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Tail Tendon Break Time for the Assessment of Aging and Longitudinal Healthspan in Mice

Anne S. Haefke and Collin Y. Ewald*

Department of Health Sciences and Technology, Institute of Translational Medicine, Eidgenössische Technische Hochschule (ETH) Zürich, Schwerzenbach, Switzerland
*For correspondence: collin-ewald@ethz.ch

[Abstract] The successful evaluation of longevity-promoting interventions requires the identification of reliable biomarkers of healthspan and lifespan. Non-enzymatic glycation end-products accumulate on collagens during the aging process, leading to the crosslinking and stiffening of collagen fibers. In murine species, the glycation of collagen during aging can be indirectly quantified by the Tail Tendon Break Time (TTBT) assay. Importantly, longitudinal linear increases in TTBTs across age are decreased by multiple established longevity-promoting interventions. Here, we discuss experimental considerations for the TTBT assay and provide an adapted version of the original TTBT protocol from Harrison and Archer 1978. We propose that TTBT remains a useful and minimally-invasive readout of longevity, especially when considered in combination with other longitudinal healthspan assaying protocols.
Keywords: AGE, Healthspan, Longevity, Biomarker, Collagen, Cross-links, Glycation, Tendon

[Background] The field of gerontology research and the identification of geroprotectors in the form of pharmacological interventions require reliable methods for quantifying an animal's biological age and assessing their health status during aging (Sprott, 2010; Kennedy *et al.*, 2014; Richardson *et al.*, 2016; de Magalhaes *et al.*, 2017; Bellantuono, 2018; Bellantuono *et al.*, 2020). The availability of a biological marker, defined as a characteristic that can be measured objectively and that can differentiate between the normal biological process of aging and altered aging processes due to interventions would be of major benefit to geroscience (Biomarkers Definitions Working Group, 2001; Thompson and Voss, 2009). To date, commonly used biomarkers have often required comparing the lifespan and healthspan of the animals. Unfortunately, however, studies that directly require measurements of the lifespan of a mouse model require two to four years to complete. Therefore, the identification of a biomarker capable of reflecting the differences in biological aging processes at earlier stages remains critical (Levine *et al.*, 2018). Here, we discuss the measurement of Tail Tendon Break Time (TTBT) as an indirect readout for age-related collagen crosslinking. We find that while TTBT might not fulfill all criteria as a bona fide biomarker of aging, TTBT remains a valuable indicator of the murine aging process during longitudinal healthspan assessments.

Collagens are structural proteins produced and secreted by cells to form scaffolds in the extracellular matrix. Collagens can be long-lived structural proteins, especially in acellular tissues; for example, collagens have been found to have a half-life time of more than one hundred years in human eye lenses (Birch, 2018; Ewald, 2019). The general decline of protein turnover during aging leads to the accumulation of non-enzymatic glycation on proteins, including collagens, due to a phenomenon called

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the Maillard reaction (Avery and Bailey, 2006). In this process, non-enzymatic glycation results in an increase in non-enzymatic crosslinking of collagen (Avery and Bailey, 2005; Snedeker and Gautieri, 2014) and the addition of side-chain modifications. This process is accelerated under increased temperatures and under conditions in which there is higher availability of glucose. The adducts and crosslinking of Advanced Glycation End products (AGEs) on collagens during aging cause significant changes in physical properties of collagen fibers, such as increased tissue bending stiffness, decreased tissue viscoelasticity, and reduced susceptibility towards denaturing agents (Avery and Bailey, 2006; Snedeker and Gautieri, 2014; Gautieri *et al.*, 2017). The increased resistance of collagen fibers to denaturing agents can be directly linked to biological aging (Paul and Bailey, 1996). Thus, the TTBT assay, which measures the resistance of a single tail tendon fascicle to elongation under the creep load of a single tail tendon fascicle during the denaturation of the fiber by concentrated urea, provides an indirect measure of the amount of collagen crosslinking (Harrison and Archer, 1978). The measurement of TTBT can be performed at least four times in an animal's lifetime by using each of the animal's four individual tendon bundles once. Tendon fascicles can be harvested from living animals using local anesthesia, which offers the possibility for longitudinal sampling (Harrison and Archer, 1978). Furthermore, a mouse's tail can be stored at -20 °C without influencing the mechanical properties of its collagen fibers (Goh *et al.*, 2008).

For almost seventy years, TTBT assays have been employed to assess tendon stiffness during the aging process (Verzar, 1955). Numerous studies have reported that older animals exhibit increased TTBTs when compared to younger animals of the same sex and strain (Olsen and Everitt, 1965; Harrison and Archer, 1978; Sell and Monnier, 1997). A comparison between the longer-lived mouse species *Peromyscus leucopus* with the shorter-lived species *Mus musculus*, for example, reveals a more rapid increase in TTBT in the shorter-lived species across age (Harrison *et al.*, 1978). Furthermore, short-lived mouse strains exhibit longer TTBTs than long-lived strains at the same chronological age (Harrison *et al.*, 1984; Higgins *et al.*, 1991; Heller and McClearn, 1992; Sloane *et al.*, 2011a and 2011b). Thus, a positive relationship between age and tail tendon breakage time has been found consistently across studies in rodents.

If non-enzymatic AGE-crosslinking of aging collagens is an age-related pathology, then interventions that slow aging should reverse or prevent collagen crosslinking. In keeping with that hypothesis, by 1965, it had been found that hypophysectomized rats had both delayed signs of aging and a shortened TTBT comparable to about half of the chronological age of the control group (Olsen and Everitt, 1965). Moreover, interventions like caloric restriction, which are known to increase an animal's lifespan, resulting in a shortened TTBT when compared to *ad libitum* fed animals from the same strain (Harrison *et al.*, 1984; Sell and Monnier, 1997). In contrast, short-lived obese mice exhibit an increased TTBT compared to nonobese controls (Harrison *et al.*, 1984). Similarly, a more youthful or decreased TTBT is found in long-lived Snell dwarf mice when compared to their shorter-lived sibling controls (Flurkey *et al.*, 2001). TTBT assays have also been used to demonstrate the effect of lifespan-altering interventions involving hypothalamic programming, in which shorter-lived MBH-IKK-β mice exhibited greater TTBTs than both the control group and the longer-lived MBH-IκB-α mice (Zhang *et al.*, 2013). Taken together,

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interventions that slow aging and increase lifespan slow collagen aging, as indicated by a more youthful TTBT.

Given that TTBT increases with age and this increase is slowed by longevity interventions, TTBT values have been considered as a biomarker of aging. The TTBT meets eight of the nine criteria for a biomarker of aging, including being minimally invasive, insensitive to anesthesia, and highly reproducible. TTBTs increase substantially with an animal's age, and the age-related changes in TTBT can be slowed or reversed by longevity interventions.

Some caveats to the use of TTBT as a biomarker of aging remain, however. For example, TTBT values show minimal predictive power for predicting the length of the animals' lifespan. Using 23 recombinant inbred strains (C57BL/6J and DBA/2J, *i.e.*, BxD RIs), Sloane and colleagues have found that neither the rate of change in TTBT nor absolute TTBT values correlate significantly with the rate of increase in lifespan of BxD RIs (Sloane *et al.*, 2011a). As no robust univariate statistical relationship between TTBT and lifespan was found, TTBT might not be suitable for direct correlations between chronological age and TTBT values (Sloane *et al.*, 2011a). Moreover, many of the interventions that lower TTBT also lower glucose levels and temperature (Sloane *et al.*, 2011a), which may directly lower the accumulation of AGEs and crosslinking of collagens, and therefore lead to a lower TTBT independent from their effects on aging. Finally, it remains unclear whether AGEs on collagens are actively prevented under conditions in which lifespan is prevented, or whether they are actively removed by these interventions. Nevertheless, we argue that the sensitivity of TTBT to aging as well as to longevity interventions makes it an important factor in the assessment of qualitative, age-related changes, even though TTBT might not be suitable as a bona fide biomarker of aging (Sloane *et al.*, 2011a).

In this work, we review the knowledge gained by TTBT assays across the past 70 years. We discuss the advantages and potential short-comings of TTBT and its suitability as an assay to the aging process. We find that while TTBT is an established assay for the study of age-related changes to collagens and extracellular matrix, TTBT should be used in combination with additional assays, such as collagen deposition during aging (Teuscher *et al.*, 2019). TTBT should be seen as a qualitative assessment of age-related changes. We therefore advise use the TTBT in combination with other longitudinal healthspan assays, as described by Bellantuono and colleagues (Bellantuono *et al.*, 2020) when assessing healthspans.

General technical considerations for TTBT

For a general design of a study for the longitudinal assessment of health and lifespan that compares a longevity-promoting intervention (*i.e.*, geroprotector) to a control, please see Bellantuono and colleagues (Bellantuono *et al.*, 2020). When crafting a study's design for longitudinal sampling, keep in mind the requirement of anesthesia and the limited number of times TTBT can be performed. The time brackets to measure significant changes in TTBT show different ranges in different mouse species (Harrison *et al.*, 1984; Higgins *et al.*, 1991; Heller and McClearn, 1992; Sloane *et al.*, 2011a and 2011b). In general, for C57BL6 mice of the ages of 2 to 5 and 5 to 10 months, the TTBT is about double the time

for each of these mouse age brackets (Higgins *et al.*, 1991). From 6 to 17 and 17 to 27 months, the TTBT is about double or triple in time, respectively (Sloane *et al.*, 2011b). For other mouse strains, such as DW/J or DBA, the TTBT measured at 6 and ~18 months of age can increase by about 6 or 18-fold, respectively (Flurkey *et al.*, 2001; Sloane *et al.*, 2011b). Furthermore, fascicle sampling might impact other healthspan assays, such as rotarod performance or the Howlett/Rockwood frailty assay, for which the tail helps with balancing or assess tail curling, respectively. Caution in the preparation and execution of measuring TTBT is advised due to the following critical issues:

1. The non-enzymatic glycation reaction depends on the amount of circulating sugar and the temperature of the tissue (Harrison and Archer, 1978). Thus, researchers need to consider whether their intervention of interest will affect blood glucose levels. Furthermore, the body (rectal) temperature of mice is about 6 °C warmer than at the base of the tail and 9 °C warmer than the middle part of the tail. Due to this temperature difference, it is recommended to use fascicles from the middle part of the tail (Harrison and Archer, 1978). In addition, even subtle microenvironmental temperature differences create significant variations, depending on whether dorsal or ventral fascicles were used (Higgins *et al.*, 1991). To minimize individual variation, housing temperature and the location of fascicles harvested from the tail should be kept constant. The difference in age-related increases in TTBT is usually far greater than these individual TTBT differences, however.
2. TTBT depends on the rate of penetration of the urea solution into the fascicle, which is proportional to the surface area per volume ratio (Harrison and Archer, 1978). Thus, a split fascicle will break faster, but a double fascicle will break similar to a single fascicle (Harrison and Archer, 1978).
3. TTBT depends on the urea solution temperature. The lower the temperature of the urea solution, the longer the TTBT. Around 45 °C is recommended for the solution (Harrison and Archer, 1978).
4. TTBT is not altered by anesthesia, fascicle length, dryness, or constant urea concentration (Harrison and Archer, 1978). The latter point is important since water will evaporate from the urea solution, and water needs to be re-added during longer measurements.

Materials and Reagents

1. 1,000 ml glass beaker (Duran, catalog number: 21 106 54)
2. Petri dish, 90 x 14 mm (Roth, catalog number: N221.2)
3. Pipette tips, 10-100 µl (Greiner Bio-One, model number: GB685290)
4. Animals:
   a. 4-months old male C57BL/6 mice
   b. 30-months old male C57BL/6 mice
5. Deionized water (Sigma-Aldrich, catalog number: 38796-1L)
6. Potassium phosphate monobasic, KH_2PO_4 (Sigma-Aldrich, catalog number: P9791-1KG)
7. Sodium tetraborate, $Na_2B_4O_7$ (Sigma-Aldrich, catalog number: 221732-500G)
8. Surgical Suture (Ethicon, catalog number: V3030H)
9. Urea (Sigma-Aldrich, catalog number: 15604-1KG)

10. 1 M HCl (Sigma-Aldrich, product number: H 9892)
11. 7 M urea solution (pH 7.5) (see Recipes)

Note: All reagents can be stored at room temperature (23 °C).

Equipment

1. 2 g weight (Roth, catalog number: N152.1)
2. -20 °C freezer
3. Dissecting stereomicroscope (Tritech Research, model: SMT1)
4. pH meter (Mettler Toledo, catalog number: 30019028) with pH electrode (Mettler Toledo, catalog number: 51343105)
5. Pipette, 10 µl (Eppendorf, model: EP3121000023)
6. Permanent marker (edding, model: 404)
7. Scissors (Hilbro Stainless, catalog number: 10-402)
8. 60 cm height Retort iron stand with clamps
9. Surgical Tweezers/Forceps (Hilbro International, catalog number: 12.0012.10)
10. Thermometer (ThermoWorks, model: USB-REF)
11. Tripod with a height of 27cm (Rollei, catalog number: 20797)
12. Video camera (Sony, catalog number: HDR-CX240E) with a suitable memory device
13. Water bath, Interior: 52 cm length x 28 cm width x 14 cm depth (Salvis, catalog number: 333198)

Software

1. Prism 8 (GraphPad)

Procedure

A. Setup preparation (needs to be done once, can be reused after the first run)
   1. Prepare a retort iron stand with clamps to hang the sutures and place it above the water bath. The use of clamps is necessary as the height of the setup should be adjustable to allow easy alterations of the tendon's immersion depth (Figure 1).

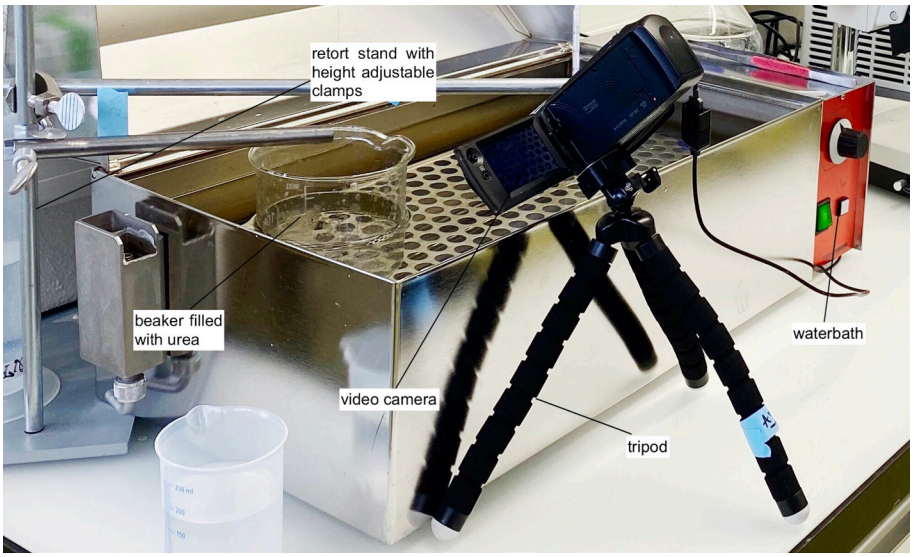


Figure 1. Final experimental setup. The picture depicts the retort iron stand with clamps to hang the tendons above the water bath, submersed in a beaker of urea solution. The camera is used to determine the rupture time of the tendon.

2. Attach a surgical suture to a 2 g weight using regular knots.
3. Set the temperature of the water bath to 45 °C ± 0.1 °C and use a thermometer to verify the temperature. The temperature should be monitored carefully, as an increase in temperature will shorten the TTBT and lead to imprecise measurements.
4. Place a beaker containing a 7 M urea solution with a pH of 7.5 (see the instructions for the preparation of urea solution in the Recipes section) inside the water bath. Make sure that the water level of the water bath is at least 1 cm above the urea level, to prevent a temperature gradient in the urea solution. You might have to adjust these levels to allow a steady stand for the beaker. It is important to exchange the urea solution and to adjust its pH to 7.5 by using 1 M HCl before measuring the next tendon fascicle.
5. Prepare a setup to determine the rupture time of the tendon. Position a camera on a tripod to clearly see the weights hanging in the urea and to record the whole experiment. The tendon breaking time is defined as the time between suspension of the fascicle in the urea bath to the time the weight drops to the bottom of the beaker.

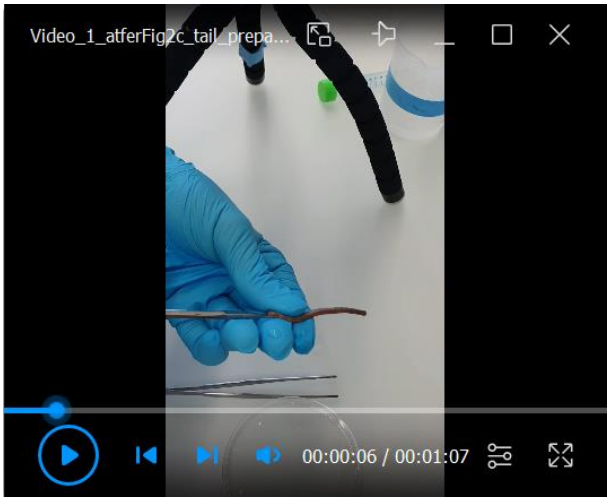
B. Isolation of a single tail tendon fascicle

Fascicles can be harvested directly from anesthetized living animals (Harrison and Archer, 1978). Alternatively, tails can be cut off close to the body from euthanized mice and stored in a 15 ml Falcon tube at -20 °C. It is possible to store the cut-off tails in vacuum-sealed plastic bags. Particular attention should be paid to mark the ventral or dorsal side of the cut-off tails. For some mice, the fur appears lighter or darker for the ventral or dorsal part of the tail. This protocol describes the procedure for mouse tails stored at -20 °C.

1. Take the mouse tail out of the -20 °C freezer. Place the tail into an empty Petri dish for thawing.

After approximately one minute, the tail should be flexible and fully thawed. It is possible to identify the ventral side of the tail by squeezing the cut-off tail ending and most of the time a blood drop comes out from the ventral artery (Figure 2A).

2. Use surgical scissors to cut the skin (*ca.* 1 cm incision) longitudinal at the proximal end of the tail (Figure 2B). Use surgical tweezers or forceps to grab the edge of the cut of skin and carefully peel off the skin along the tail (Figure 2C, Video 1). Once the skin is removed, four tendon bundles are visible. Each bundle of fascicles can be used to isolate tendon fascicles, which allows for multiple measurements from the same animal. To help keep track when re-measuring the same tail, make a mark with a permanent marker between tendon bundles at the dorsal or ventral area (Figure 2D). Different TTBTs have been reported for dorsal and ventral tendon fascicles (Higgins *et al.*, 1991). Therefore, only compare tendons from either dorsal or ventral origin to each other or be aware of these differences when analyzing your results.



Video 1. Removing the skin from the tail

3. Assure that you remove fascicles from the middle part of the tail, to avoid deviations in fascicle breaking time due to the temperature gradient in a mouse's tail (Harrison and Archer, 1978).
   a. Prepare a Petri dish filled with distilled H_2O at room temperature. Covering the bottom of the dish with the water is sufficient.
   b. Cut off 2-3 cm of both the caudal and cranial end of the tail to gain access to the mid-tail section. Again, mark the ventral side of the tail.
   c. From the resulting tail of the mid-tail section, use the caudal end to pull out fascicles with a surgical tweezer and place the fascicle bundle into a Petri dish filled with distilled H_2O. It is not necessary to remove the whole fascicle bundle.
   d. Carefully isolate a single fascicle (Figure 2E, Video 2) out of the removed fascicle bundle, using a surgical tweezer. Use a dissecting microscope to assure you have isolated a single fascicle. The isolated fascicle should have a smooth and straight appearance. It should be approximately 1 cm in length and be around 0.1 mm in diameter. Discard double or split

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fascicles.

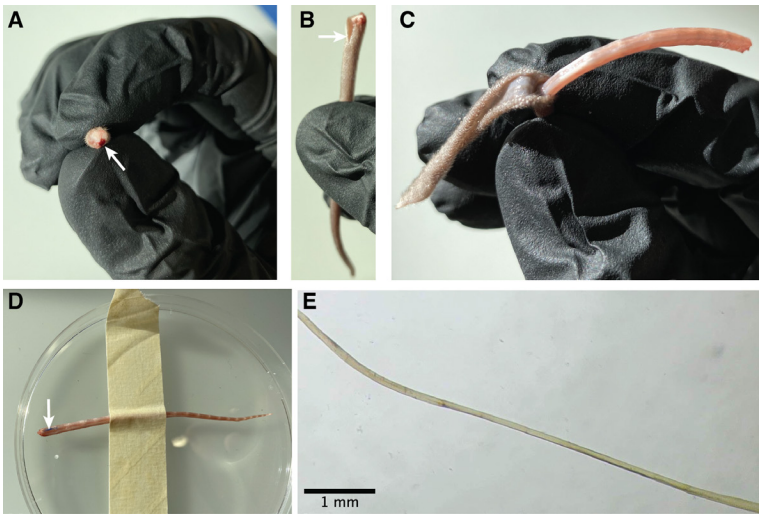
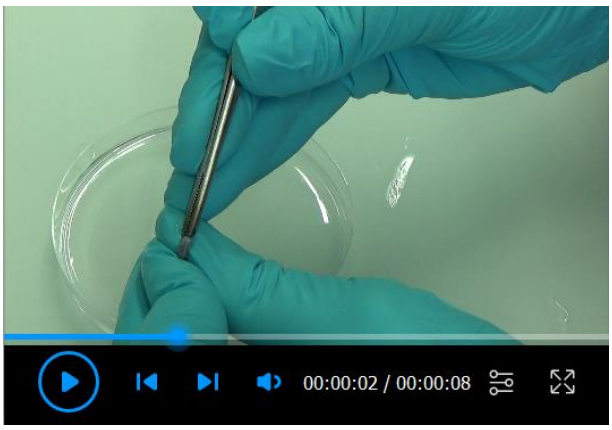


Figure 2. Tail tendon fascicle isolation. A. Squeezing tail ending. Blood drop (white arrow) appears from the ventral artery. B. Incision to the skin (white arrow) at the tail ending. C. Peeling off the skin. D. Marking the ventral side with a blue permanent marker (white arrow). E. A single tail tendon fascicle under a dissecting microscope



Video 2. Isolating a single fascicle

C. Hang the single tail tendon fascicle into the urea bath.
   1. Prepare the surgical slipknot on the suture attached to the 2 g weight as well as on the suture you use to hang the tendon into the urea bath (Video 3).

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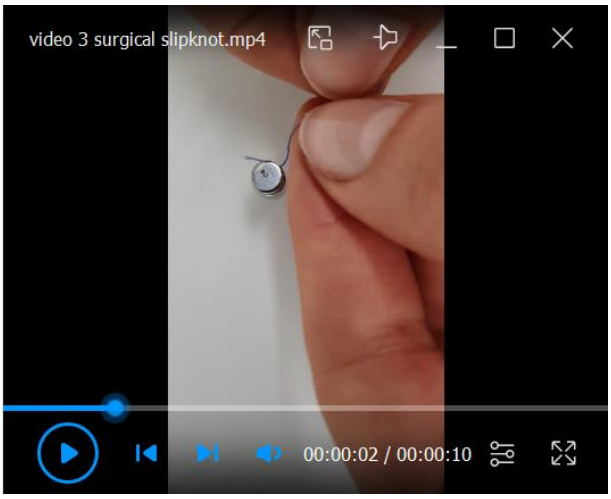


Video 3. Preparation of the surgical slipknot

2. First, attach the tendon to the 2 g weight. Take up the tendon with a tweezer and hang it through the opening of the surgical slipknot. As soon as the fascicle is in place, tighten the knot (Figure 3, Video 4).

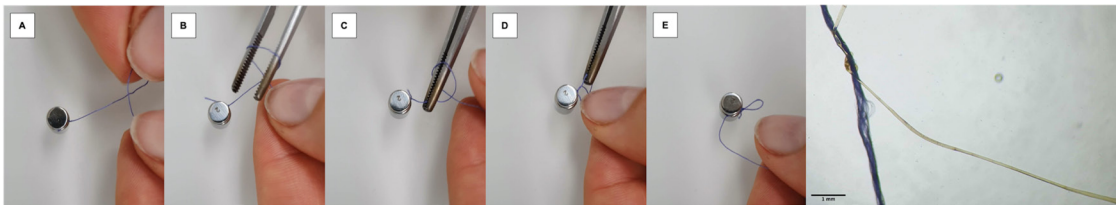
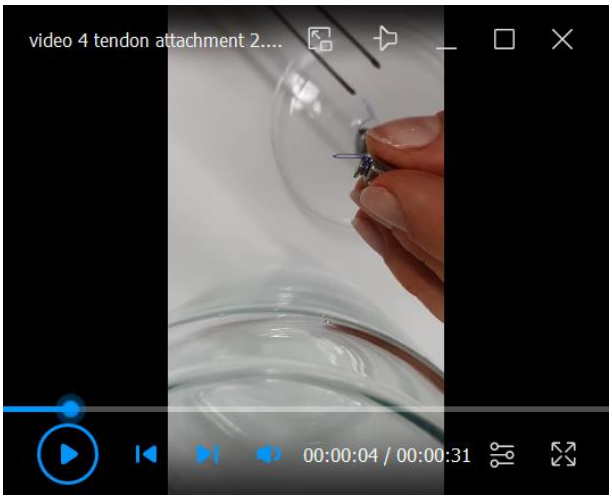


Figure 3. Step-by-step preparation of a surgical slipknot. Form a loop in the suture (A) and reach through that loop using forceps (B). Use the forceps to grab the suture near the attached weight (C) and pull that part of the suture through the prepared loop (D). The final surgical slipknot result is depicted in E. Repeat the procedure for the other end of the suture to attach the fascicle (Video 4).



Video 4. Attachment of the tendon to the surgical slipknot

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3. Repeat the procedure for the surgical suture placed above the urea bath (Video 5).

Video 5. Final attachment of the tendon and placement in the urea bath

4. Check whether the temperature of the urea has reached 45 °C before submersing the tendon.
5. Gently place the weight in the urea bath and adjust its height so that the tendon is fully submersed in urea (Figure 4).

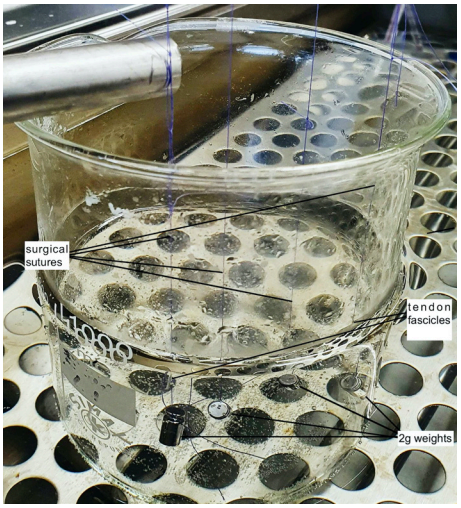

Figure 4. Tendon attached to a 2 g weight and submersed in a beaker filled with urea solution. It is possible to have 4 tail tendons in the same urea bath to measure, for instance, a control vs 3 other conditions in parallel.

D. Measurement and readout of the experiment
   1. Start the video recording, making sure you can clearly see the weights (Figure 5).

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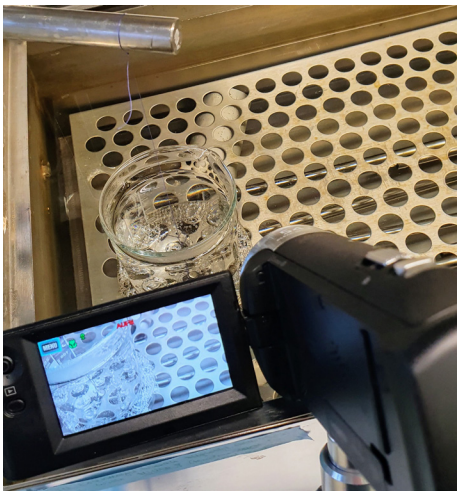

Figure 5. Experimental setup using a video camera to determine the exact TTBT

2. At the start of each experiment, label the urea level on the sidewall of the beaker using a waterproof marker. Check the urea level every hour and gently refill the beaker with water if the urea level has dropped below the mark. To avoid turbulences, pipette the water down the sidewall of the beaker. Refiling the beaker is important, as only water evaporates, and thus the urea concentration would change if the beaker is not refilled regularly (Harrison and Archer, 1978).
3. After the weight has dropped to the bottom of the beaker, assure both ends of the suture have some remaining tendon on them. This step is important to avoid false results due to the tendon slipping through the knots.
4. Stop the recording and check when the rupture took place. Note down the TTBT.

Data analysis

The rupture time in minutes can be analyzed and plotted (mean and individual data points) using GraphPad Prism 8 software. In the example below, Figure 6 depicts the age-related difference in TTBT. 30-months old mice exhibited an increased TTBT of 91 ± 12 min, when compared to 4-months old mice (mean TTBT 9 ± 4 min).

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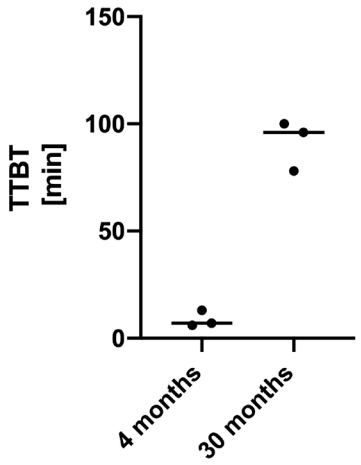

Figure 6. TTBT [min] in mice of different age groups

Recipes

1. 7 M urea solution (pH 7.5) (Harrison and Archer, 1978)
   a. Dissolve 600 g Urea (Sigma-Aldrich) in 900 ml of distilled water
   b. Add 60 ml of 0.1 M KH_2PO_4 solution
   c. Add 40 ml of 0.05 M $Na_2B_4O_7$ solution
   d. When fully dissolved, measure the pH of the urea solution
   e. Adjust the pH of the urea solution to 7.5 by adding 1 M HCl if necessary (this needs to be done before each measurement)

   The prepared solution will have a volume of 1.4 L and should be stable for around two weeks. If the solution turns cloudy, it should be discarded immediately.

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Moreover, we want to acknowledge the previous work performed by Harrison and Archer (1978), which served as a foundation for our adapted protocol.

Competing interests

The authors, ASH and CYE, declare no competing interests.

Ethics

All animal studies conformed to the Swiss animal protection laws and were approved by the veterinary office of the Canton of Zürich, Switzerland (License nr. ZH092/19).

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