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Serum vitamin D concentrations in rabbits (*Oryctolagus cuniculus*) are more affected by UVB irradiation of food than irradiation of animals

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ABSTRACT

Rabbits kept under ultraviolet B (UVB)-irradiation respond with increasing serum vitamin D (25(OH)D) concentrations, but it is unknown whether irradiation of the animals or their feed contributes more. Twenty-four New Zealand White rabbits were divided into three groups for a four-week period: the control group (C) received no UVB-exposure and non-irradiated hay (ergocalciferol (vitamin D_2) concentration 2.22 μg/100 g dry matter). The direct exposure group (D) was provided with 12 h of UVB-irradiation daily and fed the same hay as group C in shaded areas to prevent UVB-irradiation thereof. The indirect exposure group (I) did not receive direct UVB-irradiation but was fed hay of the same batch that was exposed to 12 h of UVB-irradiation (vitamin D_2 6.06 μg/100 g dry matter). Serum 25(OH)D_2, 25(OH)D_3, ionised calcium, total calcium, phosphorus, and magnesium concentrations were measured weekly. There was no systematic effect on serum mineral concentrations. The serum 25(OH)D_2 concentrations were significantly higher in group I compared to groups C and D from the second week onwards. 25(OH)D_3 concentrations increased only in group D, with significant differences to both other groups from the third week onwards, yet at lower magnitudes than the noted increase of 25(OH)D_2 in group I. Total 25(OH)D concentrations were highest in group I, intermediate in group D and lowest in group C. Serum total 25(OH)D concentration was more affected by UVB-irradiation of rabbits' feed than by direct irradiation of the animals themselves. If rabbit serum total 25(OH)D concentrations should be managed, diet manipulation rather than animal UVB-exposure appears to be more effective.

Introduction

Vitamin D synthesis in the skin after ultraviolet B (UVB)-irradiation has been widely studied in humans and animals (Watson and Mitchell, 2014, Karppinen et al., 2017, Hymøller and Jensen, 2010, Nemeth et al., 2017). In this endogenous vitamin synthesis UVB-radiation (wavelength 290–315 nm) penetrates the skin and converts 7-dehydrocholesterol (provitamin D_3) to previtamin D_3, which isomerizes to vitamin D_3 (cholecalciferol) (Holick et al., 1981). An overdose via this route is however not possible as both provitamin D_3 and vitamin D_3 are photosensitive (Holick et al., 1981; Lehmann and Meurer, 2010).

Nutrition is another source for vitamin D. Vitamin D_3 is found in many animal products, which serve as an important dietary source of vitamin D for omni- and carnivorous species (How et al., 1994; Schmid & Walter, 2013). Endophytic fungi contaminating plant material contain ergosterol in their cell membranes, which is transformed to ergocalciferol (vitamin D_2) after UVB-exposure. This comprises the main nutritional source of vitamin D for herbivores (Jäpelt & Jacobsen, 2013). Both vitamin D_2 and D_3 are transported to the liver, where they are converted to 25-hydroxyvitamin D_2 (25(OH)D_2) and 25-hydroxyvitamin D_3 (25(OH)D_3), respectively. These together form total 25-hydroxyvitamin D (25(OH)D), which is considered the best indicator of vitamin D status (Holick, 2002).

Few studies report endogenous vitamin D synthesis in rabbits. Mellanby and Killick (1926) observed that young rabbits did not develop rickets after being exposed three times weekly to mercury vapour lamps for 10 weeks. Several authors observed an increase in rabbits' vitamin D concentrations after exposure to artificial UVB-irradiation compared to

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baseline values (Molitor et al., 2023) and control group rabbits (Emerson et al., 2014; Watson and Mitchell, 2014).

Mellanby and Killick (1926) were the first to observe that irradiation of feed (but not the animal) prevented rickets in a young rabbit. Vitamin D_2 produced in hay after UVB-exposure might serve as an important source of vitamin D, but the effect of vitamin D_2 from UVB-irradiated hay on rabbits' serum 25(OH)D_2 and total 25(OH)D concentration has not been assessed so far. This is, however, relevant as a Finnish study indicated the diet as a more relevant source of vitamin D for pet rabbits than daily outdoor access during summertime (Mäkitaipale et al., 2019). Therefore, we aimed to study how rabbits' serum vitamin D concentrations respond to separate UVB-irradiation of either the animals or their feed.

Material and methods

Study design

This four-week experiment was approved by the Cantonal Veterinary Office Zurich, Switzerland (No. 35593; ZH041/2023). We compared the effects of direct (irradiation of the animal) and indirect (irradiation of hay) UVB-exposure on rabbits' serum 25(OH)D_2, 25(OH)D_3 and total 25 (OH)D concentrations. Animals were randomly assigned to three treatment groups; control group (C) received neither a UVB-light source nor UVB-irradiated hay. The direct exposure group (D) was provided with 12 hours of UVB-irradiation but was fed in shaded areas to prevent an effect on the hay. The indirect exposure group (I) did not receive a UVB-light source but was fed hay that had been UVB-irradiated for 12 h.

Animals, husbandry and feeding

Twenty-four intact juvenile female rabbits aged five weeks were purchased from a specific pathogen free breeding facility (Charles River Laboratories, Châtillon, France). Until the beginning of the study at the age of 27 weeks, the animals were habituated to their experimental environment and handling procedures.

The rabbits were kept in one room with three enclosures. Each enclosure was equipped with five hay racks, 12 nipple drinkers for *ad libitum* water access, a salt lick, wooden shelters whose roofs served as elevated platforms, two toys, varying gnawing opportunities, and a bedding of wood shavings. A window provided natural light; artificial lighting of the room was set to a constant 12 h cycle.

Until the age of 15 weeks, rabbits received hay for *ad libitum* consumption complemented with a pelleted diet (3525 Ranger Kaninchen Spezial, Granovit, Kaiseraugst, Switzerland; vitamin D_3 added at 8 µg/100 g dry matter per manufacturer's information). From 16 weeks of age onwards, the pelleted diet was discontinued, and different hay types were fed *ad libitum*, with the sole prevision of the experimental hay by 25 weeks of age. The latter comprised a balanced second cut from an artificial pasture, with an estimated 30 % consisting of legumes (predominantly *Trifolium repens*). The experimental hay had been artificially dried indoors and stored with no direct sunlight exposure, and contained crude protein, neutral detergent fibre, calcium and phosphorus at 17.3, 47.3, 1.09 and 0.39 % of dry matter.

Animals (aged 27 weeks at the initiation of the study) were weighed on the first day of each experimental week. The hay provided to each group, and the corresponding leftovers on the next morning were weighed daily.

UVB-irradiation

For both hay and animal irradiation, Arcadia© Pro T5 UVB kits with D3+ Dessert 24 Watt, 12 % UVB lamps (Arcadia Reptile, West-Sussex, UK) were used. To achieve a similar maximal intensity of UVB-exposure to the hay (spread on the ground) and to the rabbits at dorsum level, lamps were fitted at a height of 77.5 cm and 88.0 cm, respectively. Hay was irradiated for twelve hours in batches of 1.5–2 kg, spread over a surface of 310 cm×60 cm. UVB-exposure was provided with four lamps, rendering an irradiation intensity between 21 – 73 µW/cm^2 as measured weekly using a Solarmeter© 6.2 (Solar Light Company, Glenside, Pennsylvania, USA).

The enclosure of group D was fitted with four UVB lamps so that the irradiation ranged between 7 – 69 µW/cm^2 measured weekly at 11.5 cm from floor level. In this enclosure, the hay racks were placed under wooden tables. A beam on the tables' rim prevented entry of oblique rays to ensure that UVB light did not reach the roughage. Another beam on the floor prevented hay being pulled out of the hayracks to be spread into the irradiated area of the enclosure. During the first six days of the experiment, UVB-exposure was gradually increased to twelve hours per day.

To estimate the extent to which rabbits of group D were under the UVB lights (and not in shaded areas), camera surveillance with video recording of a single 12-hour exposure period was evaluated. Every five minutes, a 15 second period was watched, and individual rabbits visible under the UVB lamps were reported. For this recording, the animals were marked on their backs to facilitate recognition. An estimation of the complete exposure time during the 4 week period was beyond our logistical means.

Sampling

At the initiation of the study and thereafter at weekly intervals, blood samples were taken from all rabbits from either the *Vena auricularis* or the *V. saphena* with a 25 or 22 G needle. Samples were partly collected in a lithium heparin (0.3–0.5 mL) and partly (3.5–3.7 mL) in a plastic tube with a clot activator (Sarstedt AG & Co. KG, Nümbrecht, Germany). Each sample was protected from direct sunlight. Blood in the plastic tubes with a clot activator was centrifuged at 3461 relative centrifugal force for 15 minutes (Hettich EBA 20, Beverly, MA, USA). Serum was collected and stored at -20 °C.

Prior to feeding, representative samples of all batches of hay were taken. During the entirety of the experiment, samples of the radiated and non-radiated hay were collected, homogenised by grinding through a 1 mm screen (Retsch GmbH, Haas, Germany) and submitted as two pooled samples for vitamin D analysis.

Analyses

The ionized calcium (iCa) concentration was immediately analysed from lithium heparin sample on site with a commercial chemistry analyser (i-STAT® 1, i-STAT Corp, Windsor, NJ, USA). Serum samples were submitted to the Clinical Laboratory of the Vetsuisse Faculty of Zurich for determination of calcium (Ca), magnesium (Mg) and phosphorus (P) concentrations on an automated chemistry analyzer (Cobas C 501, Roche Diagnostics, Rotkreuz, Switzerland) using colorimetric assays (Roche Diagnostics, Rotkreuz Switzerland). Serum 25(OH)D_2 and 25 (OH)D_3 concentrations were determined with LC-MS/MS using the MassChrom® 25-OH-Vitamin D_3/D_2 in serum (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany) assay kit on SCIEX 4500MD mass spectrometer at University Hospital Zurich under ISO/IEC 17025:2017 accreditation. The assay was fully validated and showed an inter-assay variability for 25(OH)D_2 of 4.3 % (17.6 µg/L) and 5.5 % (41.4 µg/L) and for 25(OH)D_3 of 5.1 % (16.3 µg/L) and 5.4 % (38.0 µg/L). The intra-assay variability was 2.4 % (17.6 µg/L) and 4.9 % (41.4 µg/L) for 25(OH)D_2 and 2.6 % (16.3 µg/L) and 3.0 % (38.0 µg/L) for 25(OH)D_3. The method had a LLOQ of 1 µg/L for 25(OH)D_2 and 25 (OH)D_3, respectively. Total 25(OH)D was calculated as the sum of 25 (OH)D_2 and 25(OH)D_3.

The vitamin D_2 and D_3 content of the pooled hay samples were determined at an accredited laboratory (Eurofins Scientific Ag, Schönenwerd, Switzerland) by high performance liquid chromatography (EN 12821–2009).

Statistics

Data are displayed as means ± standard deviation. Statistical testing was performed in R (R Core Team, 2020). The initial and final weight, the change in body mass, and the relative food intake ($g.kg^{-0.75}.d^{-1}$) were compared between groups using an ANOVA (confirming normal distribution of residuals by Shapiro-Wilk-test) and Tukey's post hoc test.

We assessed the serum results using mixed effects linear models (with animal as random factor to account for repeated measurements) with the 'lmerTest' package (Kuznetsova et al., 2017), using treatment group as a fixed factor and the sampling week as an additional fixed factor, including its interaction with treatment group. Comparisons between the group values of individual weeks were performed on least square means using the 'emmeans' package. Model residuals were assessed for normal distribution using the Shapiro-Wilk test; if residuals were not normally distributed, the model was re-run using, in this sequence, log-transformation, square-root transformation, or ranked data. Correlations between serum measures were assessed by Spearman's rho. The significance level was set to 0.05.

Results

All animals remained clinically healthy during the study. The UVB-irradiation had no adverse effect.

There were no differences in the initial body mass between groups (group C: 3.45 ±0.29 kg, group D: 3.33 ±0.05 kg, group I: 3.47 ±0.20 kg; ANOVA $p = 0.336$). The relative hay intake differed significantly between the groups (ANOVA $p < 0.001$); post hoc tests indicated no difference between group C (117 ±4 $g/kg^{0.75}/d$) and group I (116 ±3 $g/kg^{0.75}/d$; $p = 0.607$), but a significantly lower intake in group D (108 ±6 $g/kg^{0.75}/d$; $p < 0.001$ in both comparisons) was noted. This, however, had no effect on the final body mass that did not differ between the groups (group C: 3.57 ±0.29 kg, group D: 3.46 ±0.13 kg, group I: 3.66 ±0.23 kg; ANOVA $p = 0.224$). Similarly, the body mass change during the experiment did not differ significantly (group C: 0.12 ±0.07 kg, group D: 0.13 ±0.07 kg, group I: 0.18 ±0.08 kg; ANOVA $p = 0.292$).

The vitamin D_2 concentrations of the non-irradiated (group D and C) and the 12 hour irradiated hay (group I) were 2.22 and 6.06 µg/100 g dry matter, respectively. The vitamin D_3 concentration did not exceed the assays' detection limit (LOQ, < 0.25 µg/100 g), in either non-irradiated and 12 h irradiated hay. Calculated vitamin D_2 intakes were 2.4, 2.2 and 6.5 $µg/kg^{0.75}$/day for C, D and I, respectively.

At the beginning of the study, there were no differences between the groups in serum 25(OH)D_2, 25(OH)D_3 and 25(OH)D concentration (Fig. 1). The serum 25(OH)D_2 concentrations of group I increased significantly over time, reaching a steady state within two weeks (Fig. 1A). Concentrations were higher compared to group D and C from two weeks onwards. One animal in group I had distinctively lower serum 25(OH)D_2 concentrations than the other animals of this group, yet presented the same pattern of immediate increase after the first week (Fig. 1A). This animal is included in the statistical evaluations reported in Fig. 1; excluding this animal would only have made differences between the groups more prominent.

25(OH)D_3 concentrations were significantly higher in group D compared to the groups' baseline after one week, compared to group I after 2 weeks, and compared to group C after 3 weeks (Fig. 1B). The total 25(OH)D concentrations were numerically higher in group I, lowest in group C, and intermediate in group D (Fig. 1C). Again, the significant increase compared to baseline occurred after the first week in group I, and at the end of the experiment in group D. There was no significant change over time in group C. There was no significant correlation between serum 25(OH)D_2 and 25(OH)D_3 (Table S1, Fig. S1).

Serum iCa as well as Ca concentrations were lower, and serum P was higher in group I rabbits at day 0 than in the subsequent weeks; in the case of P, the difference was also significant compared to the initial values of the other groups (Fig. S2). There were no other relevant differences in Ca, iCa, P and Mg concentrations between groups. Serum 25(OH)D concentrations did not correlate with any serum mineral concentrations (Table S1, Fig. S3).

The rabbit that spent most time under the UVB-lights during the 12-hour observation period also had the highest 25(OH)D_3 concentrations. For the other animals, the time spend under UVB-lights was not associated with the 25(OH)D_3 concentrations (Fig. S4).

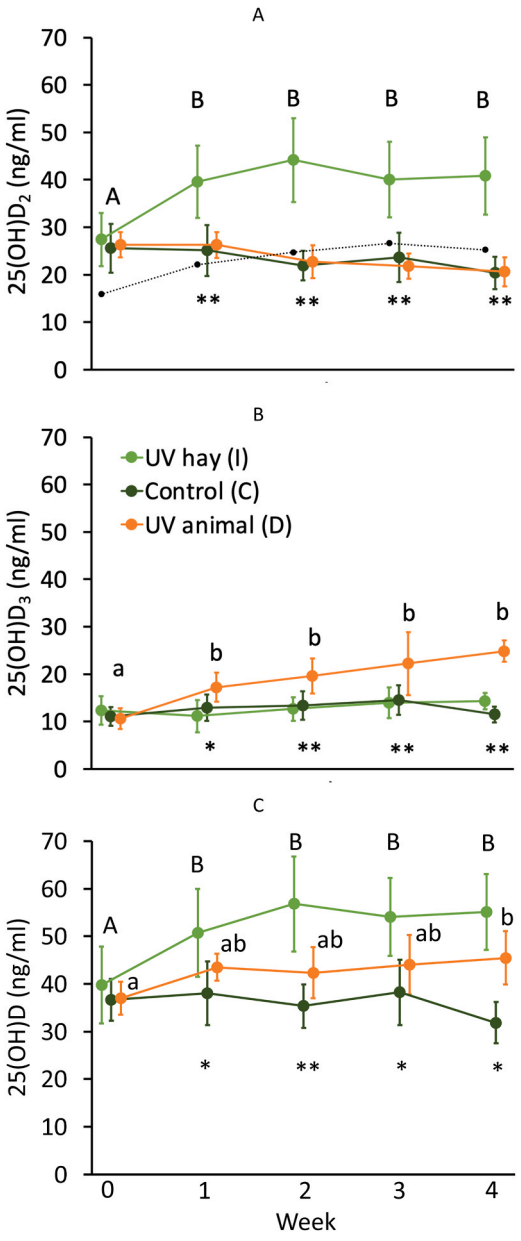

Fig. 1. Development of serum concentrations in rabbits (n=8 per group) either exposed to direct ultraviolet B (UVB) light but no exposure of the diet (group D), on a diet of UVB-irradiated hay (group I), and a control group with no direct UVB light and a diet of untreated hay (group C); (A) 25-hydroxyvitamin D_2 (25(OH)D_2), (B) 25(OH)D_3, (C) total 25(OH)D concentrations. Different letters within groups indicate significant differences over time (only occurring in group I [capital letters] and D [lower case letters]). Stars indicate a significant difference between one (*) or both (**) groups of the lower values with the group of the highest value. In (A), one outlier animal of group I is indicated separately whose 25(OH)D_2 concentrations followed the pattern of the group, but at a distinctively lower level. Statistics include this animal.

Discussion

Only in rabbits fed with irradiated hay (group I) did the serum 25(OH)D_2 concentrations increase above the baseline level. The same was true for the serum 25(OH)D_3 values of the animal-irradiated group (group D). The total 25(OH)D concentrations were significantly higher in group I rabbits compared to both other groups, indicating that feeding of UVB-irradiated hay had a stronger effect on serum vitamin D than direct irradiation of the animals did. These results are similar to findings in horses where seasonal sunlight affected the animals' serum 25(OH)D_2 via impact on the pasture rather than by elevating endogenous 25(OH)D_3 production (Azarpeykan et al. 2016).

In rabbits of group D, access to UVB-exposure for 12 hours per day increased the 25(OH)D_3 concentration from 10.6 ±2.2 ng/mL to 19.6 ±3.7 ng/mL after two weeks and to 24.9 ±ng/mL after four weeks. This increase is, however, clearly below average concentrations reported in the literature after two or three weeks with a daily exposure of 6 (32.0 ±3.6 ng/mL) (Molitor et al. 2023), and 12 h (32.8 ±8.7 ng/mL) (Watson et al. 2019) respectively. Several reasons for the noted differences are possible. Firstly, in contrast to the cited experiments, the rabbits in the present study also had access to shaded areas, so that the exposure to UVB was to some degree voluntary. Swiss animal protection legislation requires that rabbits are able to withdraw to covered areas at will. Therefore, without a precise quantification of the UVB-exposure time which was not possible in this study, caution is warranted when comparing data. The fact that the most UVB-exposed animal on the day of exposure estimation also had the highest serum 25(OH)D_3 concentrations underscores the relevance of actual exposure time.

Secondly, though both (Watson et al., 2019) and (Molitor et al., 2023) stated specifically that 25(OH)D_3 had been measured, no data on 25(OH)D_2 was provided in parallel. It is important to consider that both studies cited Emerson et al. (2014) for the methodology, who only measured total 25(OH)D concentrations. It is therefore unclear if data of these studies specifically represent 25(OH)D_3 or rather total 25(OH)D (i.e., vitamin D_2 and D_3 combined), and are higher than our 25(OH)D_3 concentrations for this reason. This is especially relevant since the hay offered in the cited studies (Emerson et al.; 2014, Molitor et al.; 2023, Watson et al. 2019) was likely exposed to the UVB-irradiation as well. Given the difference between 25(OH)D_2, 25(OH)D_3 and total 25(OH)D concentrations demonstrated in the present study, the contribution of 25(OH)D_2 from hay to total 25(OH)D concentrations in rabbits must not be underestimated.

In humans, supplemented 25(OH)D_2 is less effective than 25(OH)D_3 to increase the total 25(OH)D concentration (Armas et al., 2004; van den Heuvel et al., 2024). Nonetheless, after 25(OH)D_2 supplementation in humans, the increase of its serum concentration is accompanied by a concurrent decrease of serum 25(OH)D_3 concentration, so that the total 25(OH)D concentration remains at the baseline level (Stephensen et al.; 2012). These mechanisms were evidently not in place in the rabbits of the present study, where serum total 25(OH)D increased due to both, increased dietary 25(OH)D_2 and UVB-induced endogenous 25(OH)D_3 production.

The highest serum concentrations measured in the present study were 55 ng/mL for 25(OH)D_2 and 69 ng/mL for total 25(OH)D. The previously reported highest total 25(OH)D concentration in a domestic rabbit without vitamin D supplementation or regular UVB-exposure was 68 ng/mL (Mäkitaipale et al.; 2019). Chan et al. (1979) studied the morphological and biochemical effects of vitamin D_2 in pregnant rabbits. Over 28 days, pregnant does were injected with no, 350 μg, 3500 μg or 35 000 μg of vitamin D_2 in total. Two does of the 35 000 μg group had serum 25(OH)D concentrations above 100 ng/mL and developed aortic calcifications in addition to increased morbidity in the offspring. Serum 25(OH)D concentration above 100 ng/mL could therefore be considered toxic in rabbits. However, supravalvular lesions were already observed in some offspring from does supplemented with 3500 μg D_2, despite serum 25(OH)D concentrations below 60 ng/mL (Chan et al. 1979). Therefore, serum 25(OH)D concentrations from 60 ng/mL onwards could have unpredictable consequences, especially if maintained for longer periods.

Very low total 25(OH)D concentrations (mean 3.3 ng/mL) were reported in Finnish wild rabbits (Mäkitaipale et al., 2024), which raises the question how much vitamin D rabbits require, and whether the main function of vitamin D in rabbits is related to something else than calcium metabolism (Kamphues et al.; 1986, Uhl, 2018). Correspondingly, serum mineral concentrations were not correlated to serum vitamin D in the present study. This is also suggested by the absence of differences in serum iCa, Ca, P and Mg concentrations in the present study. If rabbits need less vitamin D for calcium metabolism, one should consider whether intoxication rather than deficiency comprises the most prominent risk in domestic rabbits.

Despite the lack of clear clinical evidence of benefits, some discussion has recently arisen regarding the use of artificial UVB-irradiation for vitamin D synthesis in pet rabbits (Molitor et al.; 2023, Watson and Mitchell; 2014). From the present study, it seems that UVB-irradiation of roughage provides a more efficient source of vitamin D for rabbits than irradiation of the animal itself. Rabbits receiving sufficient dietary vitamin D (through sun-cured hay, or a vitaminized compound feed) might not require augmentation by providing direct UVB-irradiation to render clinically sufficient serum vitamin D concentrations. To which degree rabbits, which naturally spend the day in underground burrows and forage at night, actually benefit from increased vitamin D concentrations, however, remains to be determined.

Conclusions

Vitamin D concentrations (total 25(OH)D and 25(OH)D_2) were significantly higher in rabbits fed with UVB-irradiated hay compared to rabbits fed non-irradiated hay with or without access to UVB light for the animals. It seems that dietary vitamin D_2 increases total 25(OH)D concentration more effectively compared to endogenous vitamin D_3 synthesis when rabbits are provided with a 12-hour cycle of UVB light in a practical setting.

CRediT authorship contribution statement

Johanna Mäkitaipale:Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Marcus Clauss**:Writing – original draft, Visualization, Project administration, Formal analysis, Data curation, Conceptualization. **Jean-Michel Hatt**:Writing – review & editing, Funding acquisition, Conceptualization. **Han Opsomer**:Writing – original draft, Project administration, Investigation. **Regula Steiner**:Writing – review & editing, Methodology. **Barbara Riond:**Writing – review & editing, Methodology. **Annette Liesegang**:Writing – review & editing, Methodology, Conceptualization.

Declaration of Competing Interest

The other authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2024.106149.

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