POULTRYNSECT

D2.3 Report on poultry welfare assessment (1st trial)

Deliverable 2.3

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June 2023

This project has received funding from transnational funding bodies, partners of the H2020 ERA-NETs SUSFOOD2 and CORE Organic Cofund, under the Joint SUSFOOD2/CORE Organic Call 2019. This document reflects only the author's view and the transnational funding bodies are not responsible for any use that may be made of the information it contains.

Abbreviations	
AD	Avoidance distance
BSF	Black Soldier Fly
H/L	Heterophile/lymphocyte
TI	Tonic immobility
WP	Work Package

Content

Introduction

Introduction

The POULTRYNSECT Work Package 2 "Chickens in vivo feeding trials" aims to evaluate the BSF live larvae inclusion as feed ingredient in chicken diet to reduce the feed soybean content and increase the sustainability. Animal welfare and environment issues frequently influence the consumer choices in terms of meat purchase (1, 2, 3). Therefore, considering that the use of soybean – which is the main feed ingredient in poultry diet – is nowadays critical for its unsustainability (4), the search for alternative protein sources and rearing systems is fundamental (5). Insects as the BSF could be an alternative to soybean, thanks to their nutritional profile, high feed conversion ratios and low greenhouse gases emission (6, 7, 8, 9). Various studies have already been conducted in laying hens, broilers and other avian species fed live insects evaluating the effects on birds' growth, health status, slaughtering performance and welfare (5, 6, 10, 11, 12, 13). However, no data are available about the BSF larvae provision in medium-growing chicken genotypes.

The WP2 has three different objectives:

1) perform in vivo poultry feeding trial to determine the optimal inclusion level of live HI larvae for organic chicken production;

2) assess the gender effect on performances, welfare and health of birds fed live insect larvae;

3) assess in two different genotypes model (with different growing-rate) the effect on performances, welfare and health of birds fed live insect larvae.

For the first trial, the Label Naked Neck hybrid (medium-growing broiler genotype) was reared for 82 days. Some ethological test and welfare animal-based measurements were evaluated, and video recordings performed during the trial.

This Deliverable reports the animals' welfare and behaviour assessment obtained from the first task of the *in vivo* trials performed with Label Naked Neck chicken (UNITO).

1. Material and Methods

A total of 240 twenty-day-old Label naked neck birds were purchased from a commercial rearing centre (sexed chicks, sex ratio 1:1) and transferred to the Avian Conservation Centre of Local Genetic Resources of the University of Turin (north-west of Italy) where the trial was carried out from the beginning of October to the beginning of December. The initial weight of the birds was of 515,02 g and 435,94 g for the males and females, respectively.

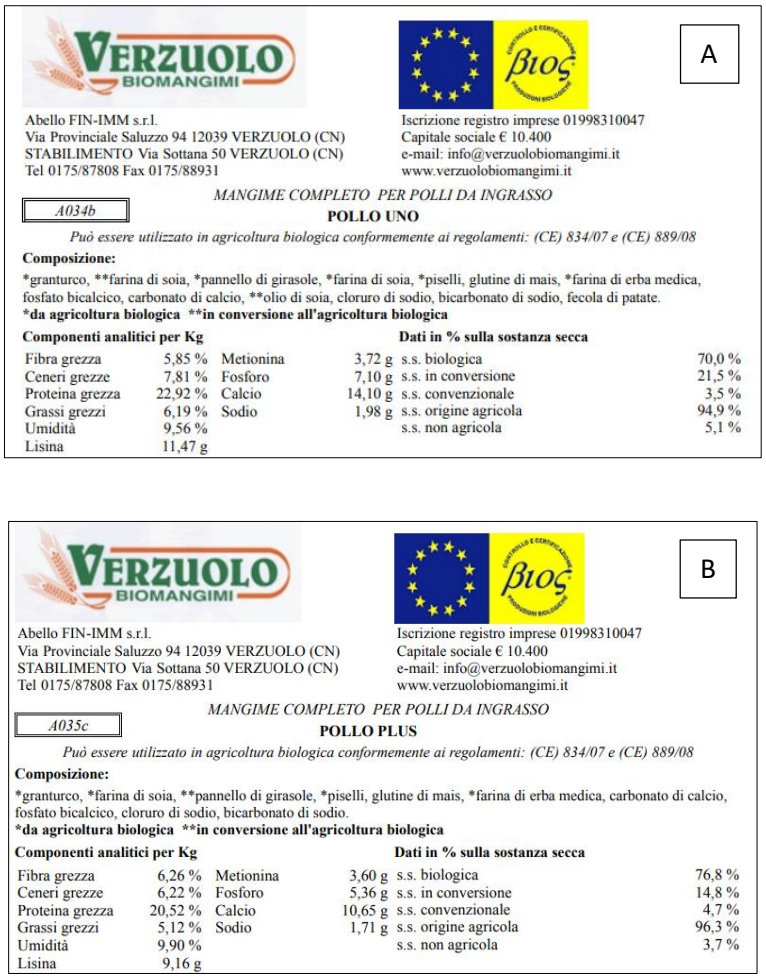
Figure 1. Females and males of Label naked neck birds

The birds were individually weighted and selected on the base of their average body weight and allotted in 24 pens. They were distributed in four experimental groups according to sex and treatment (10 chicken/pen, 60 birds/treatment):

1. males fed basal organic feed;
2. males fed basal organic feed +10% BSF supplementation;
3. females fed basal organic feed;
4. females fed basal organic feed +10% BSF supplementation.

The birds always had free access to clean and fresh water and organic feed. A first period diet was adopted until 35 days of age (22.92% crude protein, crude fat 6,19%, gross energy 18.73 MJ/kg) and a grower feed was provided from 35 to 82 days of age (20.52% crude protein, crude fat 5.12%, gross energy 18.61 MJ/kg) (Verzuolo mangimi s.r.l.). The feed composition were similar in both diets: corn, soybean, sunflower meal, soybean meal, peas, corn gluten, lucerne meal were the main ingredients (protein sources), dicalcium phosphate, calcium carbonate, soybean oil sodium chloride, sodium bicarbonate and potato flour the remain ingredients.

Feed labels of both diets are showed in Figure 2.
Natural ventilation and photoperiod (from 12L:12D in October, to 10L:14D in December) were applied for the entire duration of the trial. Outdoor access was ensured for all the birds from 49 days of age to the end of the experiment. Mortality and health status of the birds were checked and recorded daily. The animals were weighted weekly and the average weight (AW) calculated. Feed consumption was recorded and the Feed Conversion Ratio (FCR), average daily feed intake (ADFI) and average daily gain (ADG) were calculated at the end of each rearing period (20-35d, 35-82d) and for the overall period (20-82d). The FCR in the treated group was corrected on the base of the larvae dry matter (33.63%) content ingested from birds.



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Composizione:

*granturco, **farina di soia, *pannello di girasole, *farina di soia, *piselli, glutine di mais, *farina di erba medica, fosfato bicalcico, carbonato di calcio, **olio di soia, cloruro di sodio, bicarbonato di sodio, fecola di patate.
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Componenti analitici per Kg				**Dati in % sulla sostanza secca**	
Fibra grezza	5,85 %	Metionina	3,72 g	s.s. biologica	70,0 %
Ceneri grezze	7,81 %	Fosforo	7,10 g	s.s. in conversione	21,5 %
Proteina grezza	22,92 %	Calcio	14,10 g	s.s. convenzionale	3,5 %
Grassi grezzi	6,19 %	Sodio	1,98 g	s.s. origine agricola	94,9 %
Umidità	9,56 %			s.s. non agricola	5,1 %
Lisina	11,47 g				

B

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Composizione:

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***da agricoltura biologica ****in conversione all'agricoltura biologica**

Componenti analitici per Kg				**Dati in % sulla sostanza secca**	
Fibra grezza	6,26 %	Metionina	3,60 g	s.s. biologica	76,8 %
Ceneri grezze	6,22 %	Fosforo	5,36 g	s.s. in conversione	14,8 %
Proteina grezza	20,52 %	Calcio	10,65 g	s.s. convenzionale	4,7 %
Grassi grezzi	5,12 %	Sodio	1,71 g	s.s. origine agricola	96,3 %
Umidità	9,90 %			s.s. non agricola	3,7 %
Lisina	9,16 g				

Figure 2. Feed labels of the poultry diets: Label A (first period) and Label B (grower)

Behavioural observations

The video recordings were performed by means of tablets at 25 (T0), 61 (T3), and 75 (T4) days of age. A total of four replicates/treatment were recorded and three time slots (5 minutes/each) were selected to check the birds' behaviour: in the morning (9.00 a.m.), during the live BSFL provision (11.00 a.m.) and in the afternoon (4.00 p.m.), during the same day and in the same order. The collected video recordings were analysed by means of BORIS (Behavioural Observation Research Interactive Software v 7.9.7) (15). The behaviours were ordered in four macro groups: foraging related behaviours, activity behaviours, and social behaviours (Table 1). The occurrence of a specific behaviour was registered within each time slot regardless of its duration and corrected by the number of birds observed in the pen every 30 seconds. The recorded ethogram was elaborated considering the previous studies conducted (11, 12, 13, 14, 16).

Table 1. Ethogram of specific behaviour repertoire and activity of chickens.

Class	Denomination	Description	References
Foraging related behaviours	Ground pecking	Pecking at the ground	(Ipema et al., 2020a)
	Object pecking	Pecking	(Veldkamp and van Niekerk, 2019)
	Scratching	Move the litter backwards by claws	(Biasato et al., 2022)
	Eat larvae	Pecking larvae from the plates	-
Comfort behaviours	Preening	Self-feathers grooming by means of beak	(McCowan et al., 2006)
Activity behaviours	Walking	Walking/running	(Biasato et al., 2022)
	Standing	Standing stationary	(Veldkamp and van Niekerk, 2019)
	Resting	Sitting/lying stationary	(Veldkamp and van Niekerk, 2019)
	Outside	Have access to the outside paddock	-
Social behaviours	Sparring	Play fighting	(Veldkamp and van Niekerk, 2019)
	Chasing	Running after a conspecific	(Biasato et al., 2022)
	Pecking conspecifics	Pecking movements directed at a pen mate	(McCowan et al., 2006)
	Allopreening	Social preening	(Kenny et al., 2017)

Avoidance distance test

The AD test was performed to measure the birds' fear based on a human approach response (17). The operator squats on the litter closed to a group of birds for 10 seconds and counts the number of chickens within 1m (arm's length), (18), between 1m and 2m and over 2m of himself. The test was executed at 27 (T0), 41 (T1), 62 (T3), and 76 (T4) days of age between 3.00 and 4.00 p.m.

Tonic immobility (TI) test

The TI test was performed to evaluate the fearfulness level of chickens according to what was reported by (19). The test was performed in a separated area inside of the same building to avoid the eye contact with the other birds. A total of three chickens/pen were randomly selected and labelled with a second wing mark at 26 days of age. These birds were thus subjected to the TI test at 26 (T0), 39 (T1), 60 (T3), and 74 (T4) days of age. The test was performed according to the methodology adopted in previous study (19). During the test, the bird was placed on its back on a U-shaped cradle. A slight pressure was applied on the breast of the bird and the duration of the TI was recorded since the bird stopped struggling and became immobile at least for 10 seconds.

If the bird righted itself in less than 10 seconds, the test was repeated for a maximum of 3 times. If the TI was not induced within 3 attempts, the assigned score was 0 seconds. The maximum considered TI duration was 10 minutes (600 seconds). Finally, the TI induction frequency was calculated based on the number of inductions required for inducing TI (from 1 to 3 attempts) and expressed as a percentage of the total executed attempts.

Feather damage and cleanliness

The plumage condition was assessed considering the feather damage, as well as the breast feather cleanliness, at 28 (T0), 49 (T2), 63 (T3), and 77 (T4) days of age. The first parameter was scored from 0 to 5, evaluating the wings, tail, thighs and back covering conditions (20): 0 = fully feathered; 1 = rough; 2 = some broken feathers; 3 = heavily broken feathers; 4 = almost bald; 5 = bald. The feather cleanliness was instead scored from 0 to 4 (20): 0 = clean; 1 = slight change in feather coloration; 2 = marked change in feather coloration; 3 = spotted litter and faeces stuck to the feathers; 4 = marked litter and faeces stuck to the feathers.

Leg health: hock burn and footpad dermatitis scores

The leg health evaluation included both the FPD and the HB scores, and the sampling times were the same adopted for the feather condition evaluation. In particular, the FPD was scored as follows (20): 0 = no lesion; 1 = minor and superficial lesion of the skin with hyperkeratosis; 2 = moderate and superficial lesion of the skin with hyperkeratosis (less than one quarter of the foot pad affected); 3 = severe and deep lesion with hyperkeratosis (one half of the foot pad altered); 4 = severe and deep lesion with hyperkeratosis (more than one half of the foot pad altered). The HB was instead evaluated as follows (20): 0 = no lesion or mild skin rash; 1 = pronounced skin rash; 2 = moderate skin lesion and blood scabs; 3 = severe but confined skin lesion and necrotic areas (less than one half of the area altered); 4 = severe and extended skin lesion and necrosis (one half or more than one half of the area affected).

Skin neck and breast lesion scores

The skin lesion score was assessed concurrently with the feather condition. Two areas were considered regarding the skin lesions scoring: the neck and the breast. The scoring system adopted for the neck-skin lesions was design as follows (20): 0 = no lesions or less than three pecks (punctiform damage less than 0.5 cm diameter) or scratches; 1 = at least one lesion less than 2 cm diameter at largest extent or three or more pecks or scratches; 2 = at least one lesion 2 or more than 2 cm diameter at greatest extension. The breast-skin lesion protocol was instead developed considering the presence or absence of erythema: 0 = normal skin coloration; 1= intense but contained breast-skin redness (less than one half); 2 = intense and extended breast-skin redness (one half or more than one half).

Excreta corticosterone metabolites (ECM) analysis

Considering two random birds selected for the tonic immobility, fresh excreta samples were individually collected at 26 (T0), 39 (T1) and 74 (T4) days of age. Each bird was placed in a wire-mesh cage (100 cm width × 50 cm length) until at least 2 grams of fresh excreta were produced and collected in a plastic box under the cage. Subsequently, the samples were stored at −20°C for the corticosterone analysis. The ECM was executed according to previous studies (21, 22). More in detail 3 mL of 80% methanol (Sigma Aldrich, St. Louis, MO, USA) were added to 0.25 g of lyophilized excreta in an extraction tube and maintained at -20°C for 2 h to allowing the solid phase to settle to the bottom. After 2 h, the supernatant was transferred into a new vial and evaporated under the hood for 14 h. The ECM were determined by means of a multi species

enzyme immunoassay kit (K014 - Arbor Assay®, Ann Arbor, MI, United States) validated for serum, plasma, saliva, urine, dried fecal extracts, and tissue culture media. The inter- and intra-assay coefficients of variation did not exceed the 10% and the sensitivity of the assay was 11.2 ng/g of excreta. Multiple dilutions were adopted to perform the samples analyses (1:4, 1:8, 1:16, and 1:32) and all the regression slopes were parallel to the standard curve (R2 = 0.989). The mean recovery rate of corticosterone added to dried excreta was 96.5%. According to the manufacturer, the corticosterone kit presents the following cross reactivity: 100% with corticosterone, 12.3% with desoxycorticosterone, 0.62% with aldosterone, 0.38% with cortisol and 0.24% with progesterone. All the analyses were performed in duplicate, and the concentration of ECM was expressed as ng/g excreta dry matter.

H/L ratio

Blood samples were drawn from the 48 birds selected for the slaughtering at 82 days of age. For each bird, 2.5 mL of blood were stored in a serum-separating tube. A drop of blood was placed on a glass slide and the smear was obtained. The May-Grünwald and Giemsa stains (23) was used to stain the smears and a 1:200 Natt-Herrick solution used to treat the samples (24). The count of the erythrocytes and leukocytes was defined by using an improved Neubauer haemocytometer (25). A total of one hundred leukocytes, both granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) leukocytes, were counted on the glass slide and the H/L ratio was calculated.

Results and discussion

2. Results and discussion

The video recordings showed an increased foraging and exploration activity of the birds. In more details, during the live larvae provision, the ground pecking frequency resulted greater in the morning at T3 and T4 than T0, but in the supplemented birds solely (P<0.05), with higher frequencies observed in the L birds than C ones (P=0.001) (**Figure 3**). Moreover, the scratching frequency was greater in the C groups than L ones at T0, while the opposite was observed at T3 (P<0.05) (**Figure 4**). Similarly, the scratching behaviour during the afternoon was higher in the L than C groups at T3 (P<0.01) (**Figure 5**). Regarding the activity behaviours, a major number of F than M were observed outside in the C groups (P<0.01), whereas the opposite was recorded in the L ones (P<0.05) (**Figure 6**).

The TI test was not affected neither by the diet nor by the gender, despite an increase in the TI duration was observed between T1 and T3 (**Figure 7**), probably due to the gained weight of the birds. The larvae administration reduced instead the fear and increased the exploration activity of all the birds during the AD test (P<0.05). In more detail, a higher number of LF came within 1-2m from the operator than the CF ones, while no differences were observed between the M groups (**Figure 8**). Such results could be attributed to the natural greater boldness of M than F, hence the larvae provision effect might not be visible in this sex. Moreover, the higher prudence of F compared to M must be considered, being the F responsible for brooding and offspring protection, thus offering a wider margin of observation of the live larvae effect. Since the birds' damages/injuries occurred <0.05 times, the statistical analyses could not be applied. Thus, we can conclude that the live BSF larvae supplementation did not affect the feather condition, hock burn, footpad, pododermatitis and skin lesion conditions. Finally, no effects were observed in the ECM, while the H/L ratio was higher in the L than C groups (P=0.05) (**Figure 9**). Thus, such results might not be directly related to a bird's negative experience, since the animals were not exposed to an intense and prolonged stress condition and the H/L ratio broadly varies among strains. On the other hand, the competition among birds due to the larvae provision is still unknown and further research is needed.

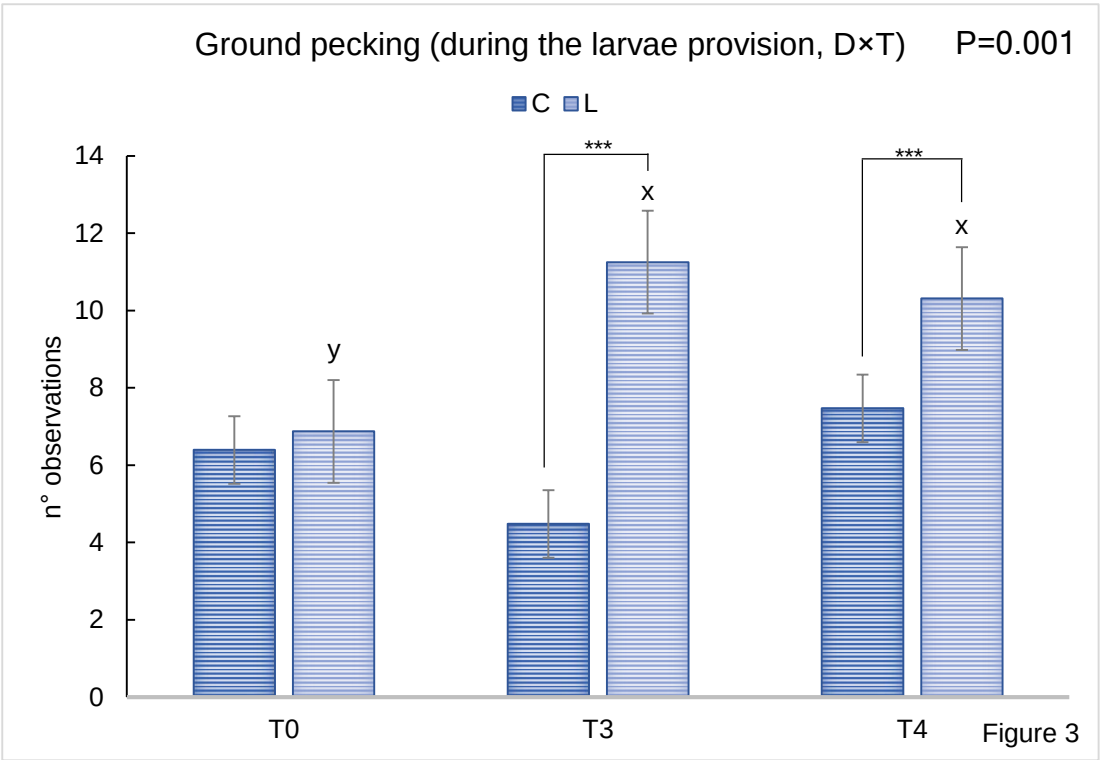
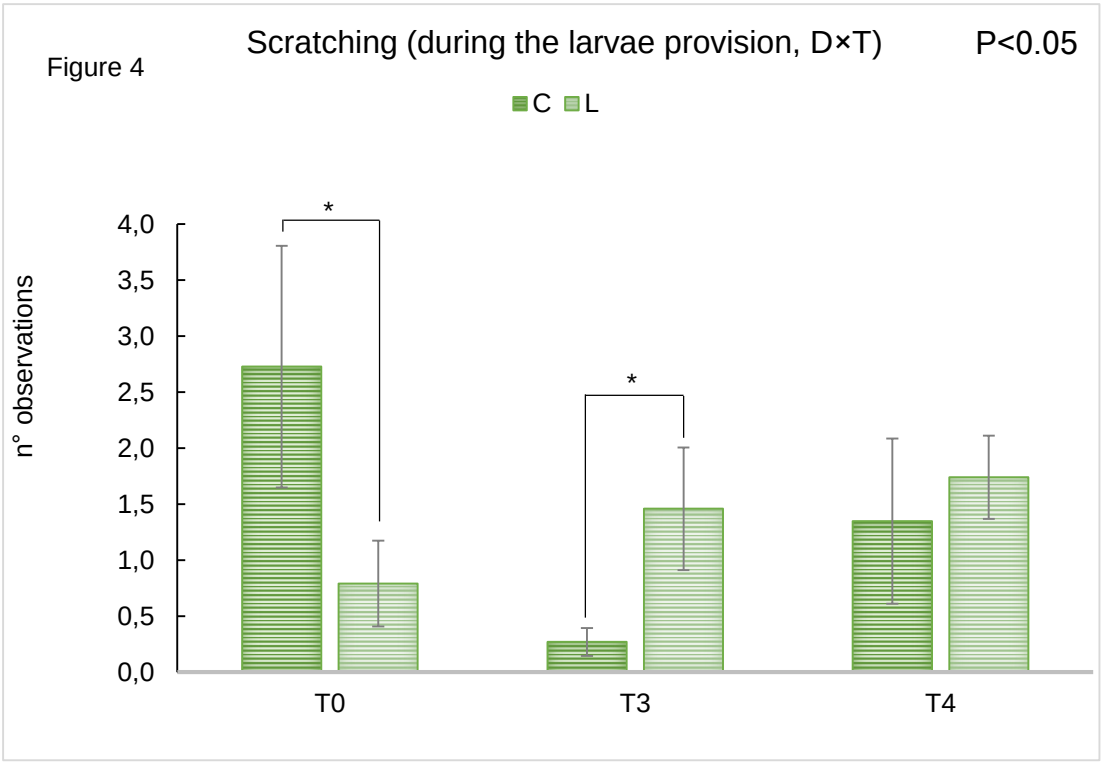


Figure 3

Figure 4



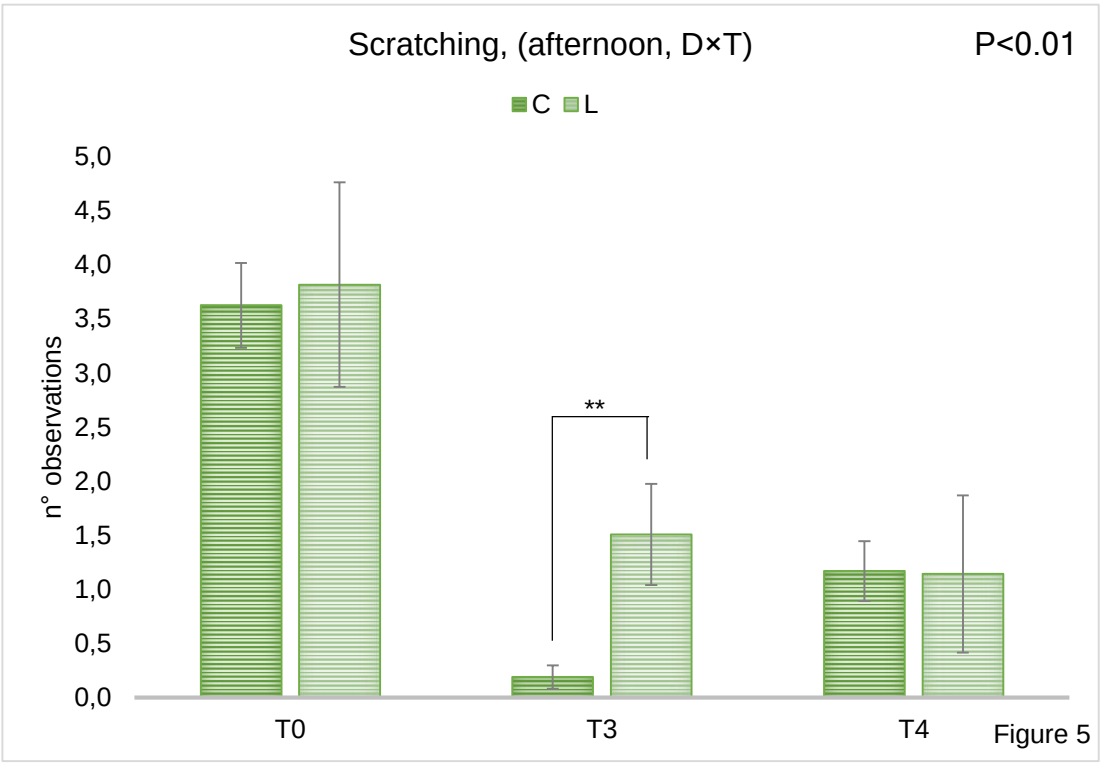


Figure 5

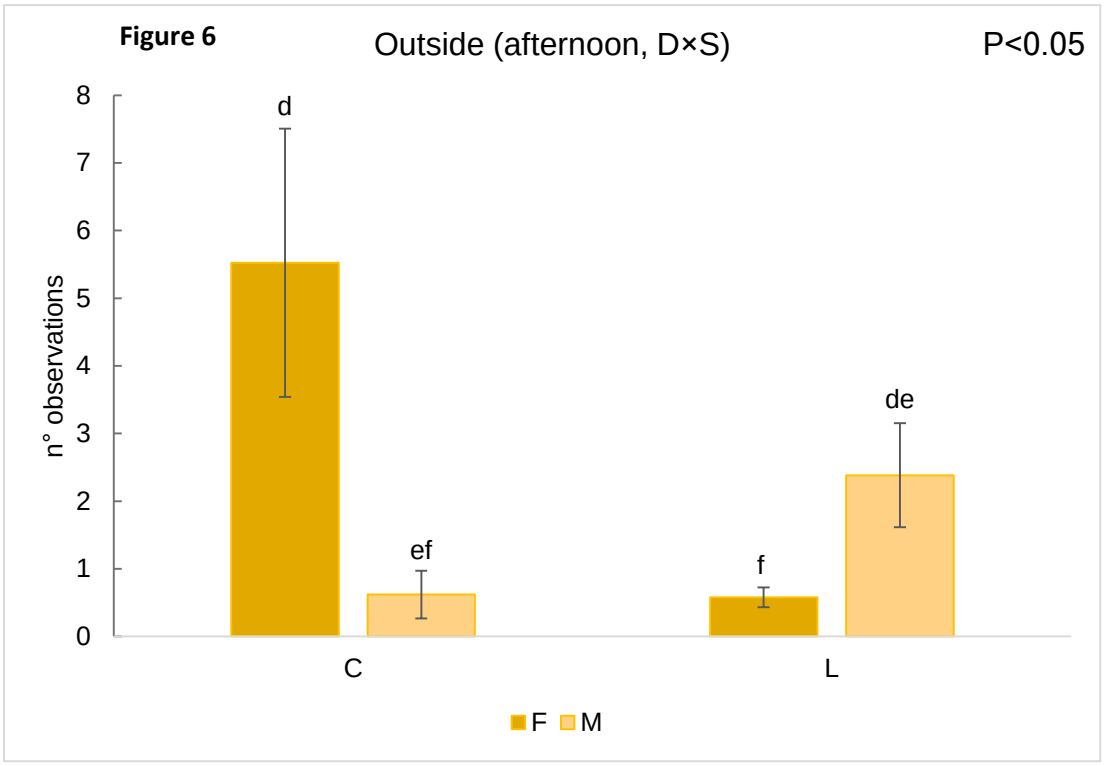


Figure 6
Outside (afternoon, D×S)
P<0.05
n° observations
8
7
6
5
4
3
2
1
0
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de
C
L
F
M

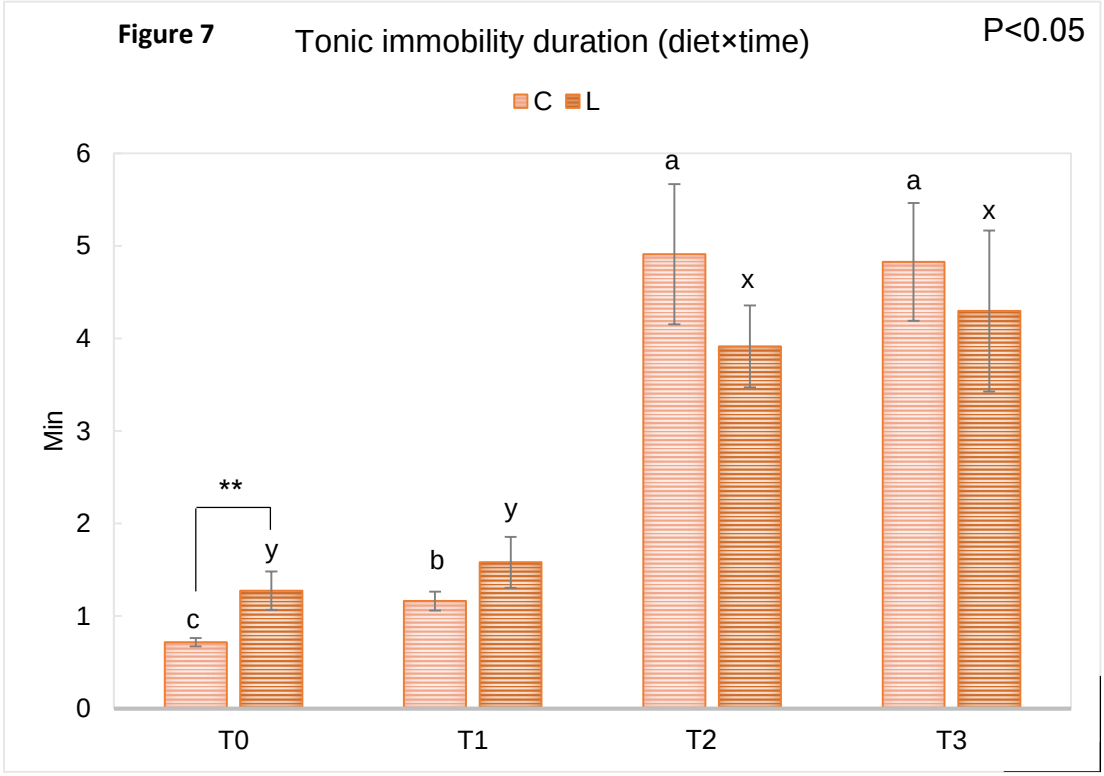
Figure 7
Tonic immobility duration (diet×time)
P<0.05
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L
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6
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4
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2
1
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a
x
a
x
T0
T1
T2
T3

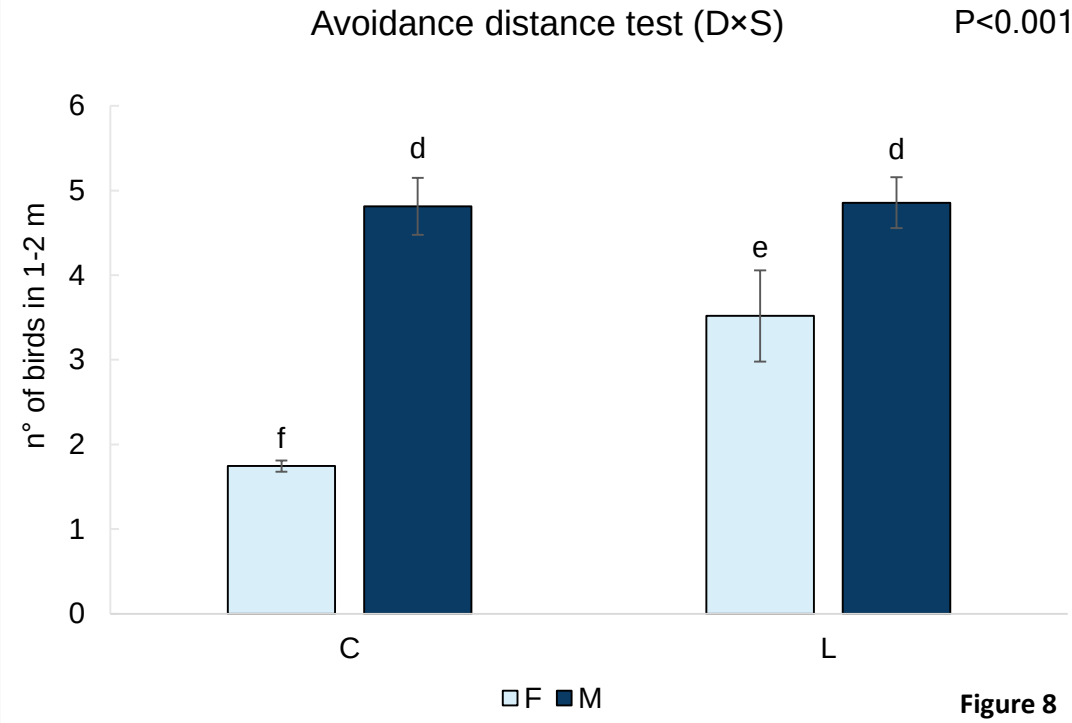

Figure 8

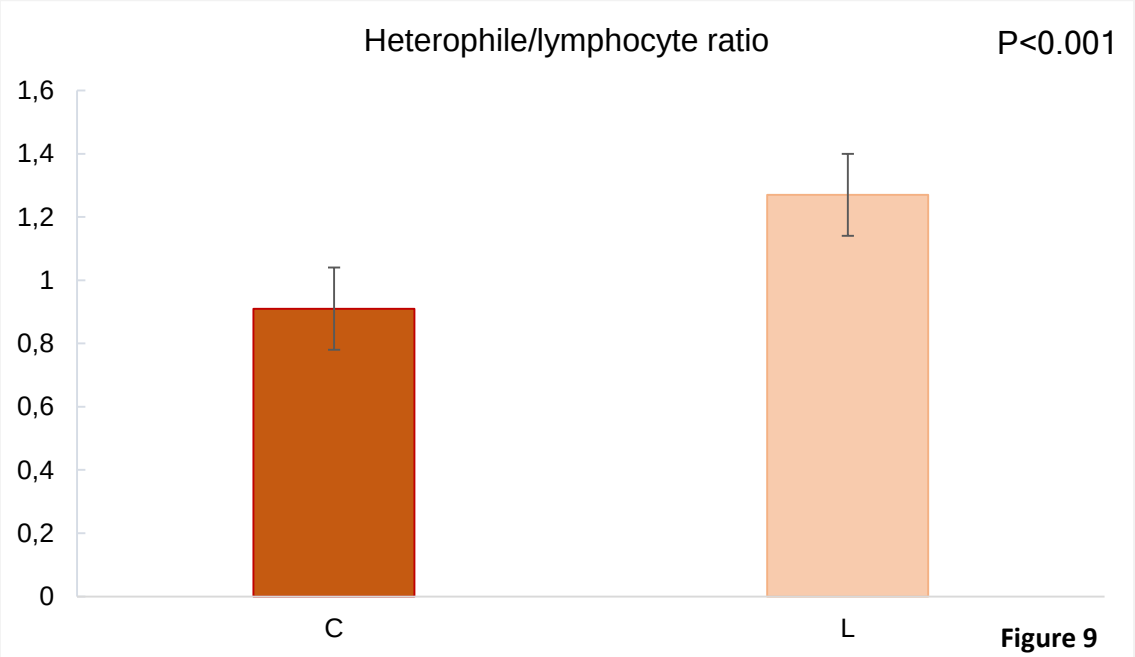

Figure 9

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This project has received funding from transnational funding bodies, partners of the H2020 ERA-NETs SUSFOOD2 and CORE Organic Cofund, under the Joint SUSFOOD2/CORE Organic Call 2019. This document reflects only the author's view and the transnational funding bodies are not responsible for any use that may be made of the information it contains.