals. The report affirmed that the animals should have the freedom to stand up, lie down, turn around, groom themselves and stretch their limbs (Brambell; 1965). These freedoms quickly developed into a more detailed and expanded list, which is currently known as the "Five Animal Freedoms".

The Five Freedoms are as follows:

1) Freedom from hunger and thirst

2) Freedom from discomfort

3) Freedom from pain, injury, or disease

4) Freedom to express normal behavior

5) Freedom from fear and distress (FAWC, 2009).

The report by Prof. Brambell led to the further study and development of welfare protocols, such as the Welfare Quality® assessment protocol for poultry, that originates from the Welfare Quality research project, financed by the European Commission in 2009. This protocol is useful to standardize different methods of assessing animal welfare and its approach is considered "animal-based", so it focuses on the animal body condition, behavior, and health conditions. As stated in the first Welfare Quality® protocol (edited by Prof. Dr. H.J. Blokhuis in October 2009), the main criteria assessed for poultry welfare are:

1) Good feeding (absence of prolonged hunger and/or thirst)

2) Good housing (thermal comfort, comfort around resting and movement possibility)

3) Good health (absence of diseases and injuries)

4) Appropriate behavior (display of social behaviors, positive human-animal relationship, positive emotional state and expression of other behaviors).

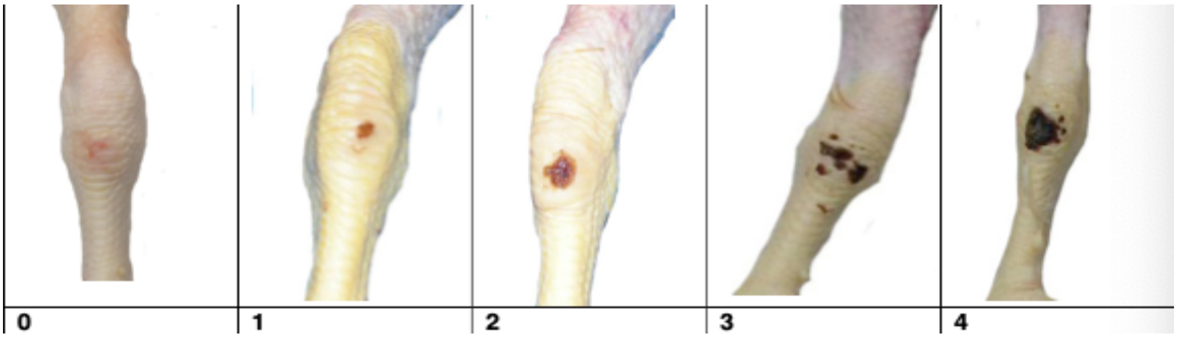
Most of these criteria can be easily measured on farm.

Good feeding criteria can be measured at the slaughterhouse or on farm (some recently built farms have scales and sensors on the ground that allow the farmer to have constantly updated data regarding the weight of birds), once the chickens are weighted. Numerous cases of emaciation in one flock might be caused by prolonged hunger, by dietary deficiencies or by an inappropriate arrangement of drinkers and trough (de Jong et al.; 2016).

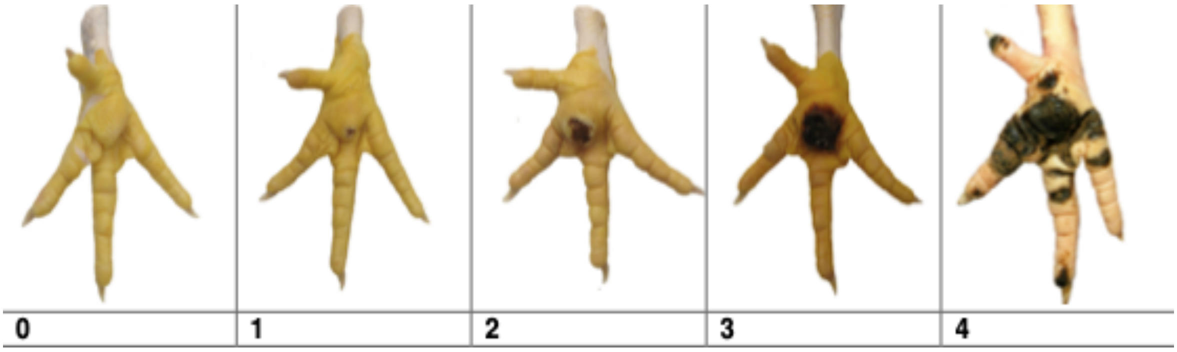
Good housing criteria are usually measured on farm, as the litter quality, dust sheet test and plumage cleanliness are parameters used to evaluate the level of comfort and hygiene provided by the housing system (Ben Sassi et al.; 2016). Also, the Welfare Quality® assessment protocol for poultry mentions ease of movement (calculated as kg/m^2) and thermal comfort. This criterion focuses on two possible events: panting and huddling. Panting is described as breathing rapidly in short gasps. It is caused by high temperatures. Huddling, on the contrary, can be observed in chickens reared in extreme cold conditions. In this case, chickens will sit together into tight groups in order to maintain their body temperature. According to the Welfare Quality® assessment protocol for poultry, poor litter quality is more difficult to manage and it leads to skin and foot lesions. On the other hand, the dust sheet test is performed in order to monitor the amount of dust in the air, providing information as well about the litter quality. The dust sheet test is performed, as reported in the Welfare Quality® assessment protocol for poultry (2009) by putting a sheet of black A4 size paper onto a clip board and above bird height on a horizontal surface. The paper sheet must be positioned when first entering the house and removed at the end of the assessment. The examiner writes with a finger on the paper to get an impression of the amount of dust on the paper and classifies the dust level found on the paper from 1 (none) to 5 (paper colour not visible).

Good health criteria are measured according to the following scheme:

- Absence of injuries: lameness, hock burn (picture 6), foot pad dermatitis (picture 7), breast blister, comb wounds and colour.
- Absence of diseases
- Mortality rates.



Picture 6: hock burn evaluation scale
Source: Colas, ITAVI



Picture 7: foot pad dermatitis evaluation scale
Source: Butterworth, University of Bristol

Appropriate behavior depends on the species that was taken into consideration but overall it can be measured through the avoidance distance test, cover on the range, free range, qualitative behavior assessment and excreta corticosterone.

The avoidance distance test is useful to evaluate the human-animal relationship. Extreme fear display may indicate stressful situations. The assessor approaches a group of chickens (at least 3 birds) in the litter area and proceeds to squat for ten seconds. The examiner counts the number of chickens at arm's length, that is standardized to 1 m. The trial is repeated twenty-one times, in different areas of the building where the birds are reared.

As quoted in the above cited Welfare Quality® assessment protocol for poultry, cover on the range is applicable to free range and extensive systems and it indicates the vegetation (e.g.: bushes, trees) or manmade shelters that birds can use for cover.

As cover on the range, also free range is applicable to free range and extensive systems. It indicated the chickens' ability to choose the environment in which it ranges and the suitability of each environment for chickens.

The qualitative behavior assessment considers the expressive quantity and quality of how chickens interact with each other and with the environment. The analyzed behaviors may be divided in two categories, that are the frequency, or point event, and the duration, or state event (Biasato et al.; 2022).

Another test useful to assess the human-animal relationship is the tonic immobility test. Tonic immobility is an anti-predator strategy, as the caught prey will remain motionless to deceive the predator into thinking that the prey has been subjugated, therefore inducing the predator to look for another prey animal (Fogelholm et al.; 2019). In this way the prey will escape the predator and increase the survival chances by 50% (Sargeant et al.; 1975). In order to perform this test, the chickens are gently placed on their backs and the examiner has to maintain eye contact with the animal while executing the test. The longer the chickens remain in the immobile state, the more fearful they are considered to be (Gallup et al.; 1972).

3.2 ETHOGRAM

Ethology is considered a scientific discipline useful to understand the biological principles of animal behavior (Mench; 1991).

Ethograms are comparable to catalogues of behaviors for each animal species since each species may display different behaviors.

The scientific community agrees on the fact that ethology is an evolving branch of science and makes a distinction between instinctive and learned behaviors (Mench; 1991). As animals react to the stimuli given by their surroundings, it can be easily assumed that almost all behaviors are adaptive (Mauldin; 1991). It is possible to categorize the behavioral pattern of animals in social behavior, aggression and cannibalism, sexual behavior and broodiness, feeding behavior and comfort behavior (Mauldin; 1991).

3.3 NATURAL POSITIVE BEHAVIORS

Among the natural and positive behaviors, we can identify the social behavior, the feeding behavior, the sexual behavior and the comfort behavior.

It is fundamental to underline that evolution and domestication strongly influenced the natural behavior of the *Gallus gallus domesticus* that, according to Charles Darwin, originated from *Gallus gallus bankiva* (picture 8).

Picture 8: bankiva chickens.
Source: www.cocincina.freeforumzone.com

Originally *Gallus gallus* lived in small groups (about 10 individuals, predominantly females) within a territory defended by the dominant male (Collias et al.; 1967). Nowadays, depending on the production line, it is common to raise chickens with the same sex in a single group with numerous individuals.

Chickens tend to establish a hierarchy within the group but in case of numerous groups in a limited area (such as in intensive farming systems) the hierarchy is not stable, and chickens may often fight in order to re-establish the hierarchy. As a consequence of this hierarchy, for what concerns the feeding behavior, it is frequent to observe the so called "pecking order", which establishes the dominant individuals that have the priority on food resources (Buitenhuis et al; 2009). As reported by J.V. Craig in his article published in 1991, agonistic pecking occurs within the first weeks of life, yet it is fully established at 8-10 weeks of age. Hormones are involved in the establishment of the pecking order, as pullets that become sexually mature early are dominant over those that mature later (Craig et al.; 1965).

Naturally chickens would spend an important amount of time foraging, including pecking and scratching. The scratching technique consists in scratching the ground with both feet and rapidly moving backwards to check for edible particles and insects that might flee. Foraging includes two phases: the first one is the "appetitive phase" and the second one is the "consummatory phase". The appetitive phase includes the research for food, hence the scratching technique. The consummatory phase is the act of picking up and swallowing food (Duncan; 1998). Even if chickens have free access to feed, they still display the foraging behavior. This statement is not valid for all breeds, as some chickens (especially broilers) are not prone to displaying the foraging behavior. This may be due to the genetic selection they underwent, in order to have calmer birds, or due to the fact that their bodies are disproportionate. Moreover, their heavy body weights put their legs under remarkable stress to allow locomotion. Consequently, scratching is quite hard to perform, and they prefer to consume feed directly from troughs.

Regarding sexual behavior, both the morphology and the behavior are involved in the male dominance. Morphologically, roosters bigger in size tend to be dominant on those with slim bodies (Gottier; 1968). The activity level is also an indicator of dominance: the time spent searching for food is positively correlated with fertility. Males tend to mate several times per day (Blohowiak et al.; 1984) and it is known that roosters have favorites, which means that a rooster may choose a single female to engage coitus with.

For what concerns comfort behavior in chickens, preening and dustbathing are quire relevant. Preening is considered as a self-grooming behavior in which the chicken runs its feathers through its beak to realign the feather barbs and then proceeds to pinch the preen gland with its beak to extract the oil and apply it on its feathers (Sandilands et al.; 2004). Other than its physiological purpose, preening may also be considered as a displacement activity in response to frustrating situations (Duncan; 1998).

As many other animal species, such as rodents from the Genus Chinchilla (Donnelly et al.; 2004), chickens do not bathe in water for the purpose of keeping their feathers clean. Dust-bathing (picture 11) is performed to remove excess oil and to control parasites, such as *Dermanyssus gallinae* (picture 9 and 10), known as the red mite (Appleby et al.; 2004).

Picture 9: *Dermanyssus gallinae*
Source: www.copyrco.it

Picture 10: *Dermanyssus gallinae* infestation
Source: www.vinayakcorporation.com

Although dustbathing might look like an unorganized sequence of movements, is it performed in a specific pattern. Firstly, the chicken raises its feathers and lays down. With its beak, the chicken scratches up the fine soil particles and dust around its body so that they are closer to the chicken's feathers. The chicken proceeds to lay on one side and it twists sideways. The chicken repeats these actions until satisfied, then it stands up, raises its feather and vigorously shakes its body in order to lose all the dust accumulated on its body (Bestman et al.; 2012).

Picture 11: chicken dustbathing
Source: www.motherearthnews.com

3.4 MALADAPTIVE BEHAVIORS

The term “maladaptive behavior” refers to those behaviors that inhibit the animal’s ability to adapt to abnormal situations and display a normal ethogram (Garner; 2005). Behavioral disorders are common in intensive farming systems. The most common maladaptive behaviors are cannibalism and severe feather pecking. Both behavioral abnormalities may lead to important economic losses to the farmers, since they can reduce the bird’s performances and increase the mortality rates (Ellen et al.; 2019).

Cannibalism and severe feather pecking may be triggered by environmental factors, such as inadequate housing conditions, or by the physiological status and genetics of the chickens (Ali et al.; 2020).

Feather pecking can be distinguished in two different behaviors, depending on the intensity of the phenomenon:

1) Gentle feather pecking: chickens gently peck the feathers of other birds, without causing any damage to the receiver (Hartcher et al.; 2015). Gentle pecking is considered a positive behavior and has an important role in social exploration (Riedstra et al.; 2002).

2) Severe feather pecking: chickens aggressively peck each other’s feathers. Severe feather pecking is an abnormal behavior in which a chicken pulls off feathers from another chicken, usually those on the abdomen, back or tail. The chicken displaying this type of behavior may also ingest the feathers. This behavior is considered to be a compulsive behavior rather than an aggressive one (Daigle; 2017).

The subject that repeatedly undergoes this painful procedure may reduce its performance because the extensive feather loss leads to the failure of proper thermoregulation, consequently the animal will increase its daily feed intake in order to compensate the dispersion of body heat (Gilani et al.; 2013). Severe feather pecking leads to skin lesions (picture 12), therefore infections may occur. In the most severe cases, if the lesions are inappropriately treated, they may lead to sepsis and death. It is then correct to assert that severe feather pecking not only leads to a decrease in performance, but also to an increase in mortality (Daigle; 2017).

Cannibalistic pecking is considered a severe type of feather pecking, culminating in blood and tissue consumption from other chickens (Craig et al.; 1996). Tissue consumption refers to the cannibalistic pecking that involves the skin and underlying tissues, which leads to severe wounds (picture 12) and eventually death (Birkl et al.; 2017). Laying hens may also display another type of cannibalism called “cloacal cannibalism”. It is defined as the repetitive pecking of the skin and tissues of the cloaca (Birkl et al; 2017), often once a prolapse occurs.

Picture 12: severe wound caused by cannibalistic behavior.
Source: www.poultryhub.org

4. THE USE OF INSECTS IN POULTRY NUTRITION: A FOCUS ON *HERMETIA ILLUCENS*

Several studies tried to substitute vegetable meal proteins completely or partially, such as soybean meal, with insects in monogastric animals, as the use of animal proteins in ruminants is forbidden. These insects can be included in the chicken diet as live larvae, dried larvae or as a meal. Insect meal can be found as whole or defatted. Larvae can either be mechanically (for instance, through high pressure) or chemically (through some specific solvents, such as n-hexane) defatted (Kim et al.; 2021).

There are different species of insects both used for food and feed all around the world, yet the European Union (to the current year) allows eight species as feed ingredients. According to the Regulation 2017/893 (Annex X) and Regulation (EU) 2021/1925, these species are:

1. Hermetia illucens (black soldier fly)

2. Musca domestica (common housefly)

3. Tenebrio molitor (yellow mealworm)

4. Alphitobius diaperinus (lesser mealworm)

5. Acheta domesticus (house cricket)

6. Gryllodes sigillatus (banded cricket)

7. Gryllus assimilis (field cricket)

8. Bombyx mori (silkworm).

Since these insects are considered farmed animals, insects producers must ensure the health of the above listed species (*Directive 98/58/CE concerning the protection of animals kept for farming purposes*). These insects may only be fed materials of vegetable origin, with some exceptions: milk and eggs (including their by-products), honey, rendered fats, blood products from non-ruminant animals are allowed (*Regulation 178/2002 General Food Law Regulation; Regulation 852/2004* and *Regulation 183/2005 contained in the Hygiene Package*)

Hermetia illucens (picture 13), commonly known as Black Soldier Fly ("BSF") is a widespread fly of the order Diptera, family Stratiomyidae. Although native to the Neotropical realm, it is a cosmopolitan species (Wang et al.; 2017). The adult subject measures ca. 16

mm and has a predominantly black body with iridescent reflections. The head is wide, with well-developed eyes. The antennae are twice the length of the head. The legs are black with lighter tarsi. BSF have membranous wings, which are overlapped and folded horizontally on the abdomen while resting (www.entnemdept.ufl.edu). This insect is a mimic fly, which means that it resembles another organisms or object. This is an anti-predator adaptation. In this specific case, BSF resemble *Trypoxylon politum*, commonly known as the Organ Pipe Mud Dauber (picture 14). It is a large, predatory wasp in the family of Crabronidae.

Picture 13: *Hermetia illucens*
Source*:* newprotein.net

Picture 14: *Trypoxylon politum*
Source*:* bugguide.net

BSF presents a peculiar life cycle: a single female lays between 206 and 639 per cycle in crevices eggs (Tomberlin et al.; 2002). The eggs hatch in about four days, then the larvae go through five larval stages. These stages last between 18 and 36 days, then the emerging larvae evolve into pre-pupae for about seven days (Holmes et al.; 2013). They now evolve into pupae (the pupal stage lasts from one to two weeks) and finally into adults, which can live up to 73 days when provided with water and food, usually sugar (Romano et al.; 2020). Adults are not able to eat, in fact they only drink water (in captivity, sugar is added to water in order to sustain the adults). On the other hand, larvae present a peculiar mouth part, which is used to consume chopped or semi-liquid food. They have a developed digestive system, and it is divided into foregut (with pharynx, esophagus, crop and proventriculus), midgut (gastric caecum and ventriculus) and hindgut (ileum, colon, rectum) and finally the anus (Bruno et al.; 2019). BSF presents a strong amylase, lipase and protease activity. The 90%

of the total enzyme activity was observed in the gut, while the remaining 10% was observed in the salivary glands (Kim et al.; 2011).

In the reproductive stage, BSF adults must be reared at a temperature between 24°C and 32°C, with a humidity between 50-70%. Adults need artificial of natural light and, as stated before, water with the addition of sugar, in captivity, or nectar (Harnden et al.; 2016). During the growth stage, larvae must be reared on organic substrates at a temperature between 27°C and 30°C with a RH of 70% (Harnden et al.; 2016).

BSF is a polyphagous species, which means that its larvae consume different kinds of substrates. BSF appreciate low pH and each substrate has its own pH level, but it is important to keep the pH of the rearing batches between 7,5 and 9,2 (Meneguz et al.; 2018). BSF are excellent bio-converters: they are in fact a detrivore and coprovore species and BSF are used to compost low value, organic substrates (food by-products, brewery by-products, manure) to quality feed.

They are not considered a pest to humans, since the adults are not attracted to habitations, and they cannot fly around as much as domestic flies. They are quite easy to catch, and they are incredibly sanitary: BSF drastically reduces the heavy metals and pathogens found in the rearing substrate, such as Salmonella spp. and *E. coli* (Erickson et al.; 2004). At the end of the production cycle, frass is obtained. Frass is an odorless, granulated residue obtained from the residual substrate and faeces and it can be used as an organic fertilizer.

Different substrates confer different characteristics to the BSF larvae, but on average they have high nutritional values:

- C.P.: 49,9%
- E.E.: 15,8%
- C.F.: 14,7%
- Gross energy (MJ/Kg DM): 21,7 (Wang et al.; 2020).

5. INSECT LIVE LARVAE AS ENVIRONMENTAL ENRICHMENT FOR POULTRY

Currently there are few studies that focus on the effects of live insect larvae. Only few studies have been published in the last few years, as the provision of larvae to livestock animals is gaining more attention. These studies mainly focus on the use of live BSF on the performance and welfare of broilers, hens and turkeys.

Specifically, Bellezza Oddon et al. (2021) analyzed the effect of BSF and yellow mealworm (*Tenebrio molitor*) live larvae on broiler performance and health status. The study involved 180 male Ross 308 broilers, which were assigned to three dietary treatments (control diet, control diet + 5% of DFI *Hermetia illucens* larvae and control diet + 5% of DFI *Tenebrio molitor* larvae). The chickens were slaughtered at 39 days of age and the experimental trial demonstrated that the inclusion of live BSF and TM larvae does not have any negative effects on bird health and growth performance. As stated by the authors, behavioral and welfare studies are required to evaluate the positive aspects of live larvae supplementation in chickens.

Biasato et al. (2022) completed such research focusing on the welfare of broilers fed live larvae. Specifically, feathering score, feet and hock health, excreta corticosterone and behavioral observations through video analysis were assessed. The provision of BSF and TM live larvae as environmental enrichment improved the welfare of chickens. The authors noticed a positive correlation between the larvae provision and the increased activity level and foraging behavior. Overall, the larvae administration led to a decrease in frustration, since insects are a natural feed source for chickens, and they would normally display the foraging behavior. Nevertheless, the feet and hock health, the feathering score and the excreta corticosterone were not affected by the live larvae administration.

The use of live larvae as environmental enrichment on chicken gut health has also been analysed to evaluate gut mucin composition, microbiota and local immune response. Colombino et al. (2021) focused their attention on the same birds from the research of Bellezza Oddon et al. (2021), collecting at slaughter, samples of duodenum, jejunum and ileum and submitting them to mucin histochemical and gene expression analysis. Specifically, the authors focused on the MUC-2 gene (the main type of secretory mucin in the intestine, which develops the mucus layer that covers the epithelium) and the IL-2, IFN-γ and TNF- α genes. The last three genes are pro-inflammatory cytokines involved in numerous warning events within the cells, such as apoptosis or necrosis. For what concerns the anti-inflammatory cytokines, the authors evaluated the IL-4 and Il-6 genes. The MUC-2 gene was similar among the three dietary treatments, while the larvae inclusion promoted the gut health by reducing the pro-inflammatory cytokines. Regarding microbiota, the caecal samples were characterized by a high presence of *Bacteroides, Faecalibacterium, Barnesiella, Helicobacter, Collinsella, Eubacterium* and *Clostridium.* These bacteria have positive effects on gut health, since they can promote nutrients absorption. For instance, *Bacteroides* present a strong hydrolytic activity, useful to degrade non-digestible carbohydrates and to produce short-chain fatty acids. Furthermore, *Clostridium* is capable of producing butyric acid, which positively influences intestinal villi structure, and it contributes to the control of occurring pathogens.

Similarly, to the above-listed experimental trials, Ipema et al. (2020a) studied the effect of live BSF larvae on the activity level and fearfulness of broilers. Ross 308 broilers were divided into: a control group, two groups with the inclusion of BSF equal to 5% of DFI (administered four or seven times a day) and two groups with the inclusion of BSF equal to 10% of DFI (administered four times a day or for prolonged period of times in transparent tubes with holes). In all the treatment groups, the foraging behavior and the activity level were increased. In particular, the broilers that were granted a prolonged access to the BSF larvae not only showed the highest activity level, but also the lowest amount of time spent in tonic immobility. This indicates a significant reduction in fearfulness. Although final weight and health were not affected, it is possible to affirm that BSF larvae improved the welfare of broilers.

Ipema et al. (2020b) proposed a similar experimental study in the same year, which consists in providing live BSF larvae to broilers, scattered on the litter, in different percentages of inclusion based on the DFI and in different frequencies. Specifically, they divided three hundred and sixty-one male Ross 308 broilers into five treatments at one day of age. The first group was the control group, which did not receive any BSF live larvae. Two groups received the larvae at either 5% or 10% of the estimated DFI (referred to as A5 and A10). Two more groups were administered the larvae two or four times a day (F2 and F4). The broilers' weights and leg health were measured on day 42. The experimental trial determined that A10F4 showed the highest rate of activity and the time spent active declined from week 4, whereas all the other treatments showed an early decline in the activity level from week 2 onwards. Hock burns occurred more in the control group than in any other group, hence lameness occurred less in the treated groups. The broilers, at the end of the trial, had similar body weights, except for those in the A10F2 group. Their body weight was lower than that of broilers allotted in the control group.

Indeed, some studies focus not only on poultry raised for meat, but also on laying hens. In fact, Star et al. (2020) published an article in which they investigate the effects of live BSF larvae provision to older laying hens. As stated before, in nature, chickens spend a significant amount of time searching for insects, hence consuming them. It is a natural behavior and the possibility of displaying this behavior in intensive rearing systems surely contributes to the improvement of animal welfare. Among the maladaptive behavior that chickens could possibly display, feather pecking is the major welfare challenge in laying hen farms. The authors examined the effects of the administration of live BSF larvae to older laying hens that did not undergo the beak-trimming procedure. The animals were divided into two groups: the control group (commercial diet) and the *Hermetia illucens* group (soy-free diet with larvae on top of it). Feathering score, egg quality and production performance were registered at the beginning of the trial (67 weeks of age) and at the end (78 weeks of age). The authors monitored the behavior of the hens through video recording systems. The experimental trial did not show any difference in feed conversion ratio, body weight increment and egg laying parameters between the two group, yet the feather condition score improved in the BSF group when compared to the control group.

Tahamtani et al. (2021) proposed a similar experiment regarding the administration of different percentages of live BSF larvae to laying hens in order to evaluate the effects on feed consumption, egg quality and hen welfare. The authors divided forty Bovans White laying hens in individual housing solutions and provided them with 0%, 10%, 20% and *ad libitum* BSF live larvae from 18 to 20 weeks of age. The authors analyzed the larvae consumption, the concentrate consumption, the hen body weight, the egg production and quality. Hens given *ad libitum* access to the BSF larvae consumed less concentrate and gained more weight. Although it might be considered as a positive aspect, the *ad libitum* access is considered as wasteful because the hens consumed more proteins than required. This could result in nitrogen leakage into the environment, leading to the excessive eutrophication of the ground and water. Regarding egg production and egg quality, such as egg weight and eggshell thickness, no differences were recorded among the four groups. The only egg quality parameter influenced by the quantity of BSF larvae administered was the egg yolk color, since the *ad libitum* group produced eggs with paler egg yolks. This is explained by the deficiency in carotenoids, which are usually provided by the natural raw ingredients in the commercial diets. The weight of the gizzard and liver were similar. The level of fearfulness remains the same.

Finally, some authors also focused on other poultry species. For instance, Veldkamp et al. (2019) published a study which focuses on the provision of live *Hermetia illucens* larvae to turkey poults. The purpose of the experiment was to assess the effects of BSF live larvae on the welfare and performance of turkey poults from 0 to 35 days of age. These poults were divided into two groups: the control group (commercial diet) and the BSFL group (poults were fed the same commercial diet + BSF larvae calculated to be 10% of the expected DFI). At 5 weeks of age, the DFI and body weight of poults in the BSFL groups were higher when compared to control groups. They also showed a significantly lower feed conversion ratio. Although the provision of BSF larvae reduced the aggressive pecking behavior, it did not completely solve the pecking problem. The authors suggest that further studies are necessary to assess whether BSF larvae may reduce the incidence of severe feather pecking.

6. AIM OF THE EXPERIMENT

The effects induced by the administration of live BSF larvae in chickens have been poorly investigated. No studies are currently available on the use of live BSF larvae in slow-growing chickens, thus making this research novel in the poultry sector.

The aim of the experiment reported in my thesis, developed on the Poultrynsect project, is to evaluate the effects of live BSF larvae on the welfare and performance of medium-growing chickens. Furthermore, this study wants to investigate if insect larvae can reduce the environmental impact of the poultry sector. The live larvae, by virtue of their characteristics, represent in fact an interesting tool to improve the sustainability of chicken meat production.

The experimental trial reported in my thesis focuses on the effects of live BSF larvae on medium-growing chickens (Label Rouge Naked Neck chickens). Specifically, the trial focuses on the welfare and performance of medium-growing chickens by analyzing different parameters, such as:

- Growth performance (live weight, FCR, average daily gain and average daily feed intake)
- Feather condition score
- Skin lesions
- Foot pad dermatitis and hock burn
- Larvae consumption time
- Tonic immobility test
- Avoidance distance test
- Blood chemistry
- Slaughter performance (slaughtering weight, ready-to-cook carcass weight, chilled carcass weight, organs weight, abdominal fat weight, meat color and pH).

7. MATERIALS AND METHODS

7.1 NAKED NECK CHICKENS

For the purpose of this experimental trial, a total of 300 Label Naked Neck chickens (Hubbard JA57 hybrid) were purchased from a commercial hatchery. This specific strain of chicken is supplied by Hubbard Breeders, an American company found in 1921 and since 2018 it’s part of the Aviagen Group. The Label Naked Neck (picture 15) is considered a medium-slow growing chicken, with a minimum slaughtering age of 81 days. The predicted marketable live weight (LW) for this strain is between 3 and 4 kg. It has been developed in order to be a free-range, rustic chicken but, according to the breeder itself, it’s also the optimal strain for extensive indoor rearing systems. They are fully colored, as the feathers are predominantly orange/red with blue/black tail and sickle feathers. The comb is developed but always kept uptight. The caruncles are excessively developed. The tarsi are yellow, although the skin is pink. According to the breeder, the average daily growth rate for this strain is 30 g (www.hubbardbreeders.com).

Picture 15: Label Naked Neck chicken, male.
Source: www.hubbardbreeders.com

7.2 *HERMETIA ILLUCENS* LARVAE CHOSEN FOR THE EXPERIMENT

Considering the fact that the Poultrynsect project has several partners, the live BSF larvae were produced by INAGRO, a privatized research center based in Rumbeke-Beitem, Belgium. Not only INAGRO is specialized in the development of precision farming technologies, as well as in recirculating aquaculture systems and insects rearing. As a partner of the Poultrynsect project, INAGRO weekly provided the larvae administered to the birds during the trial. Because the researchers' purpose was to raise the chickens according to the European organic regulations, the BSF larvae were reared on organic substrates. These larvae were then shipped in an insulated container with cool bags in order to maintain the optimal temperature during the 24 hours journey. Once at the experimental center, the larvae were sorted out (dead larvae and/or pre-pupae were discarded) and stored in ventilated containers in a climatic chamber for one week. The chosen storage temperature was 16°C to induce diapause in larvae (Spranghers et al., 2017). Each weak, a sample of BSF larvae was collected and stored at -80°C for further analysis, such as:

1) energy content

2) dry matter

3) ash content

4) ether extract

5) crude protein

6) chitin content

7) amino acid profile.

On average, the larvae showed the following composition (expressed in % as fed):

- GE (MJ/kg): 8.69
- DM: 33.63
- CP: 14,39
- EE: 9.56
- Ash: 4.34
- Chitin: 2.00 (Bongiorno et al., in press).

7.3 CHICKEN MANAGEMENT AND DIETS

The experimental trial officially began at the beginning of October 2021 and ended at the beginning of December 2021.

The sexed Label Rouge Naked Neck chicks were bought at 1 day of age, then they were transferred to the experimental facility Tetto Frati in Carmagnola (TO), Italy. This facility belongs to the Department of Agricultural, Forest and Food Science of the University of Turin, Italy. The chicks were vaccinated against Newcastle and Marek's disease and coccidiosis.

From day 1 to day 28, the birds were reared in a controlled environment (for what concerns temperature, humidity, ventilation and photoperiod). The chicks were divided into four pens (3.4m x 2.9 m), according to their sex, with rice hulls as bedding material. Each pen was equipped with feeders and drinkers and the building was equipped with automatic ventilation and illumination systems. Infrared lamps were available at all times, to ensure the optimal coop heat, until the chicks reached two weeks of age. For the first three days the chicks followed a 23L:1D light system, as suggested by the Hubbard Breeders. After three days, the dark hours were progressively increased and, at two weeks of age, the photoperiod was adjusted to recreate the natural environment. Once the chicks were 21 days old, they were individually weighted and identified with a wing mark. Then 120 males and 120 females were selected according to their weight and allotted in 24 pens (2.2 m x 3.5 m). Each pen presented an access to an outdoor area of 2.2 m x 4.5 m. The chickens were granted the access to the outdoor area from 49 days of age until the end of the trial, which is 82 days of age. Once transferred to these pens, the chickens adjusted to the natural photoperiod and ventilation. In October, the average temperature was 12.8°C (min 5°C; max 22°C) with 12h of light and 12h of dark. In November, the average temperature was 7.6°C (min -1°C; max 16°C) with a negative photoperiod of 10h of light and 12h of dark.

At 21 days of age, the 240 chickens were divided into four experimental groups (10 chickens/pen; 6 replicates/treatment):

1) control males (CM)

2) control females (CF)

3) larvae males (LM)

4) larvae females (LF).

The control groups were fed a commercial, organic feed. The larvae groups were fed the same commercial, organic feed with the supplementation of live BSF larvae. The larvae inclusion was equal to 10% of the expected daily feed intake (DFI), which was suggested by the Hubbard Breeders. Water and feed were given *ad libitum*. The commercial feeds were bought from Verzuolo BioMangimi, a company based in Verzuolo, (CN) that produces organic feeds. Two different types of feed were administered: the first one was a starter (from day 1 to 28), the second one was a grower (from 28 days of age until slaughter).

The following table describes the composition (expressed in % as fed) of the starter feed “Pollo Uno” and grower feed “Pollo Plus”:

	Pollo Uno	**Pollo Plus**
DM	90.44	90.61
CP	22.92	20.55
EE	6.19	5.02
CF	5.85	6.26
Ash	7.81	5.65
Ca	1.27	0.96
P	0.64	0.49
Na	0.18	0.15
Methionine	0.34	0.33
Lysine	1.04	0.83
AME (MJ/kg)	15.36	14.19

Table 2: composition of the feeds used during the experimental trial.

The live BSF larvae were administered from the 28th day until the end of the trial, which coincides with the slaughter.

7.4 GROWTH PERFORMANCE

The LW of each bird and feed consumption of each pen were recorded weekly, from day 28 to 81. The average daily feed intake (ADFI), average daily gain (ADG) and FCR of each pen were calculated weekly and for the experimental period (from day 28 to 81). The ADFI was calculated as fed, without considering the BSF larvae supplementation. The FCR (g/g, dry matter basis) was fixed by the dry matter amount of the (33.63%), according to the method used by Veldkamp and van Niekerk (2019).

7.5 LARVAE CONSUMPTION TIME

The larvae consumption time was recorded daily, using a stopwatch.

As the larvae were presented to the chickens in two identical red plates, the time recording started from the moment the plates touched the litter and stopped as soon as the last larva was consumed by the animals. The cut-off time was set to thirty minutes.

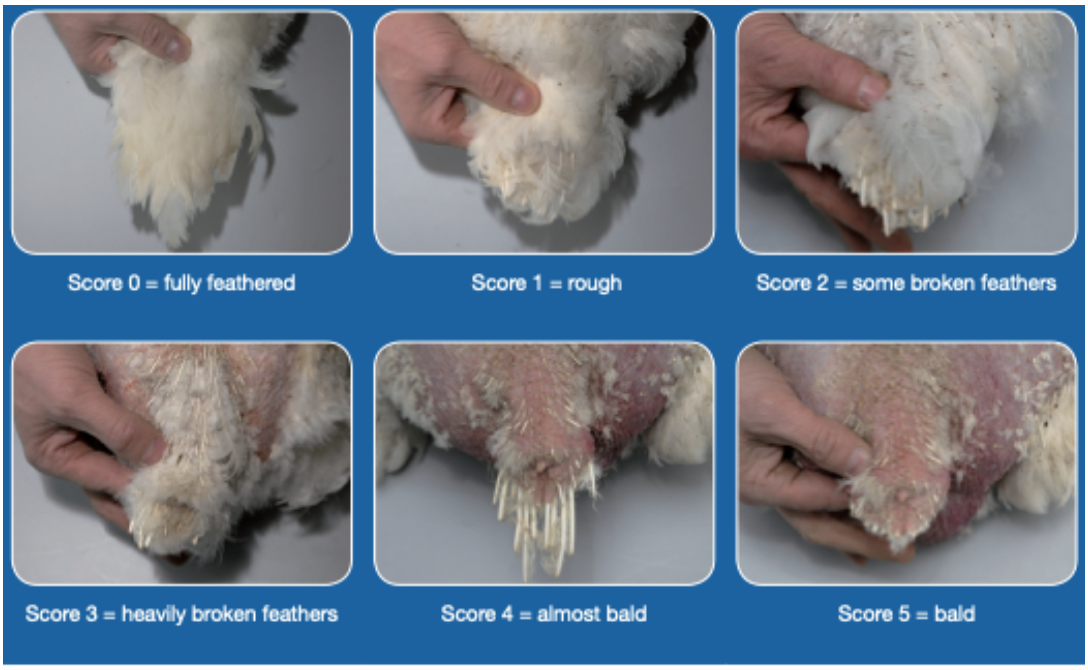
7.6 WELFARE ASSESSMENT

7.6.1 FEATHER CONDITION SCORE

Once every two weeks, all chickens were individually evaluated for the feather condition score and feather cleanliness, before being weighted.

For the purpose of this experiment, the Aviagen guidelines (December 2014) were considered for the feather condition score. This criterion is calculated on a point-cumulative system ranging from 0 (optimal condition) to 5 (worst condition) for the back (picture 16), tail (picture 17) and wings (picture 18) and from 0 (optimal condition) to 8 (worst condition) for the thigh area (picture 19).

Picture 16: feather scoring system for the back
Source: www.aviagen.com



Picture 17: feather scoring system for the tail
Source: www.aviagen.com

Picture 18: feather scoring system for the wings
Source: www.aviagen.com

Picture 19: feather scoring system for the thighs

Source: www.aviagen.com

The feather cleanliness score (picture 20) was recorded once every two weeks, following the guidelines of the Welfare Quality® assessment protocol for poultry (2009). The feather cleanliness scoring system distinguishes four levels of feather cleanliness, in fact it ranges from 0 (optimal condition) to 4 (worst condition).

Picture 20: feather cleanliness scoring system

Source: Welfare Quality® protocol (edited by Prof. Dr. H.J. Blokhuis, October 2009)

7.6.2 SKIN LESIONS

Concurrently with the feather condition score, the incidence of skin lesions was evaluated. Overall, the entire body was considered for this type of evaluation, although the main lesions were found on the neck, on the comb and on the back. Each chicken was evaluated according to the scoring system obtained by the Welfare Quality® assessment for poultry (2009):

0 points: no lesions/single, punctiform lesion (<0.5 cm)

1 point: < 1 lesion (<2 cm diameter) or ≥ 3 pecks or scratches

2 points: < 1 lesion (≥ 2 cm diameter).

7.6.3 FOOT PAD DERMATITIS AND HOCK BURN

Foot pad dermatitis (FDP), according to the Welfare Quality® assessment protocol for poultry (2009), is evaluated based on the following point-cumulative system (photographic illustration may be found on page 11, picture 7):

0 points: no sign of FPD

1 point: the pad is harder and denser, the central area presents small, black necrotic areas and a minor epithelial proliferation

2 points: significant swelling of the pad with an evident necrotic area. This area may be surrounded by white scales

3 points: significant swelling of the pad, leading to an abnormally large size of the foot. The necrosis extends to one half of the foot pad

4 points: same as score 3, nut the necrotic area extends to the other half of the foot pad.

Hock burn (photographic illustration: page 11, picture 6) is evaluated based on the point-cumulative system given by the Welfare Quality® assessment protocol for poultry (2009) as follows:

0 points: no sign of hock burn

1 point: punctiform sign of inflammation

2 points: larger inflammation area

3 points: small necrotic areas, disseminated on the joint

4 points: large and uniform necrotic areas.

7.6.4 AVOIDANCE DISTANCE TEST

As indicated by the Welfare Quality® assessment protocol for poultry (2009), the avoidance distance test was performed once every two weeks by the same operator, who entered each pen and squatted for ten seconds. The operator then measured how many chickens would approach them at arm length (1m) or between 1 and 2 m or >2 m.

7.6.5 TONIC IMMOBILITY TEST

As reported in chapter 3, the tonic immobility test was performed once every two weeks. For the purpose of this test, 3 birds/pen were chosen to perform the tonic immobility test. The chickens were gently, yet rapidly placed on their backs and the maintained eye contact with the animal while executing the test. The operator holds the animal in place by placing their hand on the sternum of the bird for a maximum of 45 seconds, while holding the head and neck with the other hand. The pressure was gradually reduced until any type of movement of the animal ceased. Once the chicken was laying still, the time recording started, and it stopped as soon as the chicken tried to stand up. If the animal moved in the first ten seconds, the test was cancelled and repeated for three times. After the third attempt, the test is considered invalid.

7.7 BLOOD CHEMISTRY

Right before the slaughtering process, blood samples were collected from 48 chickens.

A total of 5 mL of blood was collected from each chicken; 2,5 mL were placed in a K_3EDTA tube and 2,5mL in a serum-separating tube to evaluate the hematological parameters. A blood smear was performed using a drop of blood for each chicken and the smears were stained using May-Grünwald and Giemsa stains. The serum-separating tubes were centrifuged at 3500 rpm for 15 minutes at 20°C, after being stored at room temperature for 2h in order to favorite the blood clot formation. The RBC (erythrocytes, 10^6, cell/μL) and WBC (leukocytes, 10^3, cell/μL) counts were determined in an improved Neubauer hemocytometer on blood samples previously treated with a 1:200 Natt-Herrick solution (Natt and Herrick, 1952). One hundred WBC, including granular (heterophils, eosinophils, and basophils) and non-granular (lymphocytes and monocytes) leukocytes, were counted on the slide and expressed as a percentage of the total leukocytes, according to Campbell (1995).

The concentrations of alanine aminotransferase (ALT, U/l), aspartate aminotransferase (AST, U/I), creatinine (CRE, mg/d), total proteins (g/dl), uric acid (mg/d), cholesterol (mg/dl), triglycerides (mg/dl), gamma glutamyl transferase (GGT, U/l), phosphorus (P, mg/dl) iron (Fe, μg/dl) and magnesium (Mg, mg/dl) were measured by using a compact liquid chemistry analyzer system (BT 1500 vet – Futurlab).

7.8 SLAUGHTER

7.8.1 SLAUGHTERING PROCESS AND CARCASS TRAITS

At 82 days of age, based on the average live weight (LW) of each pen, 48 chickens (2 birds/pen and 12 birds/treatment) were individually labelled with a shank ring, weighted and slaughtered in a commercial abattoir. The individual slaughtering weight (SW) was recorded. The ready-to-cook carcass (RTCC), corresponding to the plucked carcass without feet, neck, head and organs, weight was recorded. The absolute weight of the abdominal fat and of the following organs was recorded: heart, spleen, bursa of Fabricius and liver. These weights were then calculated as percentage of the SW. After 24h of refrigeration, the chilled carcass (CC) weight was recorded, and the weight was then recorded as percentage of the

SW. The RTCC yield (%SW), CC yield (%SW), breast yield (%CC) and thigh yield (%CC) were recorded.

7.8.2 MEAT pH AND COLOUR

The breasts and thighs were separated from the chilled carcass and studied to evaluate the meat quality parameters 24h after the slaughter.

The pH of the *Pectoralis major* and *Biceps femoris* was recorded. For each muscle, the measurements were performed on the dorsal side and under the skin, using a Crison portable pH meter (Crison Instruments, S.A., Alella, Spain) equipped with a spear-type electrode and an automatic temperature compensation probe.

The colour of the *Pectoralis major* and *Biceps femoris* (dorsal side, under skin) was measured along with the pH. The colour of the breasts and thighs were measured using a portable colorimeter Chroma Meter CR-400 Minolta (Minolta Sensing Inc., Osaka, Japan) with a 8 mm diameter measuring area, D65 illuminant and 2° standard observer. The results were expressed in terms of lightness (L*), redness (a*) and yellowness (b*) in the CIELAB color space (Commission Internationale de l'Éclairage, supplement n. 2, CIE publication n. 15; 1976).

7.9 STATISTICAL ANALYSIS

The data was analyzed by means of IBM SPSS Statistics software, V20.0.0 (IBM). The homogeneity of variance was established by means of Levene's test, and the normality or non-normality of residuals was assessed by means of Shapiro-Wilk's test. The pen was considered the experimental unit for the larvae consumption duration and growth performance (n=6/diet), while the animal was used as the experimental unit for the slaughtering performance and the blood parameters (n=12/diet). A general linear mixed model (GLMM), with a gamma probability distribution and log-link function, was performed to analyze the larvae consumption duration, where the time (i.e., the above mentioned 5 recording periods, named T1-T5) and gender were considered as fixed effects, as evaluated by pairwise comparisons, and the replicate was included as repeated measurements on the same pen. The same was performed for the tonic immobility test, considering additionally the treatment as fixed factor. Finally, a Poisson distribution was performed for the avoidance distance test, considering the gender, time and treatment. A general linear model (GLM) was used for the growth and slaughtering performances and for the blood analyses. The gender and diet between gender and diet were considered by means of a pairwise comparison. P values ≤0.05 were declared as statistically significant, while a statistical trend was defined for P values ≤0.10.

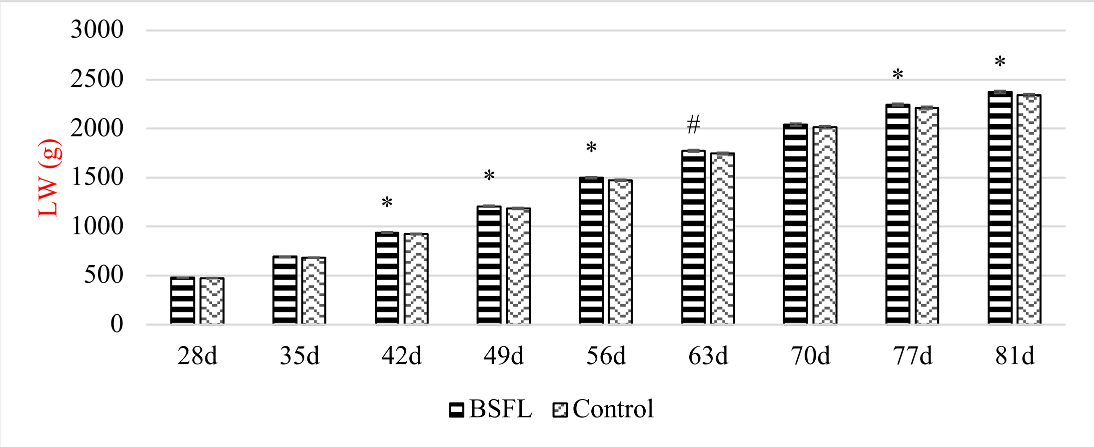
8. RESULTS AND DISCUSSION

Growth performance

The health status of the chickens was evaluated daily, and, during the experimental trial, no mortalities have been recorded.

The growth performance is reported in picture 21.

The larvae supplementation did not affect the growth performance of the chickens, except for the LW at 81 days of age: the treated birds were, on average, 32,12g heavier compared to the control birds ($p < 0.05$). On the contrary, the gender significantly influenced the growth performance parameters, with males showing a higher LW, ADG and ADFI than females ($p < 0.001$; Table 4)



Picture 21: The growth curves of the Label Rouge Naked Neck birds fed a diet supplemented with 10% live black soldier fly larvae; supplementation based on the expected daily feed intake, (28-81d; n = 6).
# indicates a statistical trend foe the control and BSFL supplemented birds (p≤0.10); * indicates a statistical difference between the control and BSFL supplemented birds (p≤0.05).

In general, the LW, ADG, ADFI and FCR resulted higher in males compared to the females ($p<0.01$). These results are similar to those reported in literature for the growth rate and the chicken breeds (Abdullah et al.; 2009). The LW was similar between the groups at the beginning of the trial, but it resulted higher at the end of the cycle ($p<0.05$) in the treated birds compared to the control birds, specifically at 42 days of age. This indicates a significant advantage in early slaughter ages. Similarly, a study found in literature, conducted by Veldkamp et al. (2019), demonstrated that the inclusion of 10% BSFL in turkeys increased the LW at 5 weeks of age. On the contrary, the results obtained in chickens vary considerably. Indeed, Bellezza Oddon et al. (2021) reported no significant influence of the 5% inclusion of BSFL on the growth performance of broilers, while Dabbou et al. (2018) reported a higher LW at 10, 24 and 35 days of age in male broilers fed 10% BSFL defatted meal. This phenomenon could possibly be explained by the fact that in the live larvae the nutrients are more diluted compared to larvae meal (containing ca. 70% of water, Ewald et al.; 2020), meaning that the live larvae effects are less visible on brief periods.

The percentage of larvae ingested based on the DFI was 10.41% in females and 8.01% in males, even though the expected DFI was identical between the two sexes (Hubbard, 2021), as well as the quantity of larvae supplied to the birds. The ADFI of females was lower compared to that of males. ($p<0.001$).

Item	**Days**	**Diet (D)**		**Gender (G)**		**SEM**		**P-value**	
		BSFL	**Control**	**Male**	**Female**	**D**	**G**	**D**	**G**
	28	479	475	515	439	2.07	2.07	0.186	<0.001
LW (g)	81	2372	2340	2742	1970	11.40	11.40	0.047	<0.001
ADG (g/d)	28-81	35.6	35.2	41.8	28.9	0.24	0.24	0.229	<0.001
ADFI (g/d)	28-81	111+9.93*	112	125	98.6	1.16	1.16	0.572	<0.001
FCR (g/g)	28-81	2.93	2.92	2.74	3.11	0.02	0.02	0.722	<0.001

Table 4: The growth performance of the Label Rouge Naked Neck birds fed a diet supplemented with 10% live black soldier fly larvae; supplementation based on the expected daily feed intake, (28-81d; n = 6).
* indicates the average amount of larvae provided (on an as fed basis, average DM: 33.63g/100g).
SEM: standard error of the mean.

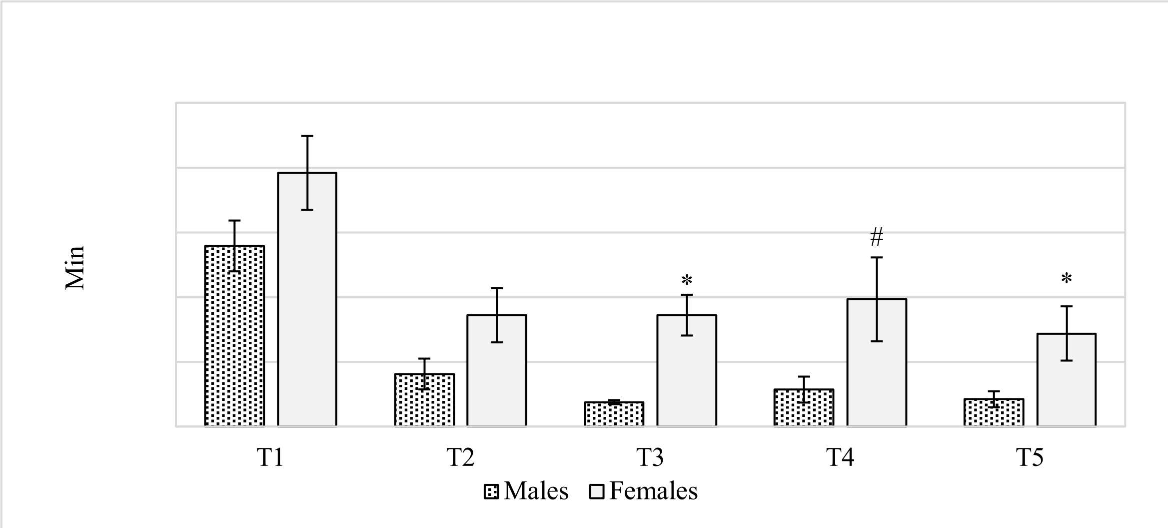
Larvae consumption time

The study aimed to provide to the Label Naked Neck Chickens an inclusion level equal to 10% of live BSF larvae, based on the expected daily feed intake (based on the data reported by Hubbard, 2021). Nevertheless, the actual BSF larvae ingestion was equal to 10.41% in females and 8.01% in males, considering the actual feed intake.

The results in terms of larvae consumption time are reported in picture 22 It is possible to affirm that the gender significantly influenced the larvae consumption time, that in fact was lower in males than females ($p<0.01$). Moreover, significant differences were recorded at T3, T4 and T5, since at T4 females consumed the larvae less quickly compared to males. On the other hand, females were faster at T5 than at T4 ($p<0.05$), meaning that chickens slowly adapt to the larvae administration, thus consuming them quickly. Interestingly, males showed an inverted trend, so the consumption time at T3 was greater compared to T2 ($p=0.066$), yet at T5 they consumed the larvae more rapidly than at T2 ($p<0.005$) (picture 21).

Chickens are naturally attracted to larvae, as live insects are a natural source of feed for chickens (Star et al.; 2020). Specifically, the attractiveness is influenced by the larvae motility (Clara et al.; 2009). Some scientific papers that describe the larvae consumption time in turkeys and broilers are available (Veldkamp et al., 2019 and Bellezza Oddon et al., 2021 respectively).

The consumption time and percentage of larvae ingested were disproportional between the two sexes. On average, males consumed 8.01% of BSF larvae in 3.64 minutes, where females spent 4.73 minutes consuming 10.41% of larvae. This could suggest that the social behavior influences the larvae consumption time. Indeed, the females establish the dominance and pecking order during the first weeks of age and they tend to maintain them throughout the life cycle (Craig, 1992). Males tend to change the pecking order and dominance more often, therefore they display fewer constant larvae consumption dynamics and tend to spend more time consuming the larvae. Both males and females spent more time consuming the larvae at the beginning of the trial, since time for adaptation to new objects is needed.



Picture 22: larvae consumption time
*P≤0.05; #P≤0.10.
T1, 28-39 days of age; T2, 40-50 days of age; T3, 51-62 days of age; T4, 63-74 days of age; T5, 75-81 days

Feather condition score

No significant results were recorded regarding the feather condition score, both for the control and the treated birds and for the two sexes. All birds scored 0 points on the point-cumulative system, meaning that the feathers were mostly clean, and the plumage was overall intact throughout the experimental trial.

The feather condition score is useful to evaluate the incidence of severe pecking behavior or any cannibalistic behavior, as the scarcity of feathers may be caused by the severe pecking behavior. This criterion considers the area on the body of the bird that lacks feathers, in particular the back, tail, thighs and wings of the chickens. As one of the purposes of the experimental trial was to assess whether the administration of live BSF larvae could possibly stimulate positive behaviors in chickens, the feather condition score is a useful indicator of aggressiveness in chickens.

Skin Lesions

No significant results were recorded regarding the skin lesions, both for the control and the treated birds and for the two sexes. All birds scored 0 points on the point-cumulative system, meaning that no lesions were recorded throughout the experimental trial.

Skin lesions are mainly caused by agonistic or aggressive behaviors. One of the most important aspects studied during the experimental trial was to determine if the administration of live BSF larvae could improve the welfare of the chickens by reducing the aggressiveness, for instance by re-directing the focus and aggressiveness of the chickens. However, the beneficial effects of the live BSFL provision cannot be proved, since the control birds did not show any skin lesion.

Foot pad dermatitis and hock burn

No significant results were recorded regarding the FPD and the hock burn, both for the control and the treated birds and for the two sexes. All birds scored 0 points on the point-cumulative system, meaning that no foot pad or upper joints lesions were recorded throughout the experimental trial.

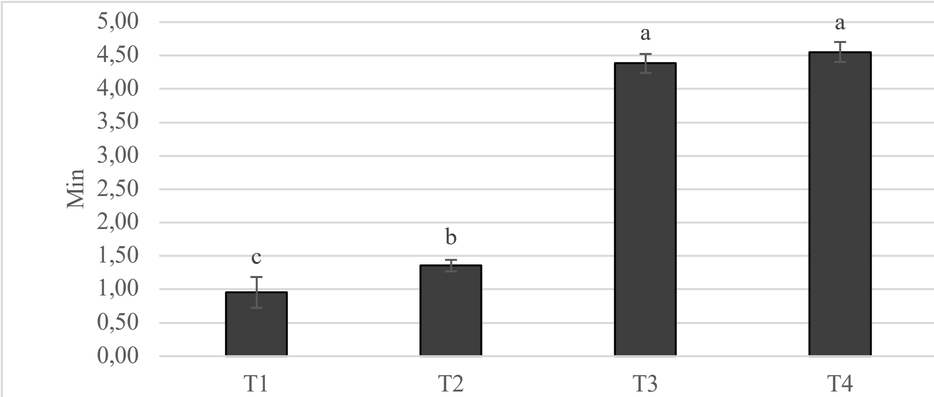
The FPD and hock burn are a form of contact dermatitis affecting the sole and the upper joints respectively (Kaukonen et al.; 2016). These conditions are mainly caused by the condition of the litter on which the chickens are reared. Excessive concentrations of feaces, dirt and ammonia could lead to the development of such diseases. However, the beneficial effects on the FPD and the hock burn of the live BSFL on provision cannot be proved, since the control birds did not show any sign of FPD and hock burn.

Tonic immobility test

Tonic immobility results are reported in picture 23.

Since no significant effects were observed between treated and control groups, as well as between males and females, the reported results are related to the different ages of the birds. During T3 and T4, all the birds spent more time in tonic immobility position, compared to T1 and T2 ($p<0.05$). These results are partially in agreement with Nakasai et al. (2013), since it discovered an increase, related to the age, in tonic immobility duration in males of a local

Japanese chicken breed. However, Nakasai et al. (2013) observed a decrease for this parameter in the females of the same local chicken breed.



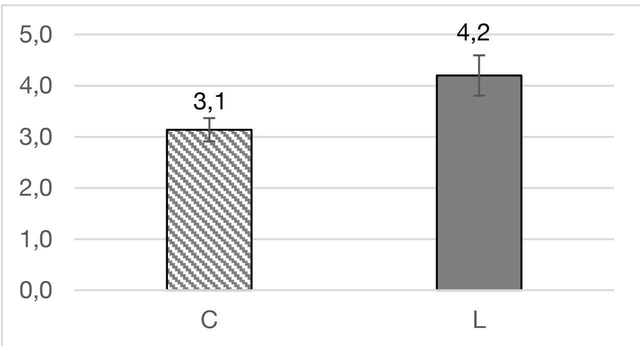
Picture 23: Tonic immobility duration at different ages (n=6).
T1: 26 d; T2: 39d; T3: 60d; T4: 74 d.
P<0.01

Avoidance distance test

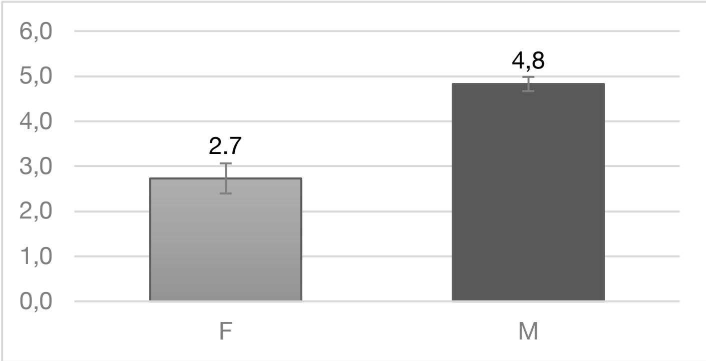
The results of the avoidance distance test are reported in pictures 24 and 25.

The results showed a significant difference between treated and control birds, since the treated groups showed a higher number of birds remaining closer (between 1 and 2 m) to the examiner (4.2 birds, on average), compared to the controls (3.1 birds, on average) ($p<0.05$).

There is also a significant difference between the two sexes, as males were less scared by the presence of the examiner compared to the females ($p<0.001$). These results are particularly interesting, especially for what concerns the BSFL administration, since it apparently reduced the fearfulness of the chickens, being an interesting tool to improve animal welfare.



Picture 24: Avoidance distance test results (distance between 1-2 m) between control birds and treated birds.
P<0.05



Picture 25: Avoidance distance test results (distance between 1-2 m) between males and females.
P<0.001

Slaughter performance

The summary of the slaughter performances is represented in table 5.

The slaughtering weight was mainly influenced by the sex, as males were significantly heavier than females ($p<0.001$). Between the control and the treatment groups there were no differences regarding the CC yield and the RTCC yield, yet there was a trend between the two genders, since the values for the CC yield were higher in males compared to those in females ($p>0.05$). Interestingly, the breast yield was higher in females ($p<0.001$) but the thigh yield was higher in males ($p<0.001$). Sexual dimorphism could be responsible for such differences.

For what concerns the organs weight, the spleen weight was lower in the control birds ($p<0.01$) and the liver was heavier in females ($p>0.05$). The heart incidence was higher in males than females ($p=0.001$) and, among the treatment groups, the control ones displayed a lower weight. The abdominal fat percentage was higher in females compared to males ($p<0.05$). The spleen and the Bursa of Fabricius are involved in the immune response system and could be considered an immune system activity indicator. Overall, stressed birds displayed a smaller spleen and Bursa of Fabricius due to the corticosterone effects (Puvadolpirod et al.; 2000). The provision of larvae could possibly have beneficial properties for the immune system, by virtue of the larvae chitin content, since it has an immunostimulant activity (Gasco et al.; 2018). The organs yield varied between sexes, as the liver incidence was higher in females than in males ($p>0.05$), as well as the heart incidence ($p=0.001$). The heart was heavier in the treated groups compared to the control ones ($p>0.05$). The increased heart incidence could possibly have a negative meaning, therefore it requires further investigations. The abdominal fat was more copious in females (p<0.05).

	Diet (D)			Gender (G)			P-value	
Item	**BSFL**	**Control**	**SEM**	**Male**	**Female**	**SEM**	**D**	**G**
Slaughter weight (g)	2441	2423	10.00	2829	2035	10.00	0.206	<0.001
CC yield (%SW)	65.2	64.7	0.28	65.3	64.6	0.28	0.213	0.091
RTCC yield (%SW)	66.2	65.7	0.30	66.3	65.6	0.30	0.256	0.132
Breast yield (%CC)	23.2	23.2	0.21	22.2	24.2	0.21	0.815	<0.001
Thigh yield (%CC)	33.9	34.0	0.20	34.8	33.1	0.20	0.700	<0.001
Spleen (%SW)	0.16	0.14	0.00	0.14	0.15	0.00	0.002	0.150
Bursa of Fabricius (%SW)	0.22	0.21	0.01	0.21	0.23	0.01	0.619	0.224
Liver (%SW)	1.87	1.85	0.04	1.81	1.90	0.04	0.739	0.087
Heart (%SW)	0.51	0.48	0.01	0.52	0.47	0.01	0.057	0.001
Abdominal fat (%SW)	1.43	1.60	0.18	1.01	2.02	0.18	0.511	<0.001

Table 5: Slaughter performance of Label Rouge Naked Neck chickens (males and females) fed a diet supplemented with 10% live BSFL; supplementation based on the expected daily feed intake (28-81d; n=12)

Meat pH and colour

Meat colour plays a fundamental role in the consumer's choice, as it is considered a selection criterion finalized to the purchase of meat (Fletcher; 2002). In fact, consumers are more likely to purchase pink and pale poultry meat (Wideman et al.; 2016). Meat colour is indeed related to the muscle pH, as the light reflecting properties of the meat and its pH are negatively correlated. Muscle pH affects the water holding capacity and the composition of meat. High pH values (Husak et al.; 2008), are proven to maintain favorable meat color and water-holding capacities. On the other hand, low pH values (close to 5.2) determine low water-holding capacities, being close to the meat isoelectric point, that inhibits the capacity of proteins to retain water (Husak et al.; 2008).

The results for the meat pH and colour are reported in table 6.

Breast and thigh pH were not affected by the dietary treatments, nor the sex (p>0.05). As demonstrated by the statistical analysis, there is not a significant difference between the two sexes, since the breast and thigh b* values were higher in females compared to males (p>0.05). On the other hand, thigh a* value was higher in males than females (p<0.05).

The b* value indicates the yellowness of the meat and an increased fat deposit in females can be assumed to explain these results. Indeed, carotenoids are mostly accumulated in the lipids, since they are fat-soluble.

On the other hand, male thighs resulted to be redder compared to the females, since the a* value indicates the redness of the meat.

	Diet			**Gender**			**P-value**	
Item	**Larvae**	**Control**	**SEM**	**Male**	**Female**	**SEM**	**Diet**	**Gender**
				Ph				
Breast pH	5.61	5.61	0.02	5.62	5.59	0.02	0.965	0.242
Thigh pH	5.85	5.87	0.02	5.89	5.83	0.02	0.489	0.052
				Color				
Breast L*	47.50	48.78	0.66	48.34	47.94	0.66	0.171	0.664
Breast a*	0.78	0.48	0.18	0.65	0.61	0.18	0.219	0.885
Breast b*	14.69	14.97	0.35	13.44	16.21	0.35	0.574	0.000
Thigh L*	35.63	35.52	0.46	35.13	36.02	0.46	0.870	0.171
Thigh a*	9.48	9.31	0.23	9.73	9.06	0.23	0.590	0.038

Table 6: Meat pH and quality results, divided for treatment and gender.

Blood traits

As reported in table 7, the leukocytes percentage was more relevant in the treated group than the control one (p<0.05). Among the leukocytes, the eosinophils rate was higher in females (p<0.01); contrarily males presented a higher monocytes rate (p<0.05). The control groups showed a higher monocytes percentage compared to the treatment groups (p<0.01) and, regarding the serum proteins and lipids, the triglycerides were greater in females than males (p<0.05). The percentage of triglycerides in females is in line with the amount of abdominal fat found in the carcasses of females, as it was more abundant compared to males. Differences among sexes and treatments were previously analyzed: some studies (Schiavone et al.; 2017, Dabbou et al.; 2018) did not report any significant difference related to the live BSFL administration., whereas other studies (de Souza Vilela et al.; 2021) demonstrated a lower WBC count in broiler supplemented with different levels of inclusion of full-fat BSFL meal, suggesting a beneficial effect on the immune system of chickens. Other studies suggest the discrepancy between the hematological traits of males and females, such as that of Peters et al. (2011), in which the hematological parameters (such as erythrocytes and leukocytes count, creatinine) were lower in females than males. On the contrary, Addass et al. (2012) reported higher hematological parameters in females. The cholesterol levels tended to be lower in the treated chickens (p>0.05), probably due to the fact that the chitin found in the BSFL reduction could have chelating effects (Prajapati and Patel, 2010)

	Diet (D)			**Gender (G)**			**P-value**	
Item	**BSFL**	**Control**	SEM	**Male**	**Female**	SEM	**D**	**G**
Erythrocytes, 10^6, cell/µL	2.32	2.22	0.23	2.49	2.07	0.23	0.770	0.191
Leukocytes, 10^3, cell/µL	31.1	23.9	2.26	26.7	27.2	2.24	0.023	0.745
Heterophils, %	47.3	42.1	2.90	47.2	42.2	2.90	0.202	0.225
Lymphocytes, %	48.1	52.8	2.65	48.2	52.8	2.65	0.212	0.218
Eosinophils, %	1.40	1.55	0.18	1.17	1.85	0.19	0.541	0.008
Monocytes, %	1.63	3.06	3.33	2.85	1.75	0.32	0.002	0.016
Basophils, %	2.90	2.67	0.32	2.54	3.05	0.32	0.617	0.269
			Serum proteins and lipids					
Total Protein, g/dL	4.61	4.73	0.11	4.70	4.65	0.11	0.405	0.743

Table 7: Hematological traits, serum proteins and lipids in both sexes of Label Rouge Naked Neck chickens fed a diet supplemented with 10% live BSFL; supplementation based on the expected daily feed intake (28-81d; n=12)

For what concerns the serum minerals and the hepatic and renal enzymes (table 8), the supplementation of live BSFL influenced the GGT (U/I) by lowering its concentration (p<0.05). GGT is an indicator of the liver health status, therefore the lower concentration in plasma suggests a beneficial effect of the live BSFL on the health status of the chickens. Overall, the administration of live BSFL did not negatively influence the blood traits of the chickens (Koide et al.; 1998).

	Diet (D)			Gender (G)			P-value	
Item	**BSFL**	**Control**	**SEM**	**Male**	**Female**	**SEM**	**D**	**G**
				Liver function				
ALT, U/l	9.95	11.7	1.03	11.08	10.6	1.03	0.220	0.755
AST, U/l	167	145	10.27	160	152	10.27	0.134	0.606
GGT, U/l	22.8	26.9	1.13	26.1	23.6	1.13	0.011	0.114
				Renal function				
Creatinine, mg/dL	0.46	0.47	0.03	0.49	0.44	0.03	0.811	0.148
Uric acid, mg/dL	8.94	8.95	0.59	8.81	9.07	0.59	0.986	0.752
				Minerals				
P, mg/dL	8.97	9.14	0.31	8.85	9.26	0.31	0.695	0.349

Table 8: Serum minerals, hepatic and renal enzymes of both sexes of Label Rouge Naked Neck chickens fed a diet supplemented with 10% live BSFL; supplementation based on the expected daily feed intake (28-81d; n=12)

9. CONCLUSIONS

The experimental trial described in the present thesis demonstrates the effectiveness of the BSFL inclusion in medium-growing chickens, specifically Label Rouge Naked Neck chickens.

The results showed a slightly increased final LW in the treated groups compared to the controls at the end of the trial, while, as expected, a higher final LW was observed in the males compared to the females, due to the sexual dimorphism.

Regarding the larvae consumption time, males were apparently more attracted to the live BSFL than females, but it is important to underline that both the sexes were interested to the larvae motility.

The meat pH and colour were not affected by the BSFL administration, suggesting the feasibility of the inclusion of this enrichment in poultry nutrition, resulting in comparable meat quality parameters.

However, no significant effects of this dietary inclusion were observed for feather condition score, skin lesions, FPD and hock burn. Generally speaking, the incidence of these phenomena was extremely low both in treated and control birds, probably related to the favorable housing conditions.

Although this is the first study that analyzed the influence of live BSFL in medium-growing chickens, promising results were obtained in terms of growth performance. Further studies are needed in order to better investigate the effects of this innovative enrichment in poultry nutrition.

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