

An index to measure the activity attitude of broilers in extensive system

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ABSTRACT In organic poultry production it is important to rear animals with a dynamic attitude to take advantage of outdoor areas. Farmers are reluctant to use such strains due to their lower productivity and older slaughtering age. However, fast growing lines grown in organic system often suffer poor health and welfare conditions. The kinetic metabolism of chickens is correlated with different types of muscle fiber: type I (in red muscles or oxidative) for prolonged and moderate movement and type II (in white muscles or glycolytic) for fast movements. Red muscle metabolism produces energy mainly by β -oxidation of Highly Unsaturated n-3 Fatty Acids (HUFA). Accordingly, kinetic activity causes higher consume of HUFA in red muscles than in white muscles, so the ratio between n-3 HUFA and their precursor C18:3 n-3 (ALA) is likely to be smaller in red than in white muscles. However, these ratios are highly affected by the environment. To reduce the effect of

environmental variables, we propose an “Activity index” as the difference between n-3 HUFA/ALA in white and red muscle within the same bird. This index, measured after slaughtering, should represent the activity performed by the chicken during its life. Given that birds in good health had the possibility of moving, the “Activity index” would measure the activity actually performed by the animals. Should birds of a given strain show a higher activity level, this would be an indication of the suitability of that strain to outdoor systems. This work verified the application of this “Activity index” on 90 birds from 6 genetic strains with known kinetic behavior reared in an experimental farm. The “Activity index” was also tested on chicken strains collected from commercial organic farms. The results confirmed that strains recognized for higher kinetic attitude actually walked more and their behavior was clearly detected by the “Activity index” estimated from their muscles.

Key words: organic production, poultry genetic line, adaptability to extensive system, movement behavior

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INTRODUCTION

In the new EU Organic Regulation 848/2018, which shall apply from January 1, 2022, the ability of animal breeds to adapt to organic farming conditions has attracted new attention. For poultry production, adaptation to outdoor rearing is an essential condition to comply with the organic rules.

In the organic system, the outdoor run for fattening poultry is a very large space (4 m²/chicken) with vegetation. Chickens with an outdoor run have a significant activity of preening, dustbathing, walking, and eating from pasture (Zhao et al., 2014). In organic rearing

system, the use of outdoor runs has a crucial role in increasing animal welfare and meat quality (Cartoni Mancinelli et al., 2017, 2020). Organic chickens in breast and drumstick muscles showed higher levels of Polyunsaturated Fatty Acids (PUFA) n-3 than animals reared indoor (Dal Bosco et al., 2016). Pastured birds acquire nutritional and wellbeing benefits (Faustin Evaris et al., 2019). However, not all the genetic lines can achieve the same benefits. Some studies report that fast-growing chickens (FG) are not suitable for organic systems. Rapid growth, high body weight, and inactivity induce several welfare and health problems in chickens (Tahamtani et al., 2018). According to Bokkers and Koene (2003), after the 8th wk of age, fast growing chickens only move to feed and drink. On the contrary, slow growing (SG) chickens can fully benefit of the outdoor run. Chickens with access to outdoor runs, had higher blood antioxidant level than indoor chickens. Consequently they showed a better oxidative response

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and meat with higher amounts of polyunsaturated fatty acids (Cartoni Mancinelli et al., 2017, 2020).

In order to avoid intensive rearing methods, EU Reg. 848/2018, while maintaining previous recommendations on the use of slow-growing poultry strains, adds the concept of outdoor rearing adaptability. As there is not a proper and standard definition on “slow growing strains,” every Member State in EU defined different criteria. The result is a wide range of interpretations, strains and daily growing limits. Describing global broiler production, Rayner et al. (2020) divided poultry breeds in fast (>50 g/day) and slow (<50 g/day) growers. Castellini et al. (2016) found poor health and welfare condition in organic birds growing >40 g/day: they had high lysozyme level in blood, indicating presence of acute and chronic inflammation.

Establishing an objective and unique criterion to define slow growth is of paramount importance. A first index of adaptability to organic farming based on 49 different traits (behavior, weights, and blood analyses) was proposed by Castellini et al. (2016). They allocated 8 strains to 3 groups: fast, medium, and SG. Their adaptability index showed significant differences among strains, but a huge variability within groups. Also, daily weight gain was not a good indicator of adaptability.

Adaptability to outdoor systems, meaning the ability to walk and pasture in outdoor runs, might be an objective index to define the ability of a genetic line to fully cope with the organic system.

Animal kinetic activity is energy consuming, the locomotor muscles obtain energy mainly from 2 sources: carbohydrate and free fatty acids. Even if the propensity of carbohydrate against fatty acids use during movement has not been completely explained, it is generally agreed that for a fast and short contracting activity muscles use glycogen, while for slow and prolonged exercise, muscles mostly use fatty acids (Brooks and Mercier, 1994).

Chicken locomotory muscles are located in the thigh and they are richer in fat compared to breast muscles (Castellini et al., 2016). Leg muscles are capable to mobilize a great quantity of fatty acids to produce energy for movement. However, in the thigh muscles great differences in terms of fiber, color and fatty acid composition have been observed (Branciarri et al., 2009; Sales, 2014). Muscle contraction, velocity and range of motion depend on fiber types (Paxton et al., 2010) and energy metabolism changes accordingly (Barnard et al., 1982).

Glycolytic fibers are indicated as type IIb or White due to lower contents of mitochondria and myoglobin (Picard et al., 2012) and use glycogen, much more readily available than fatty acids, as energy source. Oxidative fibers are identified as type I (Suzuki et al., 1985) or red for the large presence of mitochondria and high myoglobin, providing for high oxygen exchange. Oxidative fibers endure slow movement for prolonged periods (Hoppeler, 1990; Wittenberg, 2003).

Accordingly, red fibers mainly produce energy from fatty acid β -oxidation which taking place in mitochondria. This is an aerobic breaking down of fatty acids

particularly evident in High Unsaturated Fatty Acids (HUFA) (Raclot and Groscolas, 1993). Poultry metabolism of C18:3 n-3 (alpha linolenic, ALA), through a series of desaturation and elongation (Lands, 1992; Gregory et al., 2013), produces n-3 HUFA (eicosapentaenoic acid - EPA; docosapentaenoic acid - DPA and docosahexaenoic acid - DHA) with more than 20 C, β -oxidation of n-3 HUFA breaks the bond between the second carbon/beta carbon and the third carbon/gamma carbon, and predominates over n-6 β -oxidation (Kriketos et al., 1995; Mickleborough, 2013). In this view, the rate of n-3 β -oxidation in the muscle (e.g., n-3 HUFA/ALA) can adequately describe n-3 HUFA mobilization used for movement (Kriketos et al., 1995) and the resulting oxidative status.

The ratio between n-3 HUFA and ALA could be taken as an indicator of the oxidation status. This should be higher in red than in white muscles, and therefore, in animals with higher kinetic behavior (Dal Bosco et al., 2012).

Naturally, beside the kinetic activity, other confounding factors could affect this marker and thus the Activity index requires the minimization of the environmental effects to be sufficiently predictable of kinetic activity of chickens.

The aim of this work is to draw up and calibrate an Activity index on meat of chickens reared in controlled organic conditions (the same diet, management, and a known kinetic behavior). Moreover, the Activity index was also successively validated with standard organic chickens reared in commercial farms.

The trial comprises 3 main investigations:

1. The first experiment is the identification and characterization of different thigh muscles (color and fatty acids profile);
2. The second experiment provides for the formulation of an Activity index in chickens based on an experimental trial;
3. The third experiment consists in the validation of the “Activity index” in chickens reared in commercial farms.

MATERIALS AND METHODS

Experiment 1. Thigh Muscle Characterization and Activity Index

Thigh Muscles Characterization First 2 representative White and Red muscles had to be identified. Color and fatty acids analysis were performed identify the whitest and the reddest thigh muscles on which apply the index.

The left pelvic limbs of 8 male broilers “Campese by Amadori” coming from a larger population of an organic commercial farm were randomly chosen after slaughtering at 81 days of age in a commercial slaughterhouse with an average bust weight of $2,335.7 \pm 236.2$ g.

They were dissected, 6 muscles were excised and identified based on Paxton et al. (2010) as in Figure 1: *M.*

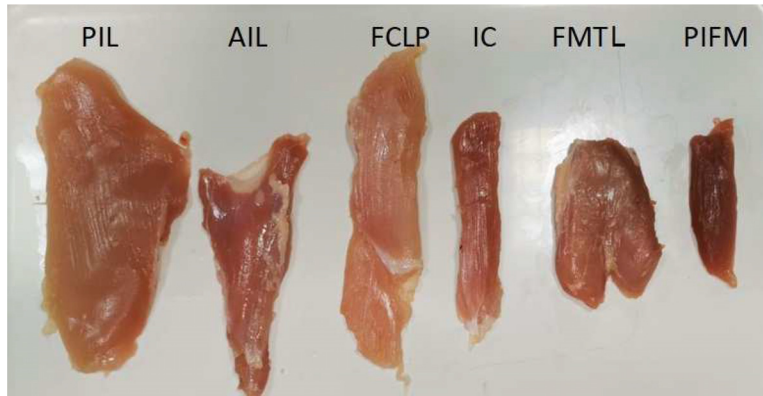


Figure 1. Six thigh muscle used for Red and White muscles determination. Note: Muscle nomenclature: M. iliotibialis lateralis postacetabularis (PIL); M. iliotibialis lateralis preacetabularis (AIL); M. flexor cruris lateralis pars pelvica (FCLP); M. iliotibialis cranialis (IC); M. femorotibialis lateralis (FMTL); M. puboischiofemoralis pars medialis (PIFM).

iliotibialis lateralis postacetabularis (PIL); *M. iliotibialis lateralis preacetabularis* (AIL); *M. flexor cruris lateralis pars pelvica* (FCLP); *M. iliotibialis cranialis* (IC); *M. femorotibialis lateralis* (FMTL); *M. puboischiofemoralis pars medialis* (PIFM). The 6 muscles were selected for their easier identification, excision and adequate size for analysis in order to confirm the results by (Suzuki et al., 1985; Hoppele, 1990; Iwamoto et al., 1993; Nakamura et al., 2004).

Muscle Color The color of the 6 muscles was determined at dissection. After sampling, muscles were halved in order to avoid interference by the epimysium translucent effect. The color analysis was measured as a 3-point average of the muscle after 1 h exposure to oxygen at 10°C. Color components were recorded using a Minolta CM-3600 D spectrophotometer (Konica Minolta Holdings Inc., Japan) in the CIELAB space (CIE, 1986) with illuminant D65 (color temperature 6504 K). White calibrations were used and lightness (L^*), redness (a^*), and yellowness (b^*) were recorded. After color determination, the samples were stored at -80°C .

Fatty Acids Assessment Six muscle samples were thawed at $+4^{\circ}\text{C}$ for 3 h. The lipids were extracted according to Folch et al. (1957). Fat obtained after evaporation of chloroform in a volumetric flask was dried in nitrogen flow and conditioned at 45°C to obtain the percentage of extracted fat. A total of 100 mg of fat extract were methylated by adding methanolic KOH, according to IUPAC procedure (1992), with C19:0 fatty acid as internal standard. Methyl-esters were injected in a GC-FID gas chromatograph (GC 6890 N, Agilent, Inc., CA) using a CP-Sil88 100 m \times 0.25 (0.2) column (Agilent Technologies) under the operating conditions described by Amici et al. (2015).

Fatty Acid Methyl Esters (FAME) standards (Supelco 37 Component FAME Mix; C22:4-n-6; C22:5-n-3 DPA; C19:0 from Sigma Aldrich (Oakville, ON, Canada) were used to identify fatty acids. Fatty acids were expressed as percentage of total FAME. Moreover, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), PUFA both n-3 and n-6, PUFA/SFA, n-6/n-3 ratio and n-3 HUFA/ALA were calculated.

Identification of Target Muscles As previously stated, the fatty acids' biosynthesis for individual or group of fatty acids and their catabolism (oxidation) could be estimated according to the precursor-to-product ratio and the ratio between n-3 HUFA/ALA (Kriketos et al., 1995) could be considered a sound marker of kinetic activity. However, to reduce the effect of environmental effect on such index (diet, grass intake, genetic strain, density, etc.) the "Activity index" was grown up as the difference between n-3 HUFA/ALA in white and red muscle within the same bird to minimize such effect and to underline the n-3 HUFA β -oxidation. Experiment 1 allowed for the identification of the 2 extreme thigh muscles in color, and therefore, in fiber type (PIL and PIFM), to be compared.

Experiment 2. Validation in Experimental Farm

Animals and Muscle Sampling This is a companion article to recent studies (Cartoni Mancinelli et al., 2020; Pulcini et al., 2021) on poultry behavior and welfare state in organic rearing system.

The trial was carried out in the experimental farm of the University of Perugia as described by Cartoni Mancinelli et al. (2020). Six hundred male chickens of 6 different genetic lines, 100 for each genetic line, were reared in compliance with Regulation CE 834/2007 and Regulation CE 889/2008. From Aviagen Breeders: Ranger Classic (RC), Ranger Gold (RG), Rowan Ranger (RR); from Hubbard Breeders: CY Gen 5 \times JA87 (CY), M22 \times JA87 (M), RedJA, known in Italy as Campese (C). The experimental protocol did not involve any animal manipulation, the animals were reared according the organic EU regulations with standard practice without compromising animal welfare and were slaughtered (81 d) in a commercial authorized plant under veterinary surveillance. Since the animals were reared in accordance with the law for commercial farms there was no need to ask Ethical Committee approval.

After slaughtering, 15 carcasses per genetic lines were randomly selected from each group of 100, weighed and dissected. The average carcass weights were 3,616.0 g RC; 3,179.3 g RG; 2,859.3 g RR; 2,930.7 g C; 3,213.3 g M, and 3,487.7 CY. From the left thigh; only PIL and PIFM muscles were excised.

The assignment of these genotypes to groups of different kinetic attitude was based on previous results of 2 companion studies by [Cartoni Mancinelli et al. \(2020\)](#) and [Pulcini et al. \(2021\)](#), where genetic types were classified as “static” (CY, M, and RC) when active behavior ranged between 0 and 11% or “active” (RG, RR, and C) when active behavior ranged between 24 and 37%. In [Pulcini et al. \(2021\)](#) the prevalence of static behavior was correlated to a more pronounced curvature of the anteroposterior axis of the tibia.

In the present work, we classified “Sedentary” genetic types that in previous companion studies showed static attitude, rarely walking in the open area and barely expressing foraging behavior, while chickens with the opposite attitude were classified “Active”.

According to these findings the animals in the present study were divided in 2 groups, Active lines (**A**) and Sedentary (**S**).

Fatty Acids and “Activity Index” Formulation Fatty acids were analyzed as described in experiment 1. After determination of n-3 HUFA/ALA in PIL and PIFM muscles, the difference was calculated for the Activity index according to the following formulation.

Activity index

$$= \text{White m} (n - 3 \text{HUFA}) / \text{ALA} \\ - \text{Red m} (n - 3 \text{HUFA}) / \text{ALA}$$

Experiment 3. Validation of the “Activity Index” on Broilers

Finally, this study focused on the Activity index validation, with its application to commercial genetic lines, reared and fed in different and unknown conditions.

In order to validate the formula, 45 animals, 81 d old, coming from different commercial organic farms, randomly collected after slaughtering in an authorized commercial slaughterhouse, were analyzed: 15 chickens (L1) of known and previously identified active genetic line 15 commercial chickens (L2) of known and previously identified sedentary genetic line and 15 commercial chickens from the FG genetic line ROSS 308 (L3). Left thigh from each bird was dissected, PIL and PIFM muscles were excised, fatty acid analyses were performed as in experiment 1, and the Activity index calculated.

Statistical Analyses

Differences between muscles from experiment 1 were evaluated by ANOVA. In this model, the 2 level factor

of muscle type was considered as fixed, whereas the animal was considered as random.

A mixed model with two fixed factors and interaction was used to analyse color and fatty acid content. Animal was considered as random, type of muscle was always accounted for, while the second fixed factor was kinetic behavior group (S vs. A) or the genetic line of the birds.

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha * \beta)_{ij} + \gamma_{k(j)} + \varepsilon_{ijkl}$$

Where μ was the common average; α_i was the effect of muscle type (PIL vs. PIFM); β_j was the effect of the genetic line or the kinetic attitude of the genetic line (S, A; or L1, L2, L3); $\gamma_{k(i)}$ was the random effect of k animal nested in each j as previously defined; ε_{ijkl} was the random error.

MIXED procedure of SAS was used (SAS, SAS institute Inc., Cary, NC) and the levels of statistical significance for multiple tests were corrected by Bonferroni test. The Root Mean Squared Error (**RMSE**) is unique for each variable because data were balanced.

The distribution of probability for each fatty acid composing the Activity index and for Activity index itself was determined with UNIVARIATE and TTEST procedure of SAS (SAS, SAS institute Inc., Cary NC).

Comparisons among means were determined using the Student t test, with significance threshold set at $P < 0.05$. Due to multicollinearity among fatty acid concentrations, Partial Least Squares analysis (**PLS**) was performed on data from Experiment 2. In PLS analysis the categorical dummy variables (0, 1) were Active (A) and Sedentary Lines (S), while fatty acids ALA, EPA, DPA, DHA, the n-3 HUFA/ALA ratio Activity index were used as independent variables. PLS analysis is able to determine the minimum set of estimation variables (latent variable, LV) to classify the dependent variable. R^2 and RMSEP (root mean square error of prediction) express the model ability to fit the data.

To avoid overestimation of the effects of mostly represented fatty acids, concentrations were transformed into $\log_{10}(1+X)$ ([Ludvigsen et al., 1997](#)). Multivariate analysis was performed by Unscramble X 10.4 version (CAMO).

RESULTS AND DISCUSSION

Experiment 1. Thigh Muscles

Color Poultry meat color is given by myoglobin content and it depends on the type of muscle and the age of the animal ([Fletcher, 2002](#)). It is affected by sex, strain, diet, fiber type, intramuscular fat, and other *postmortem* variables ([Xiong et al., 1999](#); [Northcutt and Buhr, 2010](#)). Color variation in chicken meat is very evident and muscles and fiber functions are easily detectable as white or red ([Cassens and Cooper, 1971](#); [Mir et al., 2017](#)).

The 6 analyzed muscles ([Figure 2](#)) can be categorized in extremely white (PIL), mild white (FCLP, FMTL), mild red (AIL, IC), and extremely red (PIFM), considering the redness parameter ($P < 0.001$) Yellowness

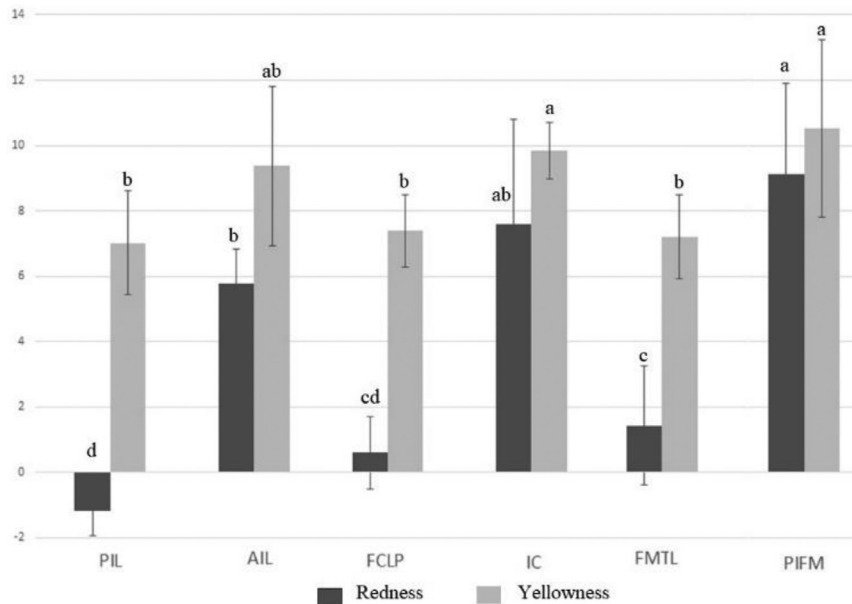


Figure 2. Redness and Yellowness parameters in different thigh muscles (mean and standard deviation). ¹Different letters indicate significant differences. ²Muscle nomenclature: M. iliobtibialis lateralis postacetabularis (PIL); M. iliobtibialis lateralis preacetabularis (AIL); M. flexor cruris lateralis pars pelvica (FCLP); M. iliobtibialis cranialis (IC); M. femorotibialis lateralis (FMTL); M. puboischiofemorales pars medialis (PIFM).

differences were similar to redness one, while no significant differences were reported for lightness ($P = 0.272$).

Our results confirm those of (Suzuki et al., 1985; Hoppeler, 1990), who described PIFM muscles as composed mostly by type I oxidative fibers, used for slow movement and prolonged period. Besides, Iwamoto et al. (1993) and Nakamura et al. (2004) represented PIL as fast-twitch muscles mainly composed by type IIb (glycolytic) fibers.

Fatty Acids Muscles in Table 1 did not show significant differences for SFA and MUFA, both performing storage functions. Nor significant difference were found for Σ PUFA, either Σ n-6 or Σ n-3. This because the concentration of linoleic acid (C18:2 n-6), which constitutes the largest n-6 PUFA, was not significantly different ($P = 0.230$) in the different muscles. Concentrations of C20:4 n-6 and C22:4 n-6 were highest in PIL, FCLP, and FMTL muscles, also the n-3 HUFA showed higher concentration in PIL, FCLP and FMTL white muscles as compared to the 3 red muscles. ALA (C18:3 n-3), the n-3 HUFA precursor, showed an opposite trend with the highest value in PIFM red muscle. This trend further enhances the differences in n-3 HUFA/ALA between the 2 types of muscles, as already described by Kriketos et al. (1995).

In our study, this ratio was significantly higher in PIL (0.87) than in PIFM (0.27) muscles, indicating a higher consumption of n-3 HUFA in red than in white muscles. These results are also consistent with Roy et al. (2007), that found PIL muscle to be prevalently glycolytic (white) and PIFM prevalent oxidative (red). We, therefore decided to choose M. iliobtibialis lateralis postacetabularis (PIL; white m.) and M. puboischiofemorales pars medialis (PIFM; red m.) to build the Activity index.

Experiment 2. Experimental Farm

Color In Table 2, the 6 poultry lines, grouped in A and S categories (Table 3), showed no significant difference in colour between A and S groups in terms of redness, lightness, and yellowness. However, significant difference in color exists between PIL and PIFM muscles ($P < 0.001$).

Fatty Acids The n-3 HUFA/ALA ratio was significantly different between A and S lines (Table 4), supporting the hypothesis that walking animals use n-3 HUFA in red muscles as fuel for movement, as already stated by Kriketos et al. (1995).

Since the diet was identical for all the chicken lines, significant differences between white and red muscles support the distinct use of fatty acids in oxidative and glycolytic muscles. Substantial differences were found in white PIL vs. red PIFM muscles.

In experiment 2, birds were fed with the same diet; when diet is unknown or different among groups, the n-3 HUFA/ALA ratio alone could be not a reliable indicator of the movement actually performed by a bird. However, the difference between n-3 HUFA/ALA ratio on the PIL and PIFM muscles from the same bird, i.e. the here proposed Activity index, should avoid most nuisance effects. The Activity index was calculated for the A (0.50) and S (0.28) groups. The difference was highly significant (F value = 20.35 with $P < 0.001$), RMSE 0.19.

In Figure 3 the probability distribution expressed as percentage, the normal and the density curves of fatty acids composing the Activity index were reported for white PIL and red PIFM muscles, both in S and A groups. All the variables showed a Shapiro-Wilk test near 1 (PW > 0.055).

Table 1. Fatty acid profile in 6 thigh muscles: (percentage on total FAME¹). Experiment 1. n = 8, DF=35.

| Fatty acids | PIL | AIL | FCLP | IC | FMTL | PIFM | RMSE | F value P value |
|--|-------------------|--------------------|--------------------|--------------------|--------------------|-------------------|-------|--------------------|
| C18:2 n-6 | 31.35 | 32.33 | 32.87 | 32.50 | 31.48 | 32.85 | 1.55 | 0.45 0.230 |
| C20:2 n-6 | 0.44 | 0.36 | 0.47 | 0.40 | 0.41 | 0.36 | 0.13 | 0.99 0.4418 |
| C20:4 n-6 | 3.91 ^a | 3.01 ^c | 3.97 ^a | 3.12 ^{bc} | 3.79 ^{ab} | 3.09 ^c | 0.49 | 6.87 <0.001 |
| C22:4 n-6 | 0.28 ^a | 0.15 ^{bc} | 0.21 ^b | 0.15 ^{bc} | 0.18 ^b | 0.13 ^c | 0.03 | 23.99 <0.001 |
| C18:3 n-3 | 2.47 ^b | 2.83 ^{ab} | 2.79 ^b | 2.78 ^b | 2.60 ^b | 3.12 ^a | 0.31 | 11.10 <0.001 |
| C20:5 n-3 | 0.09 ^a | 0.07 ^b | 0.09 ^a | 0.06 ^b | 0.08 ^{ab} | 0.05 ^c | 0.013 | 10.01 <0.001 |
| C22:5 n-3 | 1.22 ^a | 0.69 ^c | 1.04 ^{ab} | 0.75 ^c | 1.00 ^b | 0.50 ^d | 0.12 | 38.39 <0.001 |
| C22:6 n-3 | 0.83 ^a | 0.59 ^b | 0.71 ^{ab} | 0.51 ^c | 0.60 ^b | 0.39 ^d | 0.07 | 33.96 <0.001 |
| Σ SFA ² | 29.65 | 28.19 | 28.64 | 27.76 | 29.83 | 28.34 | 1.78 | 1.74 0.151 |
| Σ MUFA ² | 29.12 | 31.26 | 28.58 | 31.41 | 29.46 | 30.62 | 2.19 | 2.34 0.062 |
| Σ PUFA ² | 41.23 | 40.55 | 42.78 | 40.83 | 40.71 | 41.04 | 1.45 | 2.55 0.045 |
| Σ n-6 ² | 36.63 | 36.38 | 38.15 | 36.73 | 36.44 | 36.56 | 1.38 | 1.87 0.124 |
| Σ n-3 ² | 4.60 | 4.18 | 4.64 | 4.10 | 4.28 | 4.48 | 0.39 | 2.60 0.042 |
| Σ SFA/ Σ PUFA ² | 0.72 | 0.70 | 0.67 | 0.68 | 0.74 | 0.69 | 0.05 | 1.76 0.147 |
| n-6/n-3 ² | 7.97 | 8.72 | 8.24 | 9.12 | 8.53 | 8.19 | 0.86 | 1.12 0.369 |
| n-3 HUFA/ALA ² | 0.87 ^a | 0.48 ^c | 0.67 ^b | 0.49 ^c | 0.64 ^b | 0.27 ^d | 0.10 | 30.60 <0.001 |

Abbreviations: PIL, *M. iliobtibialis lateralis postacetabularis*; AIL, *M. iliobtibialis lateralis preacetabularis*; FCLP, *M. flexor cruris lateralis pars pelvica*; IC, *M. iliobtibialis cranialis*; FMTL, *M. femorotibialis lateralis*; PIFM, *M. puboischiofemoralis pars medialis*.

^{a-d}Different letters in the same row indicate significant differences.

¹FAME, Fatty Acid Methyl Esters.

² Σ SFA = Σ (C14:0, C16:0, C18:0, C22:0); Σ MUFA = Σ (C16:1, C18:1 cis9, C18:1 cis11); Σ n-6 = Σ (C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C22:4 n-6); Σ n-3 = Σ (C18:3n3, C20:5 n-3, C22:5 n-3, C22:6 n-3); Σ PUFA = Σ n-6 + Σ n-3; n-3 HUFA = Σ (C20:5n-3, C22:5 n-3, C22:6 n-3); ALA = C18:3 n-3.

Table 2. Color of thigh white PIL (*M. iliobtibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) muscles in A (Active lines) and S (Sedentary lines) broilers. Experiment 2. DF = 148.

| Experiment 2 | | | | | | | | |
|--------------|------------|-------|-------------|-------|------|--------------------|-------------------------|-------------------------------------|
| | PIL muscle | | PIFM muscle | | RMSE | P value A vs. S | P value PIL vs. PIFM | P value Interaction ¹ |
| | A | S | A | S | | | | |
| n | 45 | 45 | 45 | 45 | | | | |
| L* | 48.60 | 47.05 | 43.54 | 43.26 | | 4.01 | 116.55 | 1.11 |
| | | | | | 2.75 | 0.065 | <0.001 | 0.425 |
| a* | -1.01 | -1.49 | 6.95 | 5.49 | 2.92 | 4.69 | 293.34 | 1.20 |
| | | | | | | 0.048 | <0.001 | 0.275 |
| b* | 6.26 | 5.03 | 12.48 | 11.51 | 2.58 | 8.01 | 261.76 | 0.17 |
| | | | | | | 0.03 | <0.001 | 0.679 |

¹Interaction (Muscles \times Groups).

Table 3. Genetic lines used in the experimental trial. Experiment 2. n = 90.

| Genetic lines | n | Acronym | Activity behavior | Carcassweight |
|------------------------|----|---------|-------------------|----------------------------------|
| Hubbard RedJA Campese | 15 | C | A | 2930.7 \pm 298.9 ^{cd} |
| Aviagen Rowan Ranger | 15 | RR | A | 2859.3 \pm 445.4 ^d |
| Aviagen Ranger Gold | 15 | RG | A | 3179.3 \pm 277.7 ^{bc} |
| Hubbard M22xJA87 | 15 | M | S | 3213.3 \pm 469.2 ^b |
| Hubbard CY Gen5xJA87 | 15 | CY | S | 3487.7 \pm 455.4 ^a |
| Aviagen Ranger Classic | 15 | RC | S | 3616.0 \pm 210.8 ^a |

S: Sedentary lines; A: Active lines.

^{a-d}Different letters indicate significant differences ($P < 0.001$).

Table 4. n-3 fatty acid of thigh white PIL (*M. iliotibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) muscles used for estimate Activity index in active and sedentary broilers (percentage on total FAME¹). Experiment 2. DF = 148.

| Experiment 2 | | | | | | | | | |
|---------------------------|-------------------|-------------------|-------------|-------|------|------------------------------|-----------------------------------|---|--|
| Fatty acids | PIL muscle | | PIFM muscle | | RMSE | F value P value A vs S | F value P value PIL vs PIFM | F value P value interaction ³ | |
| | A | S | A | S | | | | | |
| n | 45 | 45 | 45 | 45 | | | | | |
| C18:3 n-3 | 1.58 | 1.60 | 1.83 | 1.78 | 0.13 | 0.37 0.553 | 112.7 <0.001 | 2.90 0.091 | |
| C20:5 n-3 | 0.10 | 0.11 | 0.09 | 0.09 | 0.02 | 2.56 0.132 | 45.11 <0.001 | 0.44 0.506 | |
| C22:5 n-3 | 0.84 | 0.76 | 0.46 | 0.53 | 0.15 | 0.17 0.686 | 176.9 <0.001 | 9.80 0.048 | |
| C22:6 n-3 | 0.57 ^a | 0.45 ^b | 0.29 | 0.35 | 0.11 | 2.81 0.116 | 128.5 <0.001 | 27.81 <0.001 | |
| ∑ PUFA ² | 29.53 | 29.39 | 27.55 | 28.69 | 2.17 | 1.79 0.202 | 17.16 <0.001 | 3.88 0.051 | |
| n-3HUFA /ALA ² | 0.96 ^a | 0.83 ^b | 0.46 | 0.54 | 0.16 | 0.33 0.577 | 198.2 <0.001 | 14.45 <0.001 | |

^{a,b}Different letters in the same row and muscle indicate significant differences.

¹FAME, Fatty Acid Methyl Esters.

²∑PUFA = ∑n-6+ ∑n-3; n-3 HUFA = ∑(C20:5n-3, C22:5 n-3, C22:6 n-3); ALA = C18:3 n-3.

³Interaction (Muscles × Groups)

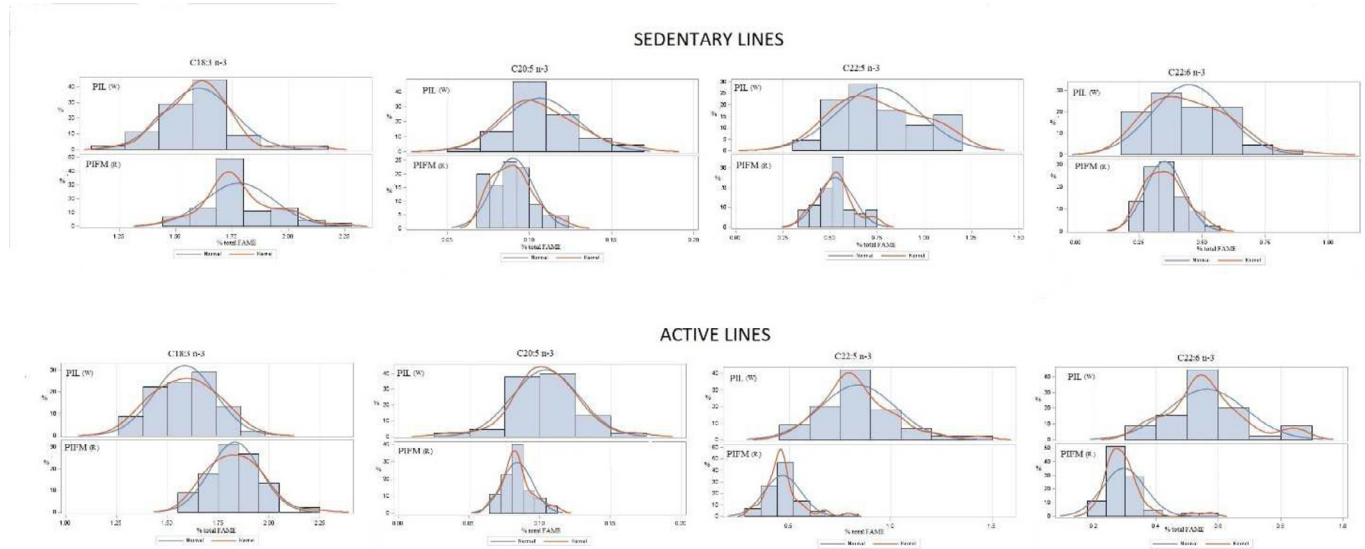


Figure 3. Probability distribution as percentage, normal and density curves of fatty acids composing the Activity index for white PIL (*M. iliotibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) in S (Sedentary lines) and A (Active lines). ¹ The blu lines indicate normal distribution; the red lines indicate kernel density estimation; ²Data were expressed as percentage of total fatty acids methyl esters (FAME).

Consistent with results described above, the two groups, A and S, were clearly separated by a PLS using 2 Latent Variables (Figure 4) with RMSRP was 0.241, RMSEV 0.288, and a R² 0.77.

Loading plot of the PLS model (Figure 5) shows the Activity index on the right together with the A group, with a strong positive association with the movement of the animals. As expected, ALA and the 3 considered n-3 HUFA showed an opposite pattern.

When a chicken is given the opportunity to walk, the Activity index expresses its kinetic attitude. To assess the kinetic behaviour of a group of birds from the same genetic line, a minimum threshold should be defined.

Among the experimental lines (Table 5), the extreme of S and A groups (RC and C genetic lines) showed significant differences of n-3 HUFA/ALA in PIL (0.69 vs. 0.94 $P = 0.028$) and PIFM muscles (0.61 vs. 0.43 $P = 0.048$).

In Figure 6, the probability distribution expressed as percentage, the normal and the density curves of Activity index in S vs. A, and in RC vs. C as extreme genotypes were reported. All the variables showed a Shapiro-Wilk test near 1 ($PW > 0.05$).

Their Activity index means were significantly different between RC (extreme sedentary strain) and C (extreme active strain) ($P < 0.001$), with 0.08 for RC and 0.51 for C, with a midrange of 0.297 (rounded to

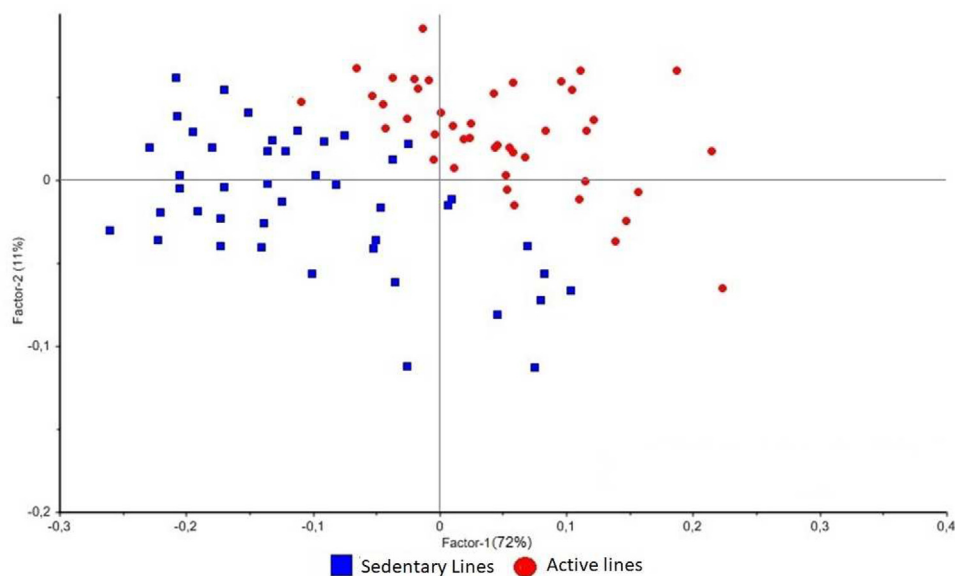


Figure 4. Plot of scores in PLS analysis for fatty acids of PIL (*M. iliobtibialis lateralis postacetabularis*) and PIFM (*M. puboischiofemoralis pars medialis*) muscles of A (Active lines) and S (Sedentary lines). Factors = PLS analysis factors assorbing major variability.

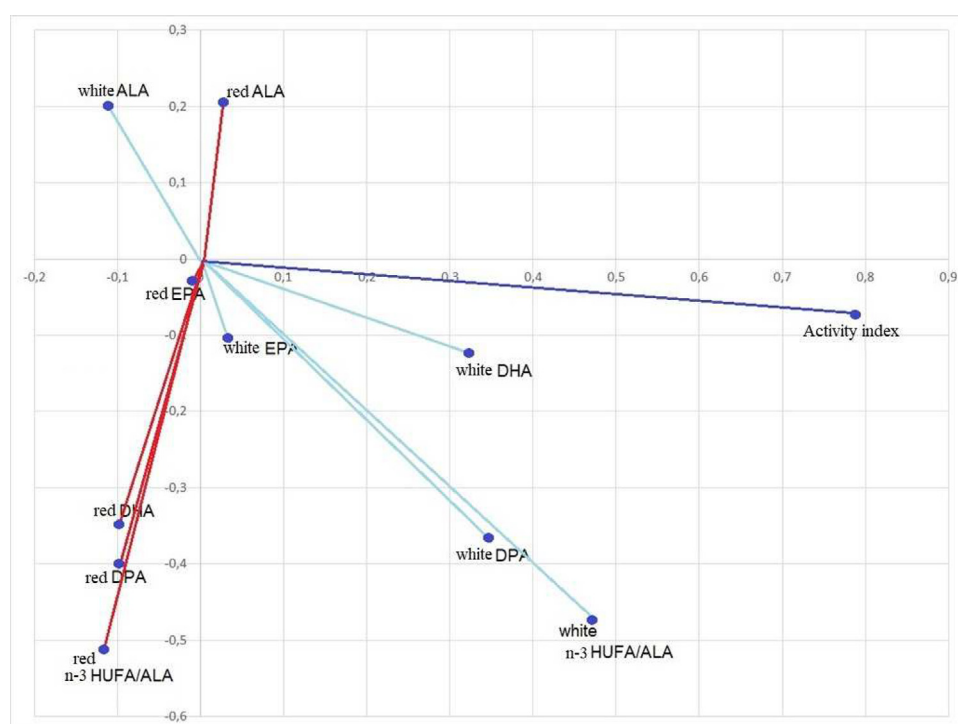


Figure 5. Plot of Loading in PLS analysis for fatty acids of white PIL (*M. iliobtibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) muscles. ¹Vector position and length for each variable indicate the capacity to absorb variability. ² ALA = C18:3 n-3 (for white PIL muscle and red PIFM muscle); EPA = C20:5 n-3 (for white PIL muscle and red PIFM muscle); DHA C22:5 n-3 (for white PIL muscle and red PIFM muscle); DPA = C22:2 n-3 (for wite PIL muscle and red PIFM muscle); n-3 HUFA = Σ (C20:5n-3, C22:5 n-3, C22:6 n-3); Activity index = n-3 HUFA/ALA (PIL) – n-3 HUFA/ALA (PIFM).

Table 5. Activity index of the 6 genotypes in experiment 1.

| | C | RR | RG | M | CY | RC | RMSE | F value P value |
|----------------|-------------------|--------------------|--------------------|--------------------|-------------------|-------------------|------|--------------------|
| Activity index | 0.51 ^a | 0.50 ^{ab} | 0.48 ^{ab} | 0.42 ^{ab} | 0.33 ^b | 0.08 ^c | 0.19 | 13.70 <0.001 |

Activity index = n-3 HUFA/ALA (PIL) – n-3 HUFA/ALA (PIFM).

^{a-c}Different letters in in the same row indicate significant differences.

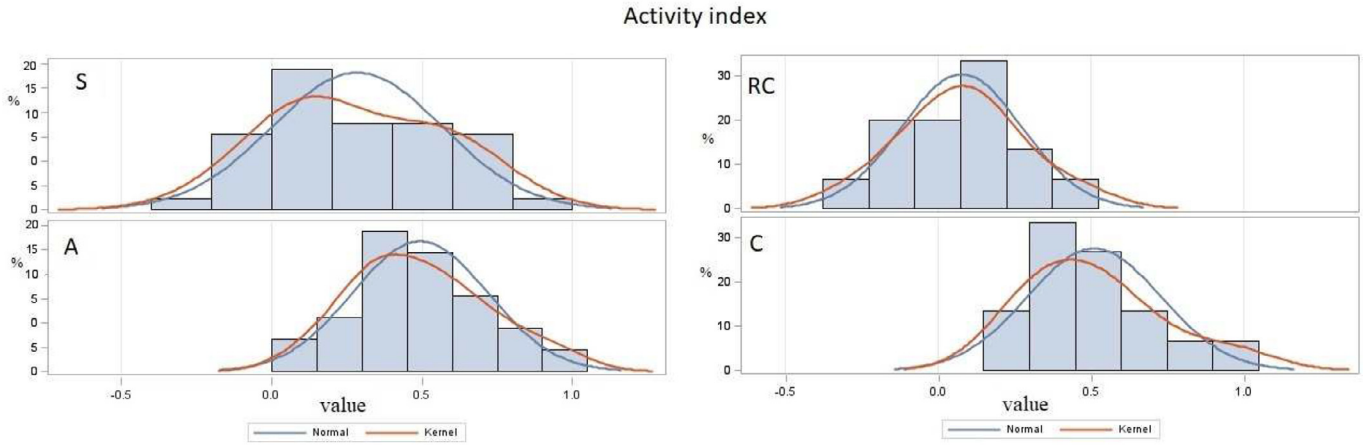


Figure 6. Probability distribution expressed as percentage, normal and density curves of Activity index in S (Sedentary lines) vs. A (Active lines) and RC (Ranger Classic) vs. C (Campese). ¹The blu lines indicate normal distribution; the red lines indicate kernel density estimation. ²Activity index = n-3 HUFA/ALA (PIL) – n-3 HUFA/ALA (PIFM).

0.3). This value could be proposed as a threshold to assess the kinetic attitude of a genetic line to be able to cope with a free-range rearing method. The idea is to offer breeders an objective way to assess the attitude of their products, after a validation on more samples and genetic lines.

Experiment 3. Validation of the Activity Index in Commercial Farms

The validation was made on three genetic lines from organic farms, raised with different diets and housing conditions: 15 chickens (L1) of known and previously identified as active genetic line 15 commercial chickens (L2) of known and previously identified sedentary

genetic line and 15 commercial chickens from the FG genetic line ROSS 308 (L3).

In Table 6 concentrations of the 4 relevant fatty acids (ALA, EPA, DPA, DHA), \sum PUFA and n-3 HUFA/ALA ratio measured on chicken of the 3 genetic lines are reported. The variability among these 3 genetic lines (L1, L2, L3) is higher than in the 2 experimental groups (A vs. S) due to different rearing conditions, but the differences between white and red muscles still are highly significant.

Figure 7 reported the probability distribution expressed as percentage, the normal and the density curves of fatty acids used in Activity index for PIL and PIFM muscles of commercial genetic lines used for the validation and Figure 8 showed the probability distribution of Activity index in the 3 commercial lines. All the variables showed a Shapiro-Wilk test near 1 (PW > 0.055).

Table 6. n-3 fatty acids in thigh white PIL (*M. iliotibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) muscles from 3 commercial chicken genotypes (percentage on total FAME¹). Experiment 3. DF = 42.

| Fatty acids | PIL muscle | | | PIFM muscle | | | RMSE | F value P value lines ⁴ | Fvalue P value muscles ⁵ | Fvalue P value inter. ⁶ |
|---------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|------|---------------------------------------|--|---------------------------------------|
| | L1 ² | L2 ² | L3 ² | L1 ² | L2 ² | L3 ² | | | | |
| C18:3 n-3 | 2.06 ^b | 1.92 ^b | 2.42 ^a | 2.29 | 2.43 | 2.47 | 0.27 | 4.66 0.018 | 20.12 <0.001 | 5.15 0.010 |
| C20:5 n-3 | 0.09 | 0.08 | 0.06 | 0.09 ^b | 0.11 ^b | 0.21 ^a | 0.05 | 5.07 0.013 | 31.47 <0.001 | 17.58 <0.001 |
| C22:5 n-3 | 0.84 ^a | 0.62 ^b | 0.64 ^b | 0.49 ^b | 0.78 ^a | 0.62 ^{ab} | 0.12 | 2.29 0.120 | 8.18 0.007 | 37.62 <0.001 |
| C22:6 n3 | 0.65 ^a | 0.67 ^a | 0.39 ^b | 0.29 ^b | 0.56 ^a | 0.36 ^b | 0.11 | 35.48 <0.001 | 57.92 <0.001 | 18.84 <0.001 |
| \sum PUFA ³ | 34.15 ^b | 37.55 ^a | 33.70 ^b | 33.68 ^b | 36.74 ^a | 34.20 ^b | 1.92 | 8.07 0.002 | 0.41 0.523 | 0.94 0.399 |
| n-3HUFA /ALA ³ | 0.77 ^a | 0.71 ^a | 0.45 ^b | 0.38 ^b | 0.60 ^a | 0.48 ^{ab} | 0.11 | 13.34 <0.001 | 56.41 <0.001 | 29.83 <0.001 |

^{a,b}Different letters in in the same row and muscle indicate significant differences.

¹FAME, Fatty Acid Methyl Esters.

²Commercial lines: L1 = Active Commercial line; L2 = Sedentary Commercial line; L3 = Fast Growing Ross 308.

³ \sum PUFA = \sum n-6 + \sum n-3; n-3 HUFA = \sum (C20:5n-3, C22:5 n-3, C22:6 n-3); ALA = C18:3 n-3.

⁴Lines L1, L2, L3.

⁵Muscles PIL, PIFM.

⁶Interaction (Muscles \times Lines).

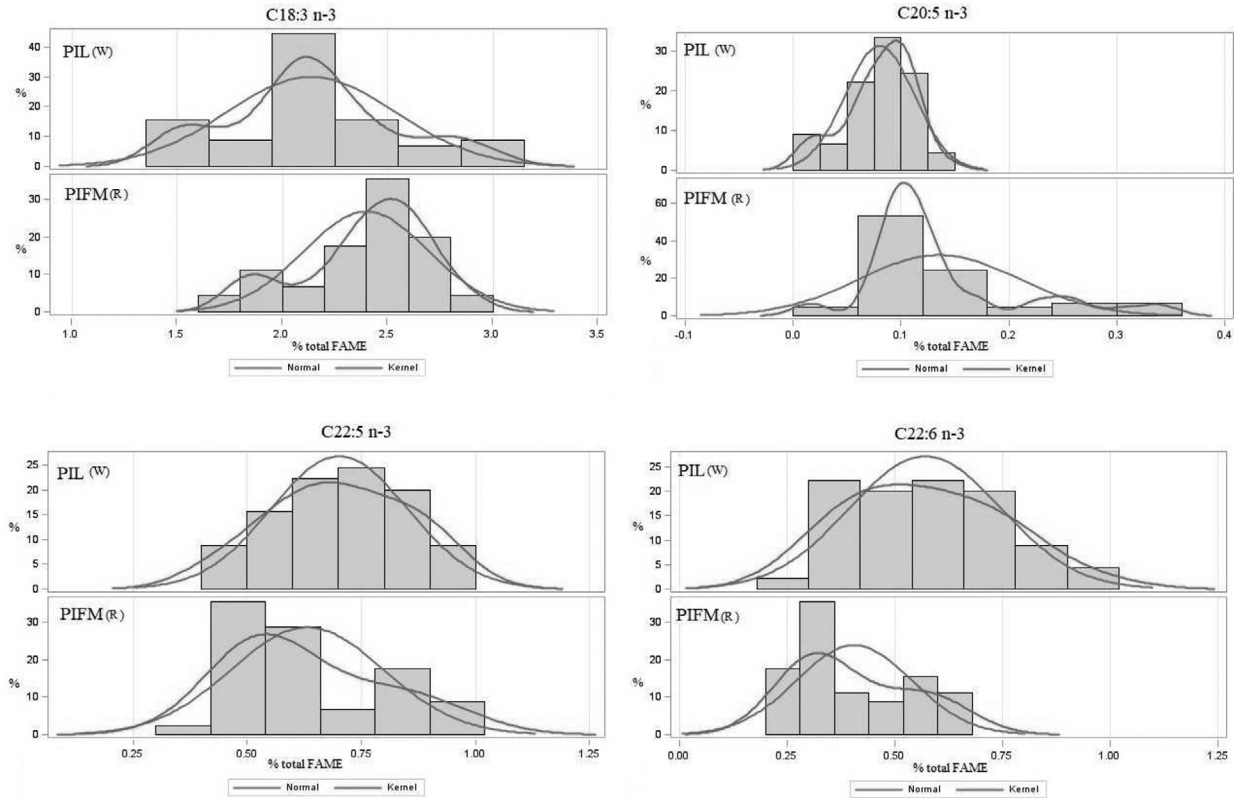


Figure 7. Probability distribution as percentage, normal and density curves of Activity index fatty acids for white PIL (*M. iliobtibialis lateralis postacetabularis*) and red PIFM (*M. puboischiofemoralis pars medialis*) muscles in three commercial lines (experiment 3). ¹The blu lines indicate normal distribution; the red lines indicate kernel density estimation. ²Data were expressed as percentage of total fatty acids methyl esters (FAME).

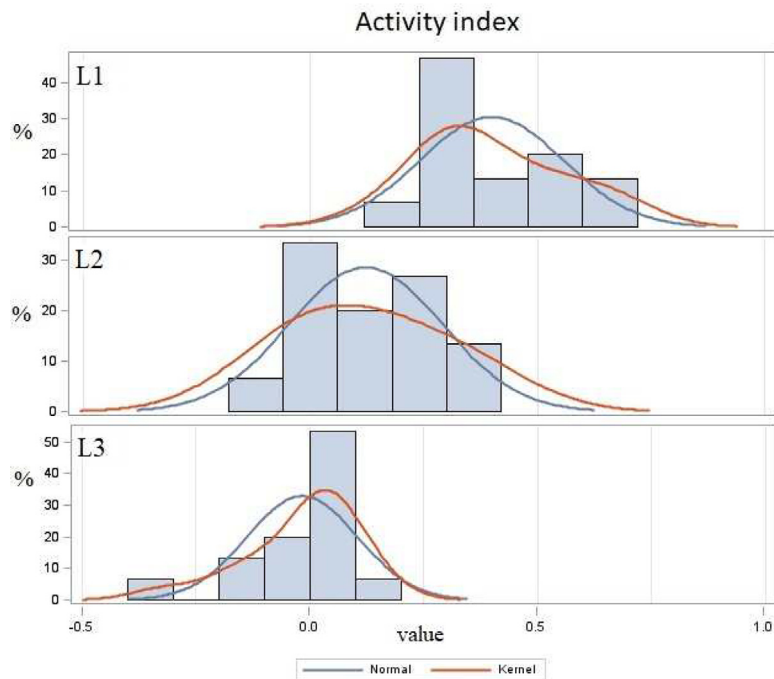


Figure 8. Probability distribution expressed as percentage, normal and density curves of Activity index in in three commercial lines² (experiment 3). ¹ The blu lines indicate normal distribution; the red lines indicate kernel density estimation. ²Commercial lines: L1 = active commercial line; L2 = sedentary commercial line; L3 = Fast Growing Ross 308; ³Activity index = n-3 HUFA/ALA (PIL) – n-3 HUFA/ALA (PIFM).

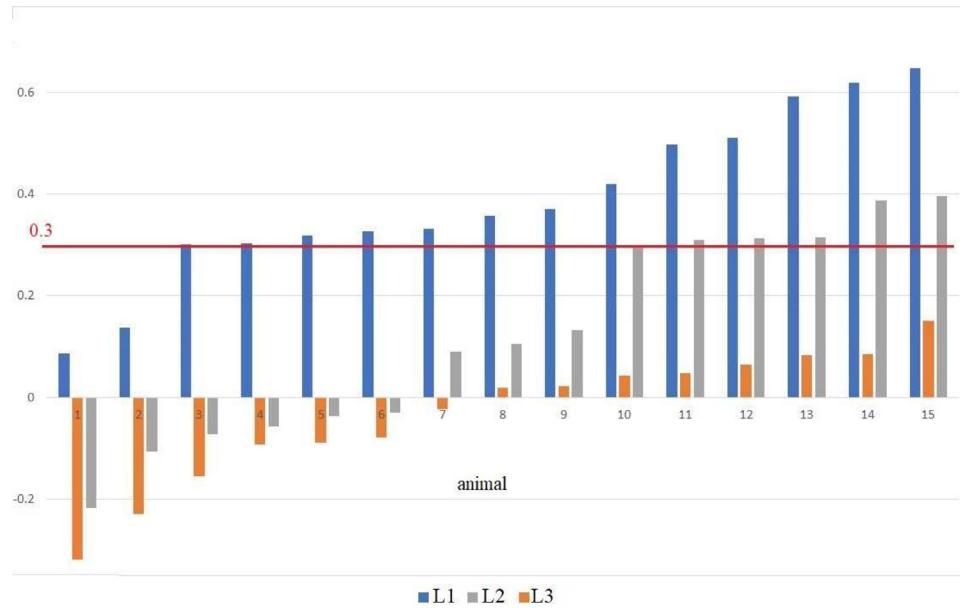


Figure 9. Activity index in the three commercial lines. ¹The red line indicate the threshold value (0.3), ²Commercial lines: L1 = active commercial line; L2= sedentary commercial line; L3 = Fast Growing Ross 308, ³Activity index = $n-3 \text{ HUFA}/\text{ALA (PIL)} - n-3 \text{ HUFA}/\text{ALA (PIFM)}$.

The Activity index clearly distinguishes the 3 commercial lines with the smallest value for L3 - 0.03, confirming its well-known sedentary attitude (Bokkers and Koene, 2003), 0.11 for L2 and 0.38 for L1 (F value 29.78; $P < 0.001$).

In Figure 9 individual birds from the 3 strains are compared: 86% of L1 lines was above the index threshold, so confirming the walking attitude of the experimental group, while only 40% of L2 and none of L3 passed the threshold of 0.3.

CONCLUSIONS

This work presents for the first time an Activity index based on β -oxidation differences between red and white thigh muscles of the same chicken to estimate their kinetic activity.

The proposed Activity index is the difference between the ratios $(\text{EPA}+\text{DPA}+\text{DHA})/\text{ALA}$ recorded in *M. iliotibialis lateralis postacetabularis* (PIL) and *M. puboischiofemoralis pars medialis* (PIFM), typically considered to be the extreme white and extreme red muscles in bird legs.

Being measured within the same animal, the index is largely independent from environmental influences, if the birds had been allowed to walk without environmental constraints. The Activity index is an attempt to measure the kinetic performances over the entire life of a bird without influences by the environment. However, this index can only be measured after slaughtering. Therefore, it can not be used for welfare assessment, while it offers an objective tool to measure the ability of a genetic line to adapt to outdoor farming. Further improvements are expected from wider implementations.

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DISCLOSURES

The authors have no conflicts of interest. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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