Genetic variation of keel and long bone skeletal properties for 5 lines of laying hens

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Primary Audience: Breeder/Layer Producers, Veterinarians, Researchers, Welfare Auditors, Breeding Companies, Parent Stock Producers, Hatcheries

SUMMARY

Fractures to the keel bone is one of the greatest problems facing the laying hen industry. With most severe effects observed in non-cage housing, frequencies are expected to dramatically increase as the industry continues transitioning away from battery cages. Incidences within commercial systems are well documented, where the main cause is believed to be high egg production and the associated need for calcium drawing on endogenous reserves (i.e., bone) leaving bone weakened and prone to fracture. The current work sought to characterize various bone mineral and biomechanical properties of 5 distinct purebred or crossbred laying hen lines (3 commercial: Bovans Brown, Dekalb White, and Institut de Sélection Animale Dual Brown; 2 non-commercial: Experimental Brown and Experimental White), following previous work that demonstrated differences in susceptibility to keel fracture using an ex vivo impact testing apparatus. The keel was then removed to undergo analysis by computer tomography; the humerus and tibia were also removed for biomechanical testing. The keel bone mineral density and moment of area correlated moderately with hen weight and susceptibility to fracture. The biomechanical properties of the tibia, but not the humerus, showed a strong relationship with hen weight. One commercial genetic line (Dekalb White) with a high susceptibility to fracture exhibited a mean tibia strength below the value expected from its mean weight. Our results suggest that for the purebred or crossbred lines other than Dekalb White, rather than properties of bone, lower mean weight may imply higher levels of activity, higher risk of collisions, and lower soft tissue protection that reflect greater susceptibility to keel fracture.

Key words: laying hen, bone health, keel, fracture, strength

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DESCRIPTION OF PROBLEM

Fracture to the keel bone of laying hens is one of the greatest problems facing the laying hen industry (Lay et al., 2011; FAWC, 2013) with frequencies expected to dramatically increase in North America as the industry transitions away from battery cages. Incidences within commercial systems are well documented (Käppeli et al., 2010; Wilkins et al., 2011; Heerkens et al., 2015; Petrik et al., 2015), where the main cause is believed to be high egg production and the associated need for calcium drawing on endogenous reserves (i.e., bone) leaving bone weakened and prone to fracture (Whitehead and Fleming, 2000; Toscano et al., 2020). Although an etiology between egg production and bone condition is reasonable, the difficulty in assessing relevant characteristics of the keel immediately before the fracture occurs has made verifying this link difficult. For instance, previous work using 2 genetic lines bred for relatively weak and strong bone manifested reduced incidence of keel fracture that mirrored the expected pattern (Bishop et al., 2000; Stratmann et al., 2016). Despite these convincing results, it was not possible to determine how behavioral differences (e.g., flightiness) between the lines affected fracture incidence. Additionally, quantification of relevant properties in the period after fracture is not beneficial, as fracture will induce substantial changes in bone morphological properties (e.g., altered bone mineral, strength, etc.). However, quantification before the fracture would require killing the hen and thus eliminates the chance to observe whether a fracture occurs.

To circumvent these problems and link keel fracture susceptibility (i.e., the likelihood that a fracture will or will not occur) with relevant characteristics, our laboratory developed an impact testing protocol (Toscano et al., 2013, 2018). The ex vivo impact procedure uses hens killed by a separate procedure within 60 s as a proxy for actual collisions. Immediately before or after the collision, relevant data can be recorded (e.g., body mass, feather condition). After collision, the keel can be visually inspected for damage and removed (with other tissues as needed) for relevant laboratory assessment (Toscano et al., 2018). The impact testing protocol was recently used to assess fracture susceptibility of 5 distinct purebred or crossbred lines and demonstrated dramatic differences between the lines (Candelotto et al., 2017). In addition to susceptibility, Candelotto et al. also quantified egg production and quality (individual and pen levels), feed consumption (pen level), and considered the results in terms of fracture susceptibility. The current study is an extension of that initial effort (Candelotto et al., 2017) and sought to characterize the keel, tibia, and humerus of the 5 genetic or crossbred lines in terms of bone mineral and biomechanical properties. We hypothesized that, across the 5 genetic lines, the properties assessed in the current study would correlate with the keel fracture susceptibility observed previously (Candelotto et al., 2017). Specifically, we anticipated the long bones of the Experimental Brown (**EB**) line (which was most resistant to keel fracture) to manifest the greatest strength, stiffness, and energy to failure; we anticipated the keel to have the greatest bone mineral density (**BMD**) and second moment of area in the horizontal and vertical planes. In contrast, we expected the Experimental White (**EW**) and Dekalb White (**DW**; which were least resistant to fracture) to demonstrate the reverse trends of these measures. The Institut de Sélection Animale Dual Brown (**DB**) and Bovans Brown (**BB**; which were moderately resistant to fracture) would have mid-range response values.

MATERIALS AND METHODS

Ethical Approval

The experiment was approved by the Cantonal Veterinary Office (Cantonal Approval: BE-15/15) and complied with Swiss regulations regarding the treatment of experimental animals.

Animals and Housing

Details of the used genetic lines and their origins, management protocols, and housing are provided elsewhere (Candelotto et al., 2017), and are discussed here in brief. Two hundred and ninety-day-old chicks of 4 specific crossbred and 1 pure line (N = 290) were brought to our research facility in Zollikofen, Switzerland. Five crossbred or pure lines were selected with the purpose of providing a broad variation in productivity and egg quality that we anticipated would relate to underlying keel fracture susceptibility. With this intent, 3 commercial (BB, DW, and Institut de Sélection Animale DB) and 2 non-commercial (EB and EW) lines were chosen. The EW line was a pure line and all others were crossbred. Upon arrival, chicks were wing-tagged with individual identification numbers and maintained in 1 pen that increased in size relative to the bird's growth. The birds were provided ad libitum food and water throughout the entire study. At 10 wk of age, all birds were assigned to 1 of 9 identical pens according to their crossbred or pure line resulting in 2 pens with 26 to 30 birds each for the crossbred or pure lines (BB [N/pen=30, 28], DW [N/pen=26, 26], DB [N/pen=30, 30], and EB [N/pen=30, 29]). Due to a sexing error of the breeding company, only 10 birds of the pure line EW were available which were all kept in a single pen. For this line, we decided that 1 pen would be more appropriate vs. 2 pens with reduced numbers.

All pens (2.0 × 3.5 m) were kept side-by-side within the same room and had visual, olfactory, and limited tactile contact between adjacent pens. Each bird was given a pen-specific colored leg band in order to identify and correct any unintended mixing between pens, though this did not occur. Each pen was provided with 2 perches (length: 2.4 m each) and a ramp (area: 85 cm × 48 cm) leading toward the perches. Also included in each pen were Vencomatic nest boxes with an interior area of 0.55 m^2, a round feeder, a round hanging nipple drinker, and a pecking stone as an occupation device. Artificial lighting was provided between 23 and 14 h of daylight/d in accordance with the management protocol. Our study was designed to limit pen-level variation by maintaining identical pens without natural lighting (as position within the barn could affect exposure) and utilized a single management protocol (Hendrix Genetics Management Guide) for feed, lighting, and egg collection. Although the 5 distinct crossbred or pure lines had individual recommended management protocols, we were keen to minimize environmental variation to the fullest extent possible and thus decided on a single management protocol, that is that for the DW line.

Bone Collection

At either 28 or 29 wk of age, all hens were prepared for impact testing by sedation with barbiturate (600 mg/kg, IP; Esconarkon, Streuli Pharma AG, Switzerland), recording body mass, and then killed by cervical dislocation. Following the impact procedure using distinct energy values (described in Candelotto et al. (2017) with more background provided by Toscano et al. (2018)), the bird was taken from the impact tester and the soft tissue surrounding the keel, tibia, and humerus was removed to allow extraction of each bone. The excised bones were then stored at −20°C until assessment.

Biomechanical Assessment

Tibia and humeri underwent 3-point bending following the American Society of Agricultural and Biological Engineers Standards 2007 (ANSI/ASAE S459 MAR1992 (R2007)) using a Zwick and Roell Universal Testing Machine with a 2.5 kN load cell after thawing for at least 24 h at 15°C. The fulcrum was extended 55 mm to get a length to bone diameter ratio greater than 10. The bones were laid in the test apparatus with the flattest side down and force was applied to the mid-shaft by a loading bar at a speed of 10 mm/min from which the force deformation curve was read (Toscano et al., 2015) and the peak force (N) recorded. Bone stiffness (N/mm) was calculated as the slope of the load/displacement curve between 0.3 and 0.5 mm. The system software also calculated the total area under the entire load/displacement curve to provide the total energy (J) required to reach structural failure.

Computed Tomography

Due to financial and time restraints as well as the objective of evaluating bones with minimal fracture damage including both old breaks and those resulting from our impact testing procedure, a subset of keels (EW: N = 2; EB: N = 6; DB: N = 7; BB: N = 3; DW: N = 1) was visually examined and selected for computed tomography assessment. The most distal 4 cm of the keel were assessed using a HRpQCT (XtremeCT II, Scanco Medical AG, Brüttisellen, Switzerland) scanner. The region was selected as it is where the vast majority of fractures occur (Casey-Trott et al., 2015; Baur et al., 2020). Approximately 800 transversal slices with an isotropic resolution of 62 microns were acquired for each specimen. The images were contoured in a semi-automatic manner using the tool proposed by Scanco Medical AG. After masking, the acquired images were analyzed using a custom-developed Matlab (Matlab V.15, Mathworks, Natick, MA) pipeline at approximately 20 (depending on the length of the bone) locations along the keel. The distance between the analysis locations was set to 2 mm. At each analysis location, 3 slices were averaged and the BMD ($mgHA/cm^3$; milligrams hydroxyapatite per cubic centimeter) and the second moment of area in cranial and sagittal axes (mm^4; as an indicator of resistance against bending in the respective planes) were calculated. A linear function relating computed tomography attenuation values (Hounsfield units) and BMD was applied to compute the average BMD values of the analyzed slices. Slope and intercept of this function were computed using a calibration phantom containing 5 hydroxyapatite rods with known densities. The calibration phantom was measured once per week by the operator to ensure the values did not drift, and if necessary, the function parameters were corrected. The second moment of area was calculated by using the equation:

$$I_y = \int_A z^2 d\ A$$

where I_y is equal to the integral over the area (A; the transversal slice of the keel at the analysis location) of z (z; the vertical distance of the element dA to the y-axis).

At locations where damage to the keel (e.g., callus, healed fracture, a line between 2 fractured parts) was believed to reduce the reliability of the response, the operator noted the point within the bone which was then treated as a missing data point and excluded from statistical modeling. The decision to exclude a slice was independent of whether the bird had been recorded as having a fracture. A table detailing the excluded sections is provided in the supplemental materials (Supplementary Table 1).

Statistical Analysis

Long bone properties were assessed using linear mixed-effects models within R Studio (version 0.98.1103, Boston, MA) with R (version 3.1.3, package lme4 [Bates et al., 2015]) including line, bone type, and their interactions as

Table 1. Sample size, least square means, and SE for assessed biomechanical properties of the humerus or tibia.

			Peak force [N]		Stiffness [N/mm]		Total energy [J]	
Bone	Genetic line	n	Mean	SE	Mean	SE	Mean	SE
Humerus								
EW		10	$230.2^{a,b}$	13.7	253.9^{b}	19	$0.547^{a,b}$	0.055
EB		58	243.9^{b}	5.3	255.3^{b}	7.3	0.621^{b}	0.021
ISA DB		60	238.7^{b}	5.6	247^{b}	7.7	0.615^{b}	0.022
BB		58	187.9^{a}	5.9	191.5^{a}	8.2	0.502^{a}	0.024
DW		52	210.1^{a}	5.8	$230.7^{a,b}$	8	0.502^{a}	0.023
Tibia								
EW		10	$223.2^{a,b}$	14.7	$207.8^{a,b}$	20.3	0.45^{a}	0.059
EB		59	310.9^{d}	5.3	324.1^{c}	7.3	0.963^{d}	0.021
ISA DB		60	287.4^{c}	5.3	272.8^{b}	7.3	0.802^{c}	0.021
BB		58	259.6^{b}	5.9	259.6^{b}	8.1	0.685^{b}	0.023
DW		52	201.2^{a}	5.9	189.7^{a}	8.1	0.488^{a}	0.023

[a–d]Means within a single column of 1 bone type (i.e., humerus or tibia) without a common superscript are different ($P > 0.05$).
Abbreviations: BB, Bovans Brown; DW, Dekalb White; EB, Experimental Brown; EW, Experimental White; ISA DB, Institut de Sélection Animale Dual Brown.

fixed effects. Body mass at the time of death was used as a covariate. Model fit was evaluated by examining residual plots. Correlations between squared coefficients of long bone biomechanical properties were also calculated. Computed tomography outputs (second moment of area and mineral density of the keel) were assessed using Mixed procedures within SAS (Proc Mixed, SAS Institute, Cary, NC) including line, slice, and their interactions as fixed effects. Crossbred line or pure line DW with reduced numbers was excluded from the analyses (for computed tomography only). All terms were initially included in the model and then removed in backwards elimination. Interaction terms were initially left in the model unless clearly non-significant ($P > 0.2$) and then subsequently evaluated using a Tukey's post hoc test ($P < 0.05$). Provided means are model estimates that incorporate statistically significant terms.

RESULTS

Biomechanical Assessment

An interaction of line and bone type was identified for all biomechanical long bones measures (Table 1). In terms of total energy to reach failure in the tibia, EW and DW ($P > 0.9$) were similar to each other but less than the other lines which were all different ($P < 0.01$). Total energy to reach failure in the humeri of BB and DW was less than DB and EB ($P < 0.01$). For peak force to reach failure in the tibia, lines EW and DW ($P > 0.9$) were similar to each other as were EW and BB, but less than the other lines which were different ($P < 0.01$). Within the humeri, peak force to failure in lines BB and DW was less than lines DB ($P < 0.04$) and EB ($P < 0.001$). For tibia stiffness, EB was greater than all other lines ($P < 0.001$); BB and DB were greater than DW ($P < 0.001$); EW was similar to all lines ($P > 0.4$) except EB ($P < 0.001$). The stiffness of humeri from BB hens was less than all lines other than DW ($P < 0.05$). Lines DW, EW, EB, and DB were similar ($P < 0.5$). Although the output of the experimental fracture procedure was not analyzed as part of this work, we have provided supplemental figures (Supplementary Figures 1–6) relating the biomechanical properties of individual hen's tibia and humerus with the severity of experimental fracture (resulting from a single collision energy, 3.248 J) for each genetic line. We have also provided figures of the correlation between each line's average tibia and humerus strength and stiffness (Figure 1) as well as the relationship between log values of tibia and humerus strength and body mass (Figure 2). In 3-point bending of the tibia, the squared correlation coefficients of the mean stiffness of the genetic lines (n = 5) were 0.97 for strength and 0.96 for energy to failure. For the humerus, the squared correlation coefficients of the mean stiffness of the genetic lines (n = 5) were 0.91 for strength and 0.51 for energy to failure.

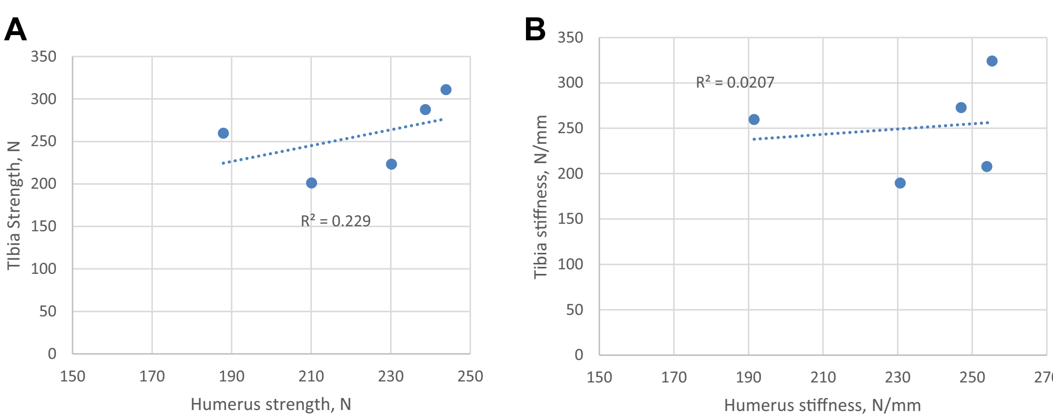

Figure 1. Correlation plots between the tibia (Y-axis) and humerus (X-axis) for biomechanical strength (A) and stiffness (B), where each point represents a genetic line or hybrid.

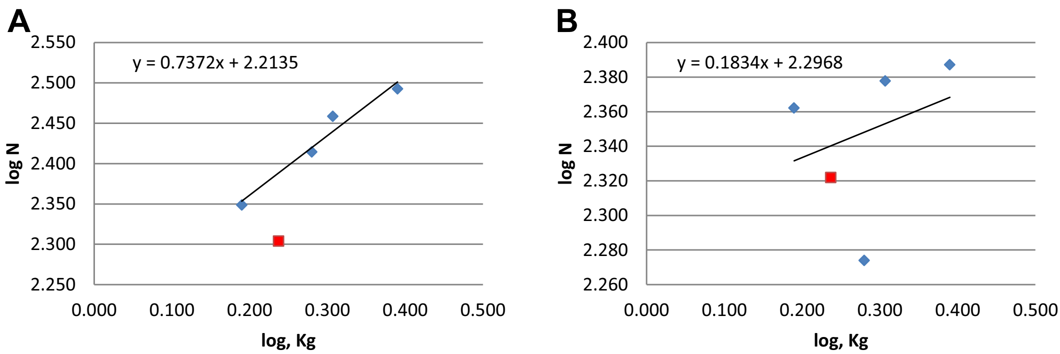

Figure 2. Correlation between logs of tibia (A) and humerus (B) bone strength (N; Y-axis) and body mass (kg; X-axis), where each point represents a genetic line or hybrid. The regression line and associated equation are calculated without the Dekalb White data point shown in red.

Computed Tomography Assessment

The best fitting model for keel BMD was found to include line ($P < 0.0001$) and slice ($P = 0.002$), though not their interactions ($P = 0.06$) (Table 2). A Tukey's post hoc comparison of BMD output revealed all lines to be different from each other ($P < 0.014$) with the exception of DB and BB which were similar ($P = 0.56$) (Table 3). Although not included in the statistical analysis due to very few numbers in the selected subset, we included results from the DW line within the table to allow for a numerical, non-statistical comparison (Table 3). Analysis of the second moment of area in the cranial and sagittal axes found the best fitting model to include the main effects of line, slice, and their first-order interactions (Table 2). Modeled data of the second moment of area in the sagittal axis for each line in relation to slice (as well as the raw data for the single sample of the DW line) are presented to aid interpretation of line differences (Figure 3).

DISCUSSION

Our results appeared to conform to what was expected as assessed by fracture susceptibility–peak force and total energy to reach failure of long bones and keel mineral density appeared to be inversely proportional to fracture susceptibility. In other words, purebred or hybrid lines that were less susceptible to fracture based on the ex vivo impact testing procedure (Candelotto et al., 2017) had long bones that were stronger and tougher (i.e., greater peak force and total energy, respectively, as defined by Melvin (1993)) and keels with greater BMD. The relationship between fracture susceptibility and properties of the mentioned bones appeared to be particularly strong at the extreme of either property. For instance, the EB line, by far the most resistant to experimental fracture, also had the greatest keel BMD, as well as peak force, stiffness, and total work to failure in the tibia. Numerically, the EB values were also larger in the humerus, though not statistically different from other lines. In contrast, the lines that manifested the greatest susceptibility to experimental fracture—the EW and DW lines—had the lowest values for keel BMD and tibia total energy and peak force to failure.

Long bone biomechanical properties did vary across bone types where—in terms of peak force and total energy—the tibia more closely paralleled the pattern of susceptibility to experimental fracture than the humerus. In the tibia, the DW and EW lines were clearly separated from the next, least resistant line (the BB) in terms of total energy, although there was no statistical difference between these 3 lines in the humerus. Given that the standard errors between bone types for these measures were similar, the greater mean difference between lines in the tibia suggests that it may be a superior target for breeding relative to the humerus. The notion that the tibia may be a more effective breeding trait to reduce keel fractures is also supported by work from Bishop et al. (2000) who developed

Table 2. Test for fixed effects of the keel's second moment of area in the cranial and caudal axes and bone mineral density assessed from computed tomography scans.

		2nd moment X			2nd moment Y			Keel bone mineral density		
Effect	NumDF	DenDF	F value	Pr > F	DenDF	F value	Pr > F	DenDF	F value	Pr > F
Line	3	335	29.83	<0.0001	338	19.19	<0.0001	338	11.14	<0.0001
Slice	1	335	1,215.02	<0.0001	338	716.88	<0.0001	338	27.65	<0.0001
Slice^2	1	335	417.04	<0.0001	338	432.78	<0.0001	338	11.76	0.0007
Slice^3	1	335	233.58	<0.0001	338	268.29	<0.0001	338	9.80	0.0019
Slice*line	3	335	4.99	0.0021	338	14.44	<0.0001	338	2.49	0.0599

Slice, slice^2, and slice^3 refer to polynomial functions for the slice factor.

Abbreviations: DenDF, denominator degrees of freedom; NumDF, numerator degrees of freedom; Pr, probability.

2 divergent laying hen lines by selecting for humerus and tibia strength (and keel radiographic density). They found that tibia strength became more differentiated between lines in comparison to the humerus as well as had greater heritability (0.30 vs. 0.45). Most critically, the 'high strength' line also appeared to have reduced broken keels. We anticipated that the humerus, given its direct muscular connections to the keel, would be a more appropriate proxy for the keel, though our results (and that of Bishop et al. (2000)) suggest this is not the case. A greater role for the tibia is also supported by the fact that calcium content in keel bones and tibia is correlated and calcium contents in keel bones negatively correlate with the occurrence of keel bone damage (Gebhardt-Henrich et al., 2017).

Reduced focus on the humerus (relative to the tibia and keel) is also supported by the observed relationships between the bone biomechanical properties of each line and body mass (and BMD). As shown by the figures depicting the correlation between the tibia and humerus for strength and stiffness, there was no correlation. Upon further examination of these properties with body mass, line-specific strength and stiffness were highly correlated in the tibia while being weaker in the humerus. This finding was in contrast to our prediction that bone strength and stiffness would correlate with each other and with body mass, a property that is well established across animals including birds (Dumont, 2010). The lack of a correlation between humerus biomechanical properties and body mass might indicate reduced use of the wings in locomotion (Biewener and Bertram, 1994). Although we would expect the tibia would increase in strength with greater activity-related loading on the legs (e.g., walking vs. wing extension or contraction) (Regmi et al., 2013; Casey-Trott, 2016), the observed relationship could also relate to the tibia's role in supporting the mass of the hens which is independent of activity. Future research is needed to determine how behavioral differences between

Table 3. Sample size, i.e., number of hens assessed, least square means, and SE for bone mineral density based on computed tomography for each of the assessed lines.

Line	n	Estimate	SE
EW	2	272^c	11.60
EB	6	376^a	6.24
DB	7	327^b	6.15
BB	3	341^b	9.59
DW	1	240	4.17

[a–c]Means without a common superscript are different ($P > 0.05$).

Slice was not found to be an effective predictor; thus, means are independent of slice. A fifth line (DW) was not included in the statistical analysis as only 1 keel was assessed, although the single (averaged) value is included in the table for comparison (BB: N = 3; DW: N = 1; ISA DB: N = 7; EB: N = 6; EW: N = 2).

Abbreviations: BB, Bovans Brown; DW, Dekalb White; EB, Experimental Brown; EW, Experimental White; ISA DB, Institut de Sélection Animale Dual Brown.

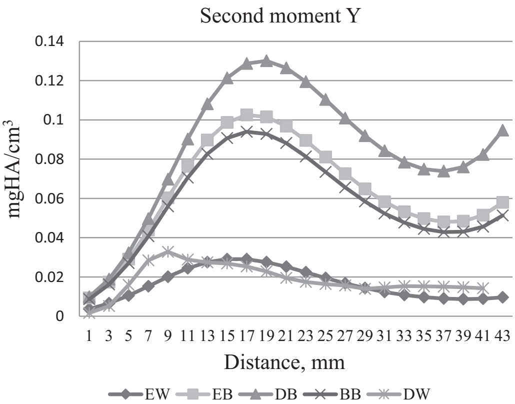

Figure 3. Second moment of area in the sagittal axis for the 4 modeled lines plus the raw value for the single DW hen. Abbreviations: BB, Bovans Brown; DW, Dekalb White; EB, Experimental Brown; EW, Experimental White; ISA DB, Institut de Sélection Animale Dual Brown.

genetic lines relate to bone properties in order to select appropriate traits and tissues.

In pursuit of exploring mechanistic explanations for variations in fracture susceptibility, comparing the lines' biomechanical properties with body mass could also be viewed from a functional perspective. Across all animal species, the respective log transformation of bone strength is expected to increase in proportion to the log of body mass (Carter and Beaupré, 2007). Interestingly, with the exception of DW hens which were the most prone to fracture, the remaining lines exhibited this relationship suggesting an agreement between form and function. The characterization of the DW line falling outside suggests its skeleton is poorly suited for loading and forces that would be experienced in comparison to the other lines. Although this observation is based on a rather limited dataset of hens within a single type of housing system and experimentally induced fractures, cross-species comparisons of this nature may be helpful in identifying causal factors for keel fracture.

The strength of a bone is understood to provide a protective effect against fracture, a concept thoroughly addressed within human (Ammann and Rizzoli, 2003) and laying hen research (Bishop et al., 2000). Stiffness is defined as the resistance of an elastic body to deformation (Currey, 2001; Forestier-Zhang and Bishop, 2016) and is rather strongly related to strength and energy to failure for a given bone geometry and loading condition. The relatively high correlation between mean tibia stiffness of the different genetic lines with strength and energy to failure supports this notion.

In summary, results of the current study emphasize the role of body weight, tibia strength, and to some extent keel BMD to objectively evaluate physiological and biomechanical properties of hens in terms of susceptibility to fracture. By assessing characteristics of likely relevance between 5 purebred or crossbred lines shown to clearly differentiate in susceptibility, the current work suggests the benefits of appropriate breeding strategies to reduce the likelihood of fractures occurring and continued research of this nature. Likely properties to be of benefit would be increased mineral density in the keel and tibial energy and strength in 3-point bending, though these should be assessed comprehensively, ideally including the influence of behavioral activity and the effect on these properties. While our results do offer a unique means of addressing keel fractures, the impact testing procedure and the design of the current study do have important limitations (some of which have already been discussed by Candelotto et al. (2017)). For instance, live hens experiencing a collision would be undergoing muscular contractions that could influence the tension on connected bone and the likelihood of fracture. Specifically for the current study, the number of hens used for computed tomography scans varied considerably between lines, was often small, and had varying levels of damage. Results will need to be replicated with larger and more uniform sample sizes, though they do provide an important reference point for those future efforts. Nonetheless, we believe our results offer important considerations for the role of bone properties in relation to keel bone fracture and the prospect of effective breeding strategies (Harlander-Matauschek et al., 2015).

CONCLUSIONS AND APPLICATIONS

1. Our results support that distinct underlying biomechanical and mineral properties of keel

and long bones reflect susceptibility to keel fracture as part of an ex vivo impact testing procedure.
2. Bone strength and toughness seemed to provide the greatest benefit to fracture susceptibility within the range of assessed collision energies and only limited benefit provided by keel flexibility.

DISCLOSURES

Teun van de Braak is employed by the company which provided the chicks as an in-kind donation and was involved in the initial planning of the study and development of the discussion.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.japr.2020.09.004.

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