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Journal

American journal of veterinary research, 84(3)

ISSN

0002-9645

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Publication Date

2023-03-01

DOI

10.2460/ajvr.22.09.0167

AJVR

AVMA

FGF4L2 retrogene copy number is associated with intervertebral disc calcification and vertebral geometry in Nova Scotia Duck Tolling Retrievers

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Received September 29, 2022.
Accepted December 23, 2022.

doi.org/10.2460/ajvr.22.09.0167

OBJECTIVES
To evaluate the effects of the chondrodystrophy-associated *FGF4L2* retrogene on intervertebral disc (IVD) calcification and vertebral geometry.

ANIMALS
22 Nova Scotia Duck Tolling Retrievers (NSDTR) with no *FGF4L2* retrogene (n = 7, wild-type dogs), 1 retrogene copy (8, heterozygous dogs), or 2 retrogene copies (7, homozygous dogs).

PROCEDURES
Computed tomography (CT) scans of the vertebral column were analyzed using computer-aided design (CAD) software. IVD calcification, vertebral column length, and vertebral geometry of the third cervical (C3), 13th thoracic (T13), and first lumbar (L1) vertebrae were compared.

RESULTS
IVD calcification was not found in wild-type dogs. IVD calcification was more frequent in homozygous dogs than heterozygous ($P = .008$) or wild-type dogs ($P < .001$) and in heterozygous dogs compared to wild-type dogs ($P < .001$). Four IVDs were subclinically herniated in 3 dogs (2 homozygous, 1 heterozygous). Calcified IVD had a greater volume and surface area in heterozygous dogs than homozygous dogs. C3 vertebral canal height-to-width ratio was greater in homozygous dogs than heterozygous dogs ($P = .044$) and wild-type dogs ($P = .010$).

CLINICAL RELEVANCE
IVD calcification and vertebral geometry can be analyzed using CAD software. The presence of 1 or 2 *FGF4L2* copies in the absence of the *FGF4L1* retrogene has an additive effect on the number of calcified IVD and a minor effect on vertebral geometry in NSDTR dogs. Data support the use of *FGF4L2* phenotyping to reduce clinical disease in segregating breeds and to monitor the introduction of wild-type alleles into fixed breed populations.

Chondrodystrophy is a genetic disorder resulting from the presence of 1 or more copies of the fibroblast growth factor 4 retrogene on chromosome 12 (CFA12) referred to as fibroblast growth factor 4 like 2 (*FGFL2*).[1–3] Chondrodystrophy results in limb shortening and premature degeneration of the intervertebral disc (IVD).[4,5] In turn, changes to the nucleus pulposus lead to IVD calcification and potential herniation.[1,6] In a retrospective, noncontrolled study of dogs from 61 breeds and mixed-breed dogs that underwent surgery to manage IVD disease (IVDD), the presence of IVD calcification was higher in dogs with 2 copies of *FGF4L2* (84.8%) than 1 copy (63.8%) compared to 0 copies (18.5%).[1] However, data from a prospective, controlled study within a single breed known to segregate the *FGF4L2* retrogene has been lacking. It is also unclear whether the presence of 1 or 2 *FGF4L2* copies influences vertebral geometry in addition to limb length. An additional *FGF4* retrogene has been reported previously on chromosome 18 (*FGF4L1*), which has also been associated with decreased limb length (chondrodysplasia)[7] but not premature IVDD.[1] In chondrodysplastic, IVDD-susceptible breeds such as Basset Hounds, Corgis,

and Dachshunds, most dogs carry 2 *FGF4L2* copies and 2 *FGF4L1* copies, while other slightly longer legged IVDD-susceptible breeds such as Spaniels, Beagles, and French Bulldogs are essentially fixed for just the *FGF4L2* retrogene.[1] This makes discrimination of the relative phenotypic impact of the 2 different *FGF4* retrogenes and gene dosage challenging. Many breeds that segregate the *FGF4L2* retrogene also carry copies of the *FGF4L1* retrogene.[1] Nova Scotia Duck Tolling Retrievers (NSDTR), however, are an *FGF4L2*-segregating breed (allele frequency is approx 0.35) but have no *FGF4L1* retrogenes and can have 0 (wild-type dogs), 1 (heterozygous dogs), or 2 (homozygous dogs) copies of *FGF4L2*.[1,2] Clinically significant IVDD is present in NSDTR dogs,[2,5] and evaluation of the effects of *FGF4L2* retrogene number on IVD calcification and vertebral geometry may have prognostic and/or mechanistic implications for IVDD and genetic approaches for reducing disease incidence.

The purpose of the prospective study presented here was to determine the influence of copy number of *FGF4L2* on IVD calcification and on the geometry of a cervical, a thoracic, and a lumbar vertebra using computed tomography (CT). We hypothesized that the number and size of calcified IVDs would increase as the number of *FGF4L2* copies increased (additive gene effect) and that the vertebral geometry of dogs with and without *FGF4L2* would differ. To test these hypotheses, *FGF4L2* wild-type, heterozygous, and homozygous NSDTR underwent CT scans of their vertebral column, and the data were compared using computer-aided design (CAD) software.

Materials and Methods

Sampling

A power analysis was conducted to determine the sample size for measurements of vertebral geometry. The first 9 vertebral column CT scans of wild-type (n = 3), heterozygous (3), and homozygous (3) NSDTR were reconstructed. Sample sizes to detect a 20% difference in vertebral canal height-to-width ratio at a power of 80% and a significance level of 0.05 for the third cervical (C3), 13th thoracic (T13), and first lumbar (L1) vertebra were calculated using an online calculation tool.[8] The calculated sample sizes were 4 for C3, 3 for L1, and 4 for T13. A decision was made to enroll a minimum of 7 dogs for each of the 3 genotype statuses (wild type, heterozygous, and homozygous).

Dogs were recruited from owners and breeders locally. Recruitment was targeted to fill the genotypic classes. Inclusion criteria were being an NSDTR and being considered healthy without systemic or neurologic disease by the owners. Exclusion criteria during screening were the presence of abnormalities indicative of a systemic or neurologic disease during physical and neurologic evaluation. All dogs were enrolled with informed client consent, and the study was approved by the University of California-Davis Institutional Animal Care and Use Committee and the University of California-Davis Veterinary Medical Clinical Trials Review Board. The enrollment period ranged from November 2018 to February 2021. Genotyping for *FGF4L2* and *FGF4L1* was done using PCR-based assays as previously described.[2] Sex and neuter status, body weight, and age at the time of enrollment were recorded. Investigators conducting data collection and analysis (CAB, DJM) were masked to the dogs' genetic status.

Intervertebral disc calcification

The dogs were sedated using a combined intravenous injection of 3 to 5 µg/kg dexmedetomidine and 0.1 mg/kg butorphanol. CT scans of the complete vertebral column were collected using a 16-slice helical CT scanner (GE LightSpeed; GE HealthCare). The slice thickness was 0.625 mm. The CT scans of the vertebral column were exported to a visualization software program (Horos v3.3.6; Horos Project) and were reviewed by a DVM student blinded to the genetic status of each dog. The DVM student initially identified presumptive calcified nonherniated and herniated IVD. The DVM student and a board-certified radiologist jointly reviewed the CT scans and confirmed the calcified IVD by consensus.

Cross-sectional DICOM images were exported into a medical segmentation software (Mimics v. 23.0; Materialise) and were segmented at a lower threshold of 226 Hounsfield units, a lower threshold previously used to select vertebral bone[9] and evaluate IVD calcification.[10] Calcified IVD were separated from vertebral endplates in individual CT slices using a *Split Mask* tool, and their mean density in Hounsfield Units was recorded. Three-dimensional (3-D) surface renderings were created and exported as *parts* (*.stl* files) into CAD software (3-Matic v. 17.0; Materialise). Volume (V, mm^3), surface area (A, mm^2), and sphericity were calculated. Sphericity was calculated using the following formula: $\frac{\sqrt[3]{36\pi V^2}}{A}$, where V was the volume and A was the surface area, resulting in a sphericity ranging from 0 to 1, where 1 would be a perfect sphere. The ventrodorsal location of calcified IVD material was recorded, based on whether it was located across the dorsal, central, or ventral third of adjacent vertebral bodies or a combination of these locations.

Vertebral geometry

Renderings of the vertebral column were prepared using the medical segmentation software (Mimics; Materialise). The length of the vertebral column was measured using 2 methods. To measure the overall length (vertebral + intervertebral space length), the distance between the cranial aspect of the dorsal arch of the atlas (C1) to the caudal aspect of the dorsal arch of the sacrum (S3) in the sagittal plane was measured in all dogs. To measure vertebral length, the length of each vertebra from C1 to S3 was measured in a subset of 6 body weight-matched dogs: 1 male and 1 female wild-type, heterozygous, and homozygous dog. Vertebral length was the length of the vertebral body, measured in the sagittal plane from its craniodorsal to its caudoventral aspect.

Within the medical segmentation software, C3, T13, and L1 vertebrae were isolated from other structures using mask editing tools. The vertebrae were exported as surface renderings (*.stl* files) to the CAD software. A Cartesian coordinate system was created for each vertebra. The origin (x = 0, y = 0, and z = 0) was at the dorsal aspect of the cranial vertebral endplate at the vertebral midline **(Figure 1)**.[11] A second point was located at the dorsal aspect of the caudal vertebral endplate within the vertebral midline. A third point was placed at the dorsal and cranial aspect of the spinous process at the midline. The 3 points were used to create the sagittal (yz) plane. The plane was rotated 90° around the longitudinal (y) axis to create the dorsal (xy) plane and around the x-axis to create the transverse (axial, xz) plane.[12] Vertebral volume, vertebral body length, width, curvature of the ventral aspect of the vertebral body, angulation of the caudal vertebral endplate relative to the cranial vertebral endplate, spinous process height, transverse process width, cranial articular surface width (C3 only), and vertebral canal height, width, length, volume, and height-to-width ratio were calculated.

To measure vertebral body length, 10 X 10-mm sketch planes were fitted to the cranial and caudal surfaces of the vertebral body. Vertebral body length was the distance between the centers of these 2 fitted planes. Vertebral body width was measured in the dorsal plane as the distance from the ventral aspect of the transverse process on the left and right of the vertebral body. Ventral vertebral body radius of curvature was measured by fitting an arc to the ventral vertebral surface in the sagittal plane using the *Circle Arc (3 Points)* function. To calculate cranial endplate angulation, a line was drawn as the intersection of the plane fitted to the cranial aspect of the vertebral body and the sagittal plane. Cranial endplate angulation was the angle between that line and the transverse plane measured in the sagittal plane. Caudal endplate angulation was measured similarly. Transverse process width for C3 and L1 was measured as the distance between the lateral aspect of the left and right transverse processes. Spinous process height was measured as the distance between the dorsal aspect of the spinous process and the dorsal plane minus vertebral canal height **(Figure 2)**.

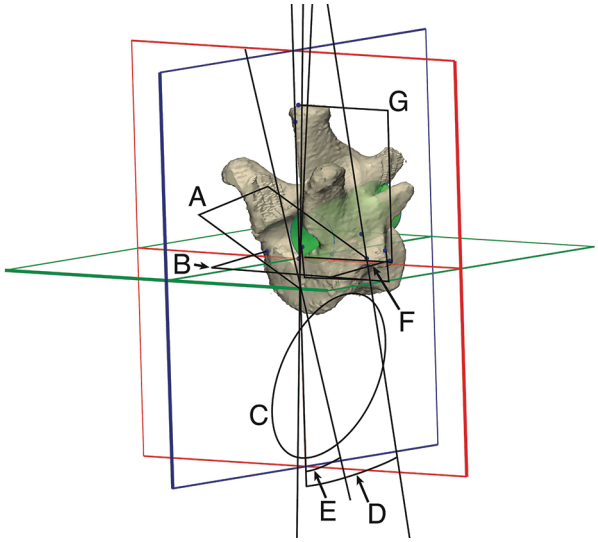

Figure 2—Representative 3-D reconstructed image of the 13th thoracic vertebra of an 8-year-old male Nova Scotia Duck Tolling Retriever showing measurements of vertebral body length (21.64 mm; A) and width (21.80 mm; B), radius of curvature of the ventral aspect of the vertebral body (17.80 mm; C), caudal endplate angulation (11.02°; D), cranial endplate angulation (17.32°; E), vertebral width at the transverse processes (32.75 mm; F), and spinous process + canal height (31.13 mm; G).

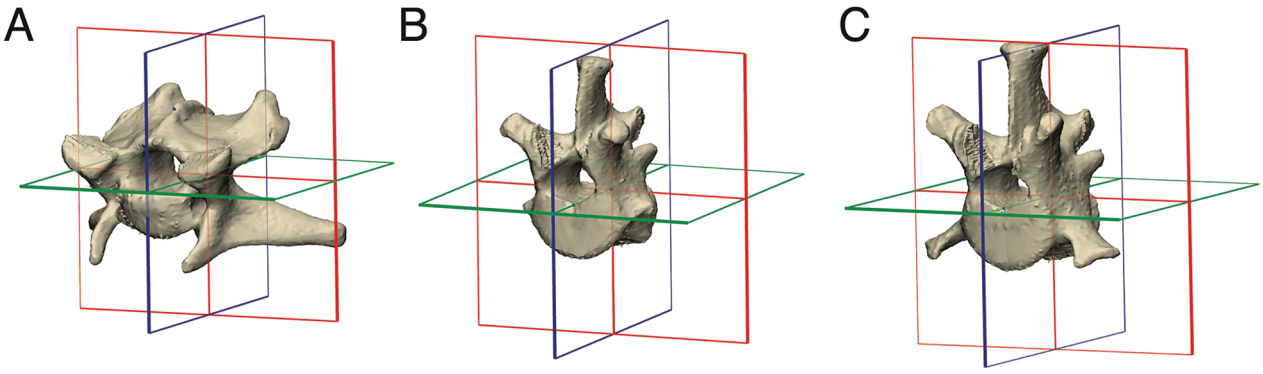

Figure 1—Representative 3-D reconstructed images derived from CT sequences of the third cervical vertebra (A), the 13th thoracic vertebra (B), and the first lumbar vertebra (C) of a 2-year-old male Nova Scotia Duck Tolling Retriever. Cartesian coordinate systems have been overlaid over each vertebra. Each coordinate system consisted of 3 axes (x, y, and z) perpendicular to each other. The sagittal plane (shown in blue) was set using 3 points at the cranial and dorsal aspect of the vertebral body (point of origin of all axes), the caudal and dorsal aspect of the vertebral body, and the cranial and dorsal aspect of the spinous process. The dorsal plane (shown in green) was set by rotating the sagittal plane 90° about the line joining the cranial and caudal points on the vertebral body. The transverse plane (shown in red) was set by rotating the sagittal plane 90° about the line perpendicular to the dorsal plane that included the point of origin. The planes are shown centered over the vertebrae for clarity.

Statistical analysis

Statistical analysis was done using statistical software (SAS 9.4; SAS Institute Inc). The effects of the *FGF4L2* genotype (wild type, heterozygous, and homozygous) on IVD calcification variables (number of calcified IVD, volume, surface area, sphericity, and density of calcified IVD) were assessed using repeated measures ANOVA for the cervical, thoracic, lumbar, sacral/caudal, and all vertebral column segments combined, with age as a fixed effect, and individual dogs as a random effect. Body weight and sex had no significant effects on IVD variables and were not included in the ANOVA of calcified IVD variables. Spearman correlation coefficients of IVD calcification variables and age were calculated. The effects of the *FGF4L2* genotype (wild type, heterozygous, homozygous) on vertebral measurements were assessed using repeated measures ANOVA with sex and body weight as fixed factors and individual dogs as a random factor. The lengths of all vertebrae were compared among the 2 wild-type, heterozygous, and homozygous dogs for the cervical, thoracic, lumbar, sacral/caudal, and all vertebral column segments combined, using an ANOVA with sex, body weight$^{1/3}$, and vertebral number as fixed effects and individual dogs as random effect. Post hoc pairwise comparisons were done using *t* tests to compare age and weight in each group. Sex distribution among groups was compared using Fisher exact test. The normality of the ANOVA residuals was assessed using the Shapiro-Wilk statistic. The ANOVA was run 2 ways. First, by looking at all possible comparisons between wild-type, heterozygous, and homozygous data. Second, by comparing wild-type with either heterozygous or homozygous data. Body weight and body weight$^{1/3}$ were included in ANOVA analyses to evaluate their impact on geometric parameters.[13] Body weight and sex were included in the model for overall vertebral column length, vertebral volume, vertebral canal volume, vertebral canal height-to-width ratio, ventral vertebral body curvature, and endplate angulation. Body weight$^{1/3}$ and sex were included in the model for overall vertebral column length, vertebral body length, vertebral canal length, vertebral canal height, vertebral canal width, vertebral body length, vertebral body width, spinous process height, transverse process width, and cranial articular surface width for C3. For nonparametric variables, data were transformed to ranks or log transformed and ANOVA was run on transformed data. Nonparametric data are reported using median (minimum, maximum), and parametric data are reported as mean ± SD. Significance was set at $P < .05$.

Results

Demographic data

No dog was excluded before enrollment because it was deemed to have a neurologic problem by its owners, and no dog was excluded during screening because of an abnormal physical or neurologic evaluation. Twenty-two NSDTR were included: 7 wild-type dogs, 8 *FGF4L2* heterozygous dogs, and 7 *FGF4L2* homozygous dogs. The mean ± SD age for all dogs was 4.3 ± 2.2 years; wild-type dogs were 3.4 ± 1.6 years, heterozygous dogs were 4.6 ± 2.7 years ($P = .336$ compared to wild-type dogs), and homozygous dogs were 4.7 ± 2.2 years ($P = .274$ compared to wild-type dogs; $P = .814$ compared to heterozygous dogs). Mean ± SD body weight was 16.3 ± 2.7 kg for all dogs combined; 16.0 ± 3.6 kg for wild-type dogs, 16.9 ± 2.1 kg for heterozygous dogs ($P = 0.447$ compared to wild-type dogs), and 15.9 ± 2.8 kg for homozygous dogs ($P = .467$ compared to wild-type dogs; $P = .970$ compared to heterozygous dogs). Thirteen female and 9 male dogs were included: 3 females and 4 males were wild type, 4 females and 4 males

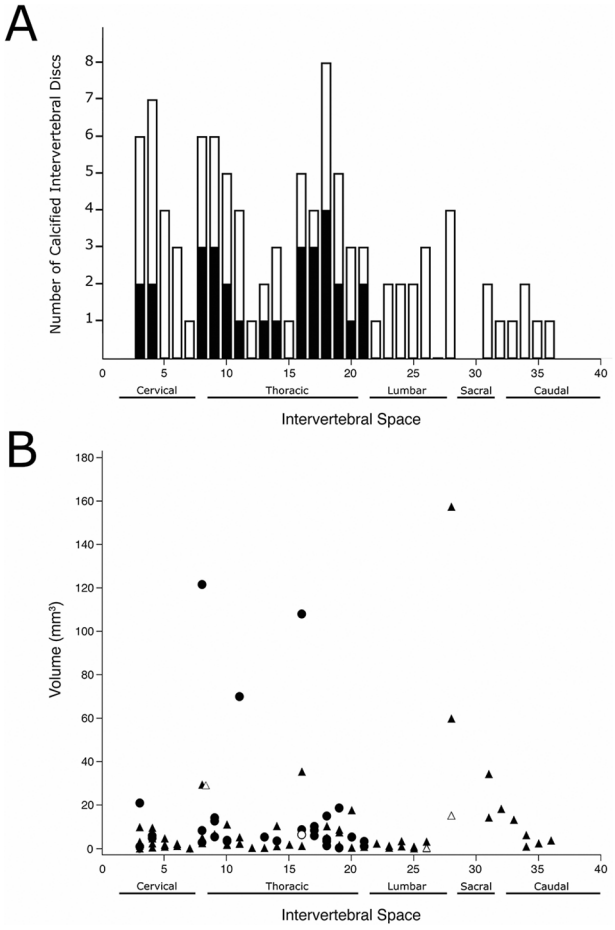

Figure 3—Histogram (A) showing the number of calcified intervertebral discs at each intervertebral space for 15 Nova Scotia Duck Tolling Retrievers with 1 *FGF4L2* copy (n = 8 dogs, black bars), and 2 *FGF4L2* copies (7 dogs, white bars). Calcification is common in the cervical, upper thoracic, and lower thoracic regions and is most common at the T10-T11 intervertebral space. Vertebral column segments are shown under the x-axis. Histogram (B) showing the volume of calcified 98 intervertebral discs (IVD) in the 15 chondrodystrophic dogs shown in histogram (A). The 30 calcified IVD in dogs with 1 *FGF4L2* copy are shown as black (calcified) or white (calcified and herniated) circles and the 68 calcified IVD in dogs with 2 *FGF4L2* copies are shown as black (calcified) or white (calcified and herniated) triangles.

were heterozygous, and 6 females and 1 male were homozygous. There was no association between sex and genetic status when analyzing all groups combined (P = .302) or when comparing wild-type and homozygous groups (P = .266).

Intervertebral disc calcification

The DVM student identified 99 presumptive calcified IVD. The DVM student and board-certified radiologist confirmed the presence of 98 calcified IVD. Intervertebral disc calcification was not detected in wild-type dogs **(Figures 3 and 4)**. Heterozygous dogs had a total of 30 calcified IVD (median per dog, 3.5 calcified IVD; range, 0 to 10; P < .001 relative to wild-type dogs). Only 1 heterozygous dog, a 1.5-year-old male, had no calcified IVD. Homozygous dogs had a total of 68 calcified IVD (median, 11; range, 5 to 17; P = .008 relative to heterozygous dogs and P < .001 relative to wild-type dogs). The highest frequency of calcified IVD (36%) was at T10-T11. All calcified IVD in the heterozygous dogs were cranial to L1. The presence of any calcified IVD on CT had a sensitivity of 94%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 88% for the presence of 1 or 2 *FGF4L2* copies. Forty-two calcified IVD were located in the dorsal third of the IVD space, 12 were in the central third, 6 were in the ventral third, 32 were in the dorsal and central thirds, 1 was in the central and ventral thirds, and 1 was in the dorsal, central, and ventral thirds. Four IVD were herniated in 3 dogs with no apparent clinical signs; 1 each at the L5-L6 and L7-S1 intervertebral spaces in a 4-year-old spayed homozygous female, 1 at the C7-T1 intervertebral space in a 5-year-old spayed homozygous female, and 1 at the T8-T9 intervertebral space in a 7-year-old spayed heterozygous female. The herniated calcified IVD were located on the ventral aspect of the vertebral canal. Age did not influence the number of calcified discs in the whole vertebral column (P = .498) or in the cervical (P = .237), thoracic (P = .167), lumbar (P = .728), or sacrocaudal (P = .681) vertebral column segments. Age also did not correlate statistically with the number of calcified IVD in the

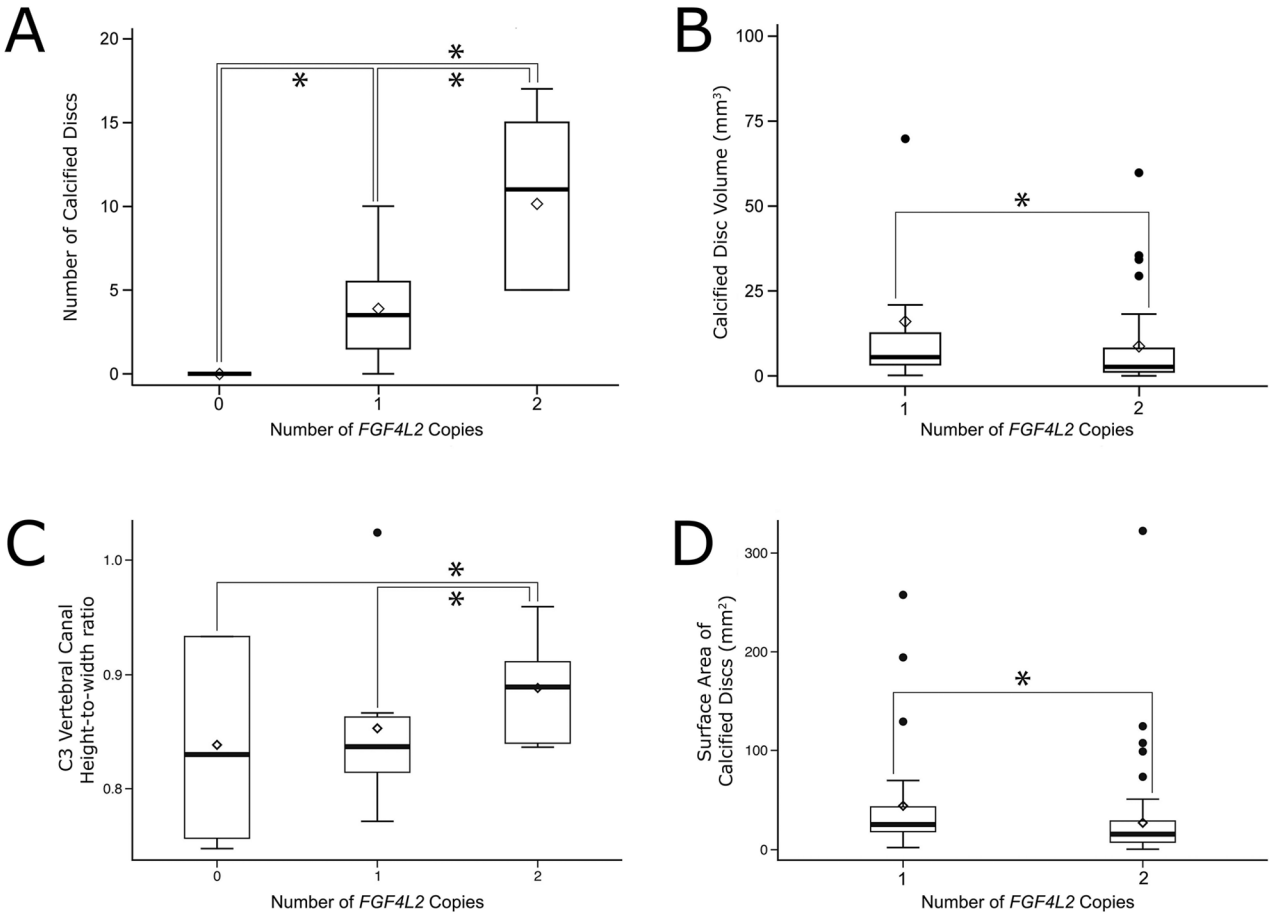

Figure 4—Box-and-whiskers plots showing the number of calcified discs (A), calcified disc volume (B), the height-to-width ratio of the vertebral canal of the third cervical vertebra (C), and the surface area of calcified discs (D) among 22 Nova Scotia Duck Tolling Retrievers with no *FGF4L2* copy (n = 7), 1 copy (8), or 2 copies (7). For each box, the thick line illustrates the median value, the diamond is the mean, the box ends are the second and third quartiles, the lower and upper boundaries are minimal and maximal data points excluding outliers, and dots represent outliers (≥ 1.5 interquartile range above or below boxes). Dogs with no *FGF4L2* copy are not shown in B and D since they had no calcified disc. *P < 0.05, statistically significant differences between groups. C3 = Third cervical vertebra.

whole vertebral column (r = 0.298, P = .178) nor in the cervical (r = 0.339, P = .123), thoracic (r = 0.349, P = .111), or lumbar (r = 0.051, P = .820) vertebral column segments. Body weight did not influence the number of calcified discs in the whole vertebral column (P = .564) nor in the cervical (P = .707), thoracic (P = .494), lumbar (P = .990), or sacrocaudal (P = .616) vertebral column segments. Sex did not influence the number of calcified discs in the whole vertebral column (P = .211) nor in the cervical (P = .925), thoracic (P = .076), lumbar (P = .377), or sacrocaudal (P = .989) vertebral column segments.

The volume of calcified IVD was larger for heterozygous dogs (median volume, 5.50 mm^3; range, 0.133 to 121 mm^3) than homozygous dogs (median, 2.65 mm^3; range, 0.005 to 157 mm^3; P = .041). The surface area of calcified IVD was larger for heterozygous dogs (median surface area, 24.8 mm^2; range, 1.55 to 257 mm^2) than homozygous dogs (median, 15.1 mm^2; range, 0.083 to 321 mm^2; P = .022). The sphericity of calcified IVD did not differ statistically among heterozygous dogs (median sphericity, 0.610; range, 0.461 to 0.828) and homozygous dogs (median, 0.655; range, 0.005 to 0.828; P = .232). The density of calcified IVD did not differ statistically among heterozygous (median density, 469 HU; range, 246 to 649 HU) and homozygous dogs (median, 420 HU; range, 234 to 715 HU; P = .531). Age statistically influenced the geometry of calcified IVD: with increased age, surface area was larger (r = 0.296, P = .003), volume was larger (r = 0.297, P = .003), sphericity was lower (r = −0.204, P = .043), and density was higher (r = 0.326, P = .001).

Vertebral geometry

Mean ± SD overall vertebral column length was 609 ± 47 mm for wild-type dogs, 623 ± 30 mm for heterozygous dogs, and 605 ± 37 mm for homozygous dogs. Overall vertebral column length did not differ significantly between genotypes (P ranging from .686 to .965 for group comparisons). For length measurements of all vertebrae, mean ± SD vertebral length was greater in homozygous dogs (24.01 ± 6.35 mm) than heterozygous (23.76 ± 6.34 mm, P = .023) and wild-type dogs (23.55 ± 5.96 mm, P = .004) and did not differ among heterozygous and wild-type dogs (P = .561). Within spinal segments, thoracic vertebral length was greater for homozygous dogs (19.36 ± 2.03 mm) than heterozygous dogs (19.13 ± 1.73 mm, P < .001) and lumbar vertebral

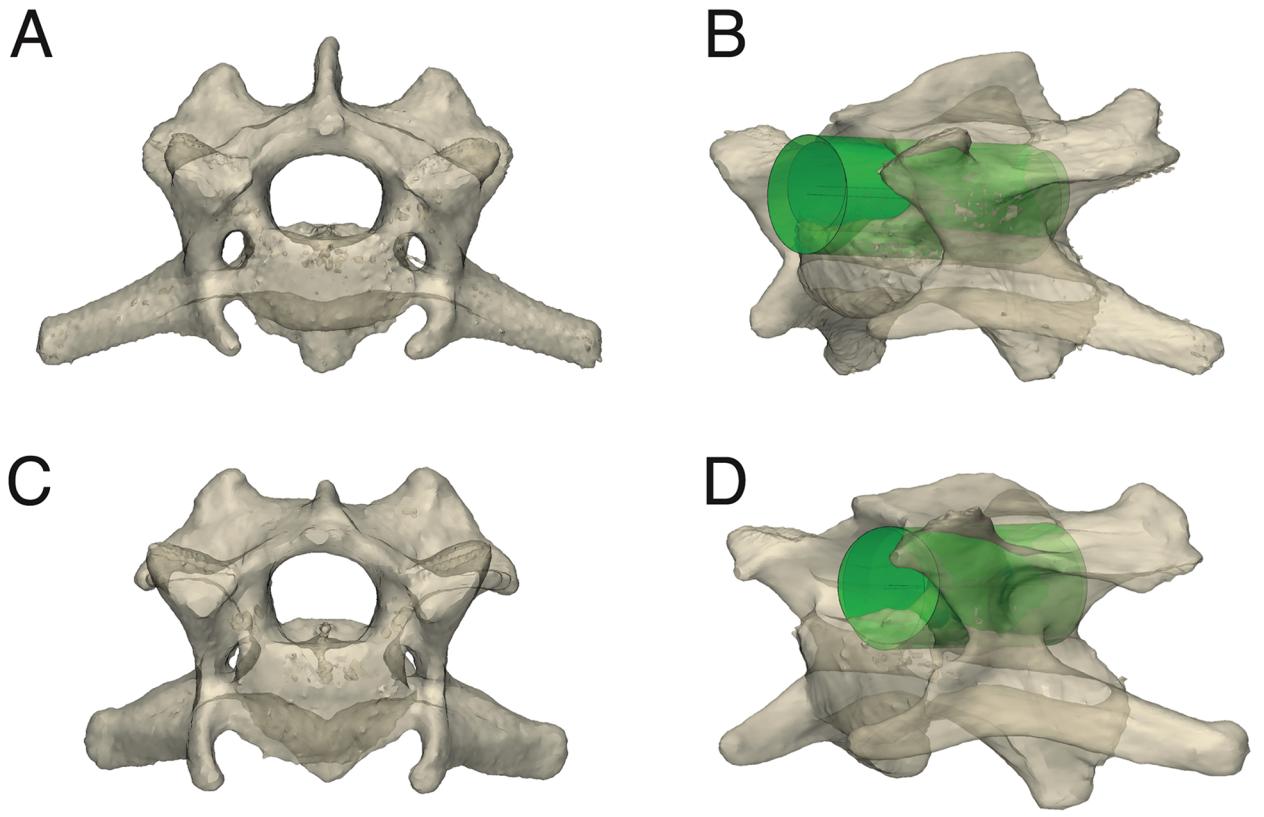

Figure 5—Representative 3-D reconstructed images of the third cervical vertebra of a 2-year-old male Nova Scotia Duck Tolling Retriever with no *FGF4L2* copy (A and B) and a 6-year-old male Nova Scotia Duck Tolling Retriever with 2 *FGF4L2* copies (C and D). The vertebral canals have different geometries: the wild-type dog has an elliptical canal (A) and the homozygous chondrodystrophic dog has a rounded canal (C). The height-to-width ratio of the spinal canal were measured by fitting 2 cylinders to each canal to determine the height and the width of the canals, respectively (B and D). These ratios were 8.65 mm/10.45 mm = 84% for the wild-type dog and 10.33 mm/10.89 mm = 96% for the homozygous dog.

Table 1—Median (range) and coefficient of variation of geometric parameters of the third cervical vertebra in 21 dogs with 0 (n = 7), 1 (8), or 2 *FGF4L2* retrogenes (7).

Parameters	0 copy	1 copy	2 copies
Vertebral canal			
Height (mm)	8.65 (8.15 to 9.58), 5.4%	8.72 (8.12 to 10.92), 9.7%	9.13 (8.12 to 10.45), 8.9%
Width (mm)	10.28 (9.28 to 12.09), 8.5%	10.55 (10.25 to 10.72), 2.2%	10.30 (9.14 to 12.16), 8.8%
Height-to-width ratio (mm)	0.83[a] (0.75 to 0.93), 9.0%	0.84[a] (0.77 to 1.02), 8.8%	0.89[b] (0.84 to , 0.96), 4.7%
Length (mm)	23.89 (22.5 to 28.28), 8.9%	23.88 (22.86 to 27.23), 5.8%	24.45 (21.95 to 27.03), 6.3%
Volume (mm^3)	1,659 (1,500 to 2,358), 19%	1,710 (1,634 to 2,296), 12%	1,791 (1,356 to 2,637), 23%
Vertebral body			
Cranial endplate angulation (°)	28.32 (20.00 to 30.08), 14%	25.76 (15.84 to 28.64), 20%	21.93 (20.62 to 30.65), 17%
Caudal endplate angulation (°)	21.69 (15.60 to 32.42), 28%	21.44 (14.68 to 28.28), 22%	21.91 (14.61 to 25.56), 20%
Cranial-caudal endplate angulation (°)	7.36 (−12.42 to 14.36), 175%	5.12 (−11.31 to 10.27), 313%	2.04 (−4.24 to 14.94), 189%
Vertebral body curvature (mm)	13.85 (10.06 to 16.20), 16%	12.79 (11.93 to 14.42), 7.1%	13.59 (9.87 to 14.95), 14%
Spinal body length (mm)	25.20 (23.98 to 29.69), 8.6%	26.17 (23.95 to 28.39), 4.9%	26.24 (23.97 to 28.32), 5.7%
Spinal body width (mm)	17.80 (15.59 to 18.91), 7.7%	17.53 (16.30 to 18.71), 4.8%	16.87 (15.19 to 18.21), 8.1%
Vertebral processes			
Cranial articular surface width (mm)	33.25 (28.18 to 36.65), 9.9%	34.02 (30.63 to 39.81), 9.7%	31.49 (30.96 to 39.51), 10%
Spinous process height (mm)	6.23 (3.48 to 9.71), 31%	6.09 (4.48 to 11.67), 36%	5.65 (3.35 to 6.52), 25%
Transverse process width (mm)	45.03 (40.61 to 56.24), 13%	45.02 (35.25 to 50.21), 10%	45.12 (41.70 to 52.32), 7.3%
Vertebra			
Volume (mm^3)	7,515 (6,458 to 12,826), 31%	8,747 (7,008 to 10,863), 15%	8,008 (6,912 to 11,389), 19%

FGF4L2 = Fibroblast growth factor 4 like 2 retrogene.

[a,b]Within a row, mean values with different letters are significantly ($P < .05$) different. Only vertebral height-to-width ratios differed significantly between groups.

length was greater for homozygous dogs (26.73 ± 2.30 mm) than wild-type dogs (25.79 ± 2.10 mm, $P = .001$). Other vertebral lengths did not differ among dog groups (P ranging from .053 to .987).

Sex, body weight, and boy weight$^{1/3}$ significantly influenced most geometric vertebral parameters. For C3, the canal height-to-width ratio was larger in homozygous dogs than heterozygous dogs ($P = .044$) and wild-type dogs ($P = .010$, **Figure 5** and **Table 1**). Other geometric variables for C3 and all geometric variables for T13 **(Supplementary Table S1)** and L1 **(Supplementary Table S2)** did not differ between wild-type, heterozygous, and homozygous dogs.

Discussion

The data presented here support the hypothesis that the *FGF4L2* retrogene, in the absence of the *FGF4L1* retrogene, has an additive effect on the number of calcified IVDs based on CT imaging. The presence of *FGF4L2* had an effect on vertebral geometry; however, it was limited to the vertebral canal.

The vertebral canal of the third cervical vertebra in homozygous dogs was rounder by approximately 5 to 10% compared to wild-type dogs, and vertebrae in homozygous dogs were approximately 1.0% longer than heterozygous dogs and 1.9% longer than wild-type dogs. In a previous study[4] in the Alpine Dachsbracke and Schweizer Niederlaufhund, 2 Swiss dog breeds segregating both *FGF4L1* and *FGF4L2* documented decreased back length associated with either retrogene being homozygous but increased length with heterozygosity. The same trend of overall vertebral column length was found in the current study in NSDTR; however, this was not statistically significant. Gross measurements in conscious dogs versus CT assessment make direct comparisons problematic; however, homozygosity was associated with a modest increase in mean vertebral length, suggesting that change in vertebral length per se may not account for this interesting morphometric observation. The mechanism for the modest geometric differences in canal geometry is not known; dog vertebrae develop primarily through endochondral ossification,[14] which is affected to varying degrees in chondrodystrophic dogs.[4,15,16] Alterations of vertebral geometry as a result of chondrodystrophy, including differences in paraspinal muscle mass geometry and C2/C3 vertebral angulation, were reported in a CT-based study[17] that compared 15 Dachshunds to 15 Labrador retrievers; however, a direct comparison between these studies is not possible because of differences in geometric analyses that were 2-D in the previous study and 3-D in the current study. The effects of the *FGF4L2* retrogene on vertebral endochondral ossification and bone development appear to be limited based on the current data, and, similar to limb length, phenotypic sequelae associated with *FGF4L2* eradication are likely to be minimal.

The additive effect of the *FGF4L2* retrogene on IVD calcification has now been demonstrated in several studies where interrogation of data from segregating populations has made analysis possible.[1,18] In addition to the current study, similar effects were shown across breeds presenting for surgically confirmed disc extrusions[1] and in Wire-haired Danish Dachshunds where genotype and calcification

screening scores were reported.[18] The number of calcified IVD has been shown to be associated with the risk of overt clinical disease in Dachshunds and Pekingese dogs,[19–22] and radiographic screening has been used in an attempt to reduce disease incidence through selective breeding, specifically within the Dachshund breed. The impact of this approach on subsequent calcification scores and clinical disease has however been limited,[23] potentially due to a combination of factors including suboptimal enrollment and compliance within the breeding population and methodological limitations. The limitations of radiographic screening including sensitivity, reproducibility, and subjectivity of screeners have been reported.[19,22,24] Sensitivity issues with radiographic screening should be controlled when applied across the test populations within the same study, however the sensitivity for CT cross-sectional imaging has been shown to be 1.0 for defining both calcification of the disc space and extruded material, compared to 0.7 and 0.3, respectively, for radiography.[25] Although a parallel radiographic analysis was not done in the current study, it is likely that findings would have been similar; the use of an optimal sensitivity imaging modality in the current study is most relevant in the failure to demonstrate any calcified IVDs in the homozygous wild-type group of dogs.

Importantly, neither genotyping of chondrodystrophy associated *FGF4L2* nor calcification scoring can define individual dogs that will have clinically overt disease (apparent pain and/or neurological deficits) and are only statistically significant biomarkers of disease predilection. Retrospective analysis using either approach will always identify animals that did not develop overt clinical disease and that would have been deemed less desirable for breeding. In 1 study,[18] genotypically selecting for wild-type long-haired dogs would have precluded 92% of dogs or 58% of dogs if heterozygous dogs were included; however, calcification breeding scores (estimated breeding values) would have also precluded 48% of dogs that did not develop overt clinical disease. It has been shown that the *FGF4L2* retrogene is sufficient to cause chondrodystrophy and associated premature IVD degeneration;[5] however, only circumstantial evidence suggests that *FGF4L2* retrogene dosage is associated with clinical disease severity. IVD calcification scores have been shown to be related to risk for "clinical" IVDD,[18–22] and the number of calcified IVD, as in the current study, is associated with genotype.[1,18]

The effect of the *FGF4L2* retrogene on IVD calcification appears to be definable in segregating populations; however, considerable variation in calcification is present even within the *FGF4L2* homozygous populations of fixed and segregating breeds alike. This is an important consideration when selective breeding strategies are considered for breeds including most Dachshunds (except Wire-haired), French Bulldogs, and Beagles where the allele is essentially fixed in the population.[1] The key question is whether the variation in calcification in homozygous *FGF4L2* populations is tractable for selection or merely represents inherent variability secondary to nongenetic influences such as neuter status, conformation, activity, and ambient temperature that have been reported or proposed to have effects on the "clinical" incidence of IVDD.[6,21,26–31] Studies[6] are ongoing to identify additional modifying loci for IVDD; however, no significant candidates have been defined to date. Considerable variability in nongenotyped studies has been reported even for different Dachshund types (Smooth-haired, Long-haired, and Wire-haired) with relatively simple phenotypes such as incidence of overt clinical IVDD,[18,21,31] and large prospective and longitudinal studies correlating genotype, imaging characteristics, and clinical disease are likely to be necessary to comprehensively define genotype effects on clinical outcome. Bruun et al[18] reported an association of calcification with genotype in Wire-haired Dachshunds where segregation of *FGF4L2* was present in the population. Interestingly, variability in total IVD calcifications was present in *FGF4L2* homozygous wire-haired dogs comparable to that seen in homozygous dogs in the "fixed" smooth and long-haired types. Also in that study, a small number (3) of smooth-haired dogs that were either heterozygous or wild-type for *FGF4L2* had only 3 calcified IVD in total, suggestively similar to findings in Wire-haired Dachshunds.

Although genetic differences do exist between the 3 Dachshund types with theoretical implications for IVDD,[6] it seems reasonable to hypothesize that a more general introduction of wild-type alleles into "fixed" Dachshund (or other fixed breed) populations would result in a beneficial reduction in IVD calcification similar to that documented in Wire-haired Dachshunds and the current study. Zero calcified discs, as seen in the limited number of wild-type NSDTR in this study, is a logical goal for the management of IVDD through breeding strategies as previously suggested:[19–22] 0 *FGF4L2* retrogenes would therefore be the logical parallel goal based on association with disease directly,[1,2,5] or with the indirect association with calcification as a surrogate marker, as seen in this and other studies.[1,18]

The effects of cross-breeding in *FGF4L2*-"fixed" breeds may not be necessarily deleterious for breed standards; cross-breeding of Dachshunds with Terrier and Pinscher breeds resulted in the Wire-haired Dachshund type and likely resulted serendipitously in the decreased *FGF4L2* allele frequency in that breed type. There should be no reason based on this precedent that similar approaches cannot be applied to other *FGF4L2*-fixed populations. Fortunately, it is likely that effects on desirable breed-defining phenotypes such as short limbs, in breeds that carry both *FGF4L1* and *FGF4L2* retrogenes (Dachshunds, Corgis, and Bassets), are likely to be minimal based on segregation studies in other breeds,[4] and the current data suggest that changes in vertebral column morphometry are likely also to be of limited impact. Clinically overt IVDD is the major driver for the eradication of underlying genetic abnormalities in chondrodystrophic dogs. However, it is likely that chondrodystrophic animals with degenerative IVD from a few

weeks of age[5,16] will have associated biomechanical sequelae and gait- and pain-related issues may be present in many animals regardless of presentation for overt owner- or veterinary-observed IVDD.

The current study is limited by small group numbers, although the sample size was determined a priori using power analysis, and the sample size was sufficient to identify clear differences between dog groups for the number of calcified IVD. However, while the precision of CAD geometric analysis allows the detection of relatively minor geometric differences among bones without and with a deformity,[32] the small sample size increased the likelihood of type 2 statistical error, where numerical differences in vertebral geometry may not have been confirmed statistically. Also, for logistical reasons relating to the analysis time required and the cost of CAD analysis, the geometric parameters in all dogs were evaluated in only 1 vertebra from each of the cervical, thoracic, and lumbar regions. It is unclear whether differences in vertebral geometry were present in other vertebrae. While the correlation between age and IVD calcification was tested, the study was not designed to evaluate the effects of age on the severity of IVD calcification, since age ranges were limited. No conclusion was drawn from the current study regarding the effects of age on IVD calcification.

NSDTR dogs were chosen specifically because of the degree of *FGF4L2* retrogene segregation within the breed and the absence of the *FGF4L1* retrogene; our findings may not be universally applicable to all chondrodystrophic breeds; however, comparable findings in other less defined studies would support generalization of the results.[1,18] Age has been shown to affect the number of radiographically defined calcified discs in Dachshunds,[33,34] and dogs in this study were not necessarily in the 24- to 48-month recommended age range utilized for calcification screening protocols.[18,21] However, age was not found to be a confounding factor, potentially because all animals fell within a relatively narrow age range (mean age ± SD for all dogs was 4.3 ± 2.2 years) with no statistically significant difference between groups. Distribution of calcified IVD in this small population of NSDTR was similar to previous reports[19,22,33,35] in Dachshunds and Pekingese, with the highest frequency in the low thoracic/thoracolumbar and cervical vertebral column (Figure 3). Data describing specific characteristics of IVD calcification are limited.[16] The nucleus pulposus is located eccentrically and dorsally in dogs and to a greater degree in chondrodystrophic dogs (when compared across breeds),[16,36] and the predominant dorsal location of calcified material was consistent with those data. Age-related increased surface area, volume, and density with decreased sphericity may be reflective of progressive pathology; however, age ranges were small and, similar to increased calcification volume in heterozygous dogs, may also be reflective of small sample numbers. The CAD geometric analysis methods presented here can be used in future research to further define bone growth abnormalities in chondrodystrophic animals and may be enhanced through automation, to allow more extensive analyses.[37]

We conclude that the presence of the *FGF4L2* retrogene has an additive effect on intervertebral calcification with limited effects on vertebral geometry. The *FGF4L2* retrogene is sufficient for the development of chondrodystrophy with associated premature IVD degeneration.[5] Correlation of genotype with calcification, which has been previously defined as a predictive factor for overt clinical disease, supports eradication of the *FGF4L2* retrogene as a means to decrease incidence of IVDD. Moreover, current data, previous morphometric studies,[4] and registration of Danish Kennel Club Dachshunds of all genotypes[18] suggest that even in genotypically fixed breeds, reduction of retrogene frequency through the introduction of wild-type alleles may have limited consequences for basic breed-defining morphological phenotypes.

Acknowledgments

The authors declare that there were no conflicts of interest. This work was supported in part by a grant from the Center for Companion Animal Health (grant No. 2018-13-F) and by the Students Training in Advanced Research (STAR) program, University of California-Davis.

The authors thank Drs. Kimberly Maciejczyk and Kathryn L. Phillips for technical assistance and Dr. Chrisoula Toupadakis Skouritakis for the illustrations.

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Supplementary Materials

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