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# The sperm hook in house mice: a functional adaptation for migration and self-organised behaviour

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## Abstract

In most murine species, spermatozoa exhibit a falciform apical hook at the head end. The function of the sperm hook is not yet clearly understood. In this study, we investigate the role of the sperm hook in the migration of spermatozoa through the female reproductive tract in *Mus musculus* (C57BL/6), using a deep tissue imaging custom-built two-photon microscope. Through live reproductive tract imaging, we found evidence indicating that the sperm hook aids in the attachment of spermatozoa to the epithelium and facilitates interactions between spermatozoa and the epithelium during migration in the uterus and oviduct. We also observed synchronised sperm beating, which resulted from the spontaneous unidirectional rearrangement of spermatozoa in the uterus. Based on live imaging of spermatozoa-epithelium interaction dynamics, we propose that the sperm hook plays a crucial role in successful migration through the female reproductive tract by providing anchor-like mechanical support and facilitating interactions between spermatozoa and the female reproductive tract in the house mouse.

### eLife assessment

This study uses ex vivo live imaging of uteri post-mating to test the role of the sperm hook in the house mouse sperm in sperm movement that would be interesting to evolutionary biologists. The significance of the work is **useful** as live imaging can reveal information not seen in fixed images. The strength of evidence is **incomplete** as they cannot directly test the role of the sperm hook in facilitating movement along the uterine wall.

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## Introduction

Since the pioneering work by Parker (Parker, 1970), postcopulatory sexual selection has been recognised as an important driving force that shapes male sperm and female reproductive track characteristics. Post-copulatory sperm competition over gamete and cryptic female choice are equivalent to male-male competition and female choice in the pre-copulatory sexual selection. Sperm competition occurs when spermatozoa from more than one male coincide within the female reproductive tract in a time window where they have a chance to fertilize the same egg (Parker, 1970). The evolutionary consequences of the sperm competition for the male traits, including baculum complexity, sperm shape and behaviour, are demonstrated in a wide range of animals from insects to mammals (Anderson et al., 2005; Lange et al., 2013; André et al., 2020). Such male adaptations secure sperm delivery to the female reproductive tract and influence sequential mating of the already mated female with other males. Cryptic female choice is a more complicated process to define as it is achieved at the various stages of the post-mating fertilization process that also occurs inside the female reproductive tract. However, post-copulatory cryptic female choice is evident where a female can exert an influence on ejaculates or even fertilised eggs that can influence male's reproductive success (Eberhard, 1996; Firman et al., 2017; Roberts et al., 2012).

Postcopulatory sexual selection results in co-evolution of the male and female traits that impact the reproductive strategies of the opposite sex (Eberhard and Lehmann, 2019; Greff and Parker, 2000). For example, copulatory plugs formed by male semen hinder sequential mating of the female with a second male and diminish the reproductive outcome of the second male (Mangels et al., 2016; Sutter and Lindholm, 2016). Females, on the other hand, by removing the copulatory plug, can counteract male strategy (Koprowski, 1992). When fluid in female reproductive tract is spermicidal, male seminal fluid neutralises the spermicidal medium and secures sperm survival inside the female reproductive tract (Holman and Snook, 2008, 2006).

Among mammals, rodent species exhibit various sperm morphological and behavioural characteristics. A notable morphological feature of murine sperm is the apical hook resulting in an asymmetrical falciform head shape that are found in most of the murine rodents (Breed, 2004; Roldan et al., 1992). The functional significance of the sperm hook that causes head asymmetry is still under debate in the field of mouse reproductive ecology. Currently, two main hypotheses attempt to explain the function of the sperm hook. One is that the sperm hook plays a crucial role in sperm competition by aiding sperm linkage (sperm train formation) that enhances straighter and faster sperm forward progression – the sperm cooperation hypothesis (Fisher and Hoekstra, 2010; Moore et al., 2002). The other hypothesis is that the sperm hook facilitates sperm-epithelium interactions in the female reproductive tract, playing a significant role in sperm migration – the migration hypothesis (Smith and Yanagimachi, 1990; Firman and Simmons, 2009). The sperm cooperation hypothesis is supported by the pioneering discovery of sperm linkage known as sperm trains (Moore et al., 2002) and suggests that sperm trains facilitate faster or straighter sperm swimming (Fisher and Hoekstra, 2010; Moore et al., 2002). However, other studies could not find supporting evidence of sperm cooperation by the sperm train or morphological changes in sperm hooks concerning the degree of sperm competition (Firman and Simmons, 2009; Hook et al., 2021). These researchers rather suggest that the sperm hook plays a crucial role in sperm migration by interacting with epithelia in the female reproductive tract.

Since thick muscle layers comprise the outer part of rodent female reproductive tract, direct observation of spermatozoa inside the female reproductive tract is a challenging feat. Therefore previous studies focused on the oviduct where muscle layers are thin and transparent, using brightfield, fluorescence or confocal microscopy (Ishikawa et al., 2016; Qu et al., 2021; Suarez,

1987). In this study, we developed an ex-vivo observation system based on a custom-built two-photon microscope (Fig. S1). This system enables the observation of spermatozoa and their behaviour inside the live female reproductive tract. While two-photon microscopy is currently the method of choice for live imaging of deep tissues (Helmchen and Denk, 2005; Benninger and Piston, 2013; Keller, 2013), it has seldom been used for studying animal reproductive ecology. Here, we demonstrate high-resolution deep-tissue imaging that allows us to observe and track sperm movement inside the female reproductive tract, including the uterus, to realise real-time tracking of spermatozoa and their migration.

Using this technology, we report newly discovered sperm behaviour and aggregation patterns that suggest various roles of the sperm hook in sperm migration inside the female reproductive tract. Based on live imaging of sperm dynamics inside the live female reproductive tract, we provide new observations and insights that suggest functions of the sperm hook that help sperm migration and cooperation. With our new observation system and results, we hope to contribute to the field of postcopulatory sexual selection in rodents by advancing methodological progress and stimulating discussion and future research on the function of sperm hook in murine rodents.

# Results

To observe mouse sperm behaviour in the female reproductive tract, we mated wild-type *Mus musculus* (C57BL/6) females with transgenic male mice (Tables S1; S2) that express DsRed at the sperm mid-piece (mitochondria) and eGFP in the sperm acrosome (Hasuwa et al., 2010). The female mice were euthanised between 0.5- and 3-hours post-mating, and their reproductive tract along with copulatory plug was excised and transferred to a sterilized Petri dish. We then conducted *ex-vivo* imaging of the live reproductive tract using our two-photon microscope (Fig. S1). **Figure 1A** is a diagram of the female reproductive tract that labels the name of each part of the tract. All observations were typically completed within 3 hours and did not exceed 6 hours post-euthanasia. Despite this observation period being longer than recommended for conventional rat transplantation surgery (Díaz-García et al., 2013), we noted live uterine movements throughout the entire observation period. When samples were transferred to incubators preheated to 37°C with 5% $CO_2$ following observation, uterine movement persisted even 24 hours post-excision. We used Fiji (Schindelin et al., 2012) and R 4.3.3 (R Core Team, 2024) for image processing and statistical analysis.

## The role of sperm hook in migration inside the uterus

We first observed sperm movement in the uterus, where most ejaculated spermatozoa are located and initial sperm selection occurs. In the uterus, we observed vigorous fluid flow caused by uterine contraction and relaxation. Consequently, most spermatozoa in the uterus were carried by this flow, adhering to the inherent flow dynamics within the uterus (Movie S1A). However, when the flow temporarily ceased, spermatozoa located near the uterine wall exhibited greater activity (Movie S1B, C). When a spermatozoon encounters the uterine wall (epithelium) during migration, the sperm hook interacted with the wall and acts as a pivot (Yu et al., 2023) that influences the direction of sperm travel (**Figure 1B, C**). Movie S2A shows two distinct types of sperm movement when transitioning from the uterus volume to the uterine wall: pro-wall-hook and anti-wall-hook directional movement (**Figure 1C**). Upon reaching the uterine wall, instead of randomly deflecting in various directions, sperm preferentially altered their heading direction such that their apical hook would face the uterine wall (pro-wall-hook direction).

To test whether the hook influences the direction of migration, we tracked spermatozoa that travelled from the uterus volume to the wall in sequentially acquired images. We found that 52 out of 63 spermatozoa (82.54%) changed their migration direction towards the pro-wall-hook direction

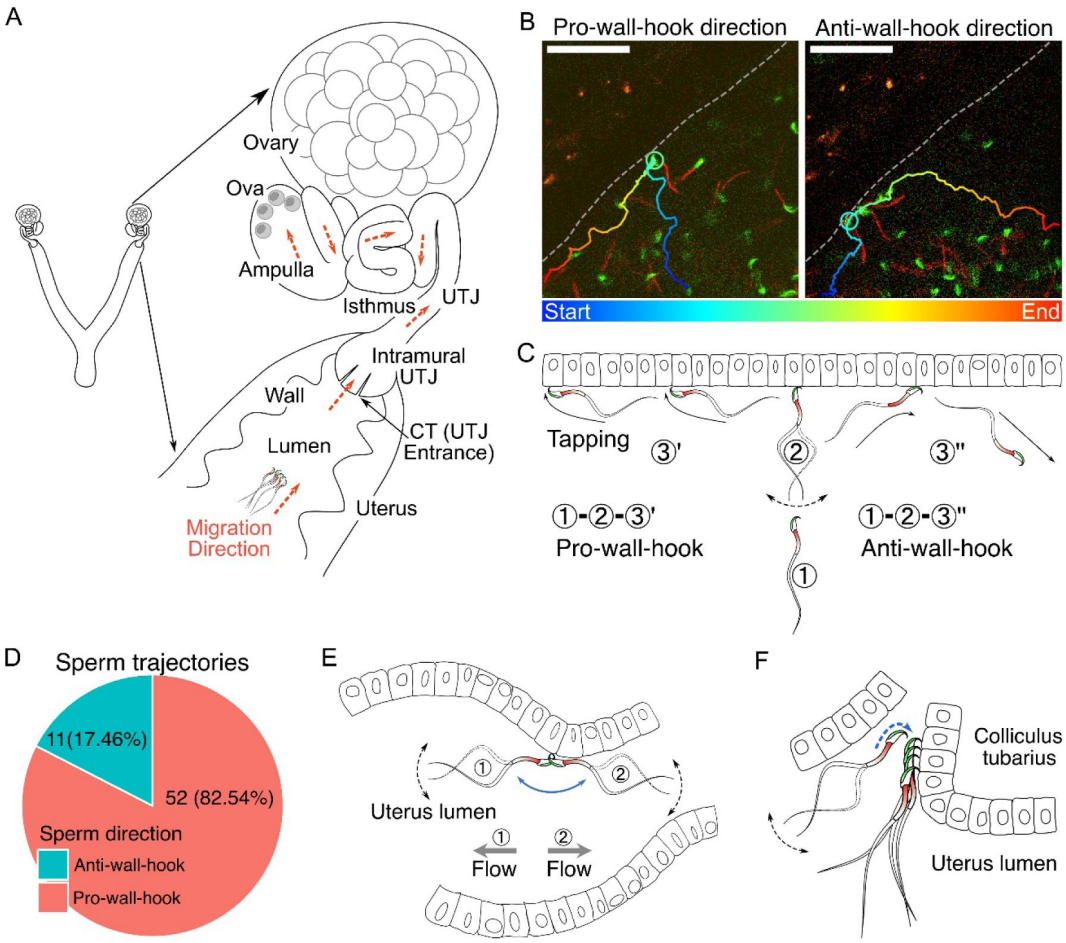


**Figure 1.**

**The structure of female reproductive tract and various sperm behaviours and interactions with epithelia in the uterus.**

(**A**) An illustration of the female reproductive tract and sperm migration direction (arrows in orange colour) inside the tract. Ova (egg cells) are in the ampulla. (**B**) Spermatozoa alter their travel direction based on their head orientation upon reaching the uterine wall (sperm hook functions as a pivot, Movie S2A). Left image shows a sperm trajectory exhibiting pro-wall-hook direction, and the right image shows anti-wall-hook direction. The trajectory is depicted in colours representing different time points. Dashed lines are guides to the eye to visualize the uterus wall. Scale bar: 50 μm. (**C**) Sperm moving direction changes over time when they reach the uterine epithelia. Numbers indicate sequences of sperm movement. (1) During sperm migration, they reach the uterine wall. (2) After reaching the uterine wall, they beat several times while facing their head towards the uterine wall. (3) Sperm beating results in a change in sperm orientation (1 to 3': pro-wall-hook, 1 to 3": anti-wall-hook). The pro-wall-hook orientation coincides with sperm travelling along the wall and the anti-wall-hook orientation coincides with sperm moving away from the wall. (**D**) When spermatozoa reach the uterine wall, their trajectories predominantly follow the pro-wall-hook direction, where the sperm hook is directed towards the uterine wall. (**E**) The sperm hook assists a spermatozoon in anchoring to the epithelia (hook as an anchor). This anchoring facilitates sperm attachment to the uterine and UTJ epithelium and prevents spermatozoa from being swept away by internal flow caused by peristaltic movement. (**F**) The sperm hook and thin sperm head aid spermatozoa in squeezing through the sperm-crowded UTJ entrance (CT) and attaching to the epithelium by acting as an anchor (Movie S3). Note that in our experiments, we do not observe the principal and terminal pieces of spermatozoa due to a lack of fluorescence in our animal model. The observed acrosome and midpiece are shown coloured in the schematic figures.

after reaching the uterine wall (**Figure 1D**). The remaining 11 spermatozoa followed the anti-wall-hook direction. A binomial test confirmed that this tendency was statistically significant (one-tailed, $p < .001$, 95% CI: 0.73, 1.00). Furthermore, when spermatozoa migrated along the uterine wall, they exhibited a tapping-like behaviour in which spermatozoa tapped their hook against the epithelium when oriented in a pro-wall-hook direction (Movie S2B). In contrast, when the sperm hook faces the luminal space, spermatozoa could not migrate along the wall and their migration trajectories followed the anti-wall-hook direction, resulting in movement away from the wall (**Figure 1D**; Movie S2A). In addition to the advantage in straighter trajectories, fluid flows are slower at the wall than at the centre of the lumen in the uterus (Zaferani et al., 2019). Therefore migration along the uterine wall will help spermatozoa to reach the entrance of the intramural utero-tubal junction (UTJ), also called colliculus tubarius (CT), where one of the most important sperm selection processes takes place (Nakanishi et al., 2004; Qu et al., 2021).

## Sperm anchoring and migration kinetics in the uterus

We observed that the sperm head was attaching to the epithelium of the CT and uterus (Movie S3A, B) when spermatozoa reached the CT. The sperm hook was affixed to the epithelium, thereby securing the spermatozoa on the epithelia. We defined this securing of the spermatozoa on the epithelia by the sperm hook as an anchor-like function of the hook (Movie S3A, B). This anchoring also helped prevent spermatozoa from being swept away by mucosal flow (**Figure 1E**; Movie S3B). When spermatozoa attached to the CT and squeezed through other spermatozoa by hooking, the apical hook always faced the epithelium (**Figure 1F**; Movie S3A). Once spermatozoa successfully clung onto the CT, anchoring to the epithelia prevented them from being pushed out by competing spermatozoa or from being swept away by fluid flow (Movie S3B).

To compare sperm migration kinetics during sperm swimming relative to their distance from the uterine wall, we tracked sperm migration trajectories by employing the TrackMate plugin in ImageJ (Ershov et al., 2022; Tinevez et al., 2017). After successful tracking using the customised tracking option in TrackMate, we computed various sperm kinetic parameters. These parameters included the curvilinear velocity (VCL), straight-line velocity (VSL), and linearity of forward progression (LIN), which are commonly used in computer-assisted sperm analysis, CASA (Amann and Waberski, 2014). Briefly, VCL was calculated as the total distance travelled divided by total travel time, VSL as the distance between initial and final positions of the sperm trajectory divided by total travel time, and LIN as the ratio of VSL to VCL, which can range from 0 to 100%, with 100% representing a perfectly straight line. We also introduced a new kinetic parameter called straight line-to-sideward movement ratio (SWR), defined as track displacement of a sperm trajectory divided by maximum sideward movement distance. Refer to Fig. S2A, B for our definition of the uterus wall and a schematic description of the sperm migration kinetic parameters, as well as Methods for more details.

Our sperm tracking analysis revealed that spermatozoa located close to the uterine wall moved faster, exhibiting higher VCL and VSL (**Figure 2A**; Table S3; Movie S1B, C). However, LIN and SWR did not significantly vary depending on sperm's distance from the uterus wall (**Figure 2B**). In contrast to the non-significant changes in LIN and SWR relative to the distance from the uterine wall, when spermatozoa swam parallel to the wall, they not only moved faster (higher VCL and VSL; **Figure 2A**), but also followed a straighter path (higher LIN and SWR; **Figure 2B**). Given that the internal fluid flow around the uterine wall is slower (Zaferani et al., 2019), and considering the hydrodynamic factors that attract spermatozoa to the wall when swimming near it (Alvarez et al., 2014; Elgeti et al., 2010), migration along the wall would be an efficient strategy for sperm movement within the uterus. We also examined the impact of both individual males (coded as 'Male ID') and females (coded as 'Date') on the sperm kinetic parameters in all models by visualising the effects of these two random variables (Fig. S3). We confirmed that the effects of the two random variables were consistent across all significant models, indicating validity of our model estimations.

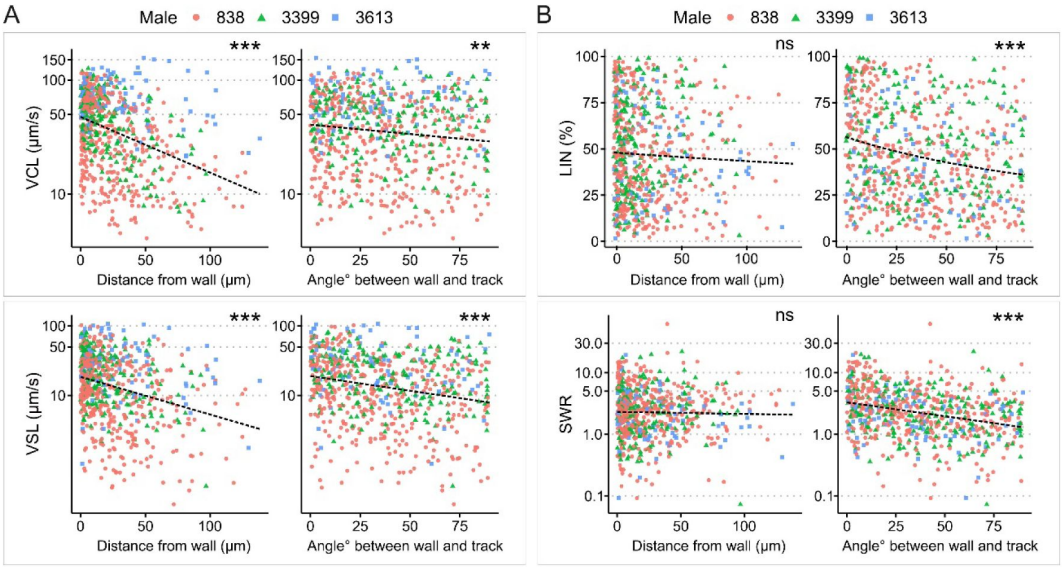


**Figure 2.**

### Analysis of sperm kinetic parameters relative to the distance and angle between the sperm trajectory and uterine wall.

(**A**) Both VCL (top) and VSL (bottom) decreased with an increase in the distance between the sperm trajectory and the uterine wall. Similarly, VCL and VSL decreased as the angle between the sperm trajectory and uterine wall increased. (**B**) The distance between the sperm trajectory and uterine wall did not significantly affect LIN (top left) and SWR (bottom left). However, both LIN and SWR decreased when the angle between the sperm trajectory and uterine wall increased. Data from different males are represented in different colours and shapes. The dotted lines indicate regression lines from simple regressions to aid visual interpretation. Check model estimates for more details and precise interpretation of the models (Table S3; Fig. S3A, B). The y-axis of each figure is displayed in log-scale except for LIN. Images for sperm tracking were acquired around the area labelled as 'Wall' in the uterus in **Figure 1A**. Sperm trajectories used in this analysis are presented in Movie S1B. The effect of the two random variables (males and females) are visualised in Fig. S3.

## Structure of UTJ and sperm behaviour at the entrance of intramural UTJ (CT)

Upon sacrificing and optical clearing the tissue, we confirmed that the entrance to the intramural UTJ (CT) in the uterus consists of nearly closed narrow gaps between mucosal folds (**Figure 3A**). These narrow gaps extended to about 100 μm deeper from the entrance (Movie S4A, B), and only a few spermatozoa could pass through a gap at a time (**Figure 3A**; Movie S4C). We were not able to find evidence of passive sperm carriage, such as upsuck-like sperm carriage (Baker and Bellis, 1993), caused by peristaltic movement from the uterus into the UTJ in real-time live images (Movie S5A). Therefore, we concluded that fluid flow induced by uterine and oviduct contraction is not a major driving force for sperm entry to the UTJ through the CT.

This conclusion raises a question about how spermatozoa enter the UTJ through the CT if the UTJ entrance is nearly closed. We found head-directional sliding of spermatozoa in the almost closed inter-luminal spaces between the uterus and intramural UTJ (Movie S5B). We did not observe any sliding of spermatozoa that directs to the tail-direction. This one-directional head-forward sliding in a very narrow inter-luminal space suggests a role of the sperm hook as an anchor that prevents backward slipping by the squeezing movement from the contraction of the uterus. Such one-directional sliding is also a plausible way of sperm migration from the uterus to UTJ through the narrow gap at the CT. We also observed that when muscle contraction and relaxation occurred at the uterus and oviduct, the surfaces of two confronting mucosal folds in intramural UTJ slid against each other in opposite directions (**Figure 3B**; Movie S5B). Such an opposite directional movement of mucosal folds, for example, will occur when the uterus bends to the left due to muscle contraction on its left side and relaxation of its right side (Fig. S4). As shown in Movie S5B, the opposite movement of mucosal folds makes space between mucosal folds at the CT, providing an opportunity for nearby attached spermatozoa to enter the intramural UTJ (Fig. S4). Although application of further experimental approaches will be necessary, occasional fluctuations in the size of the luminal space between mucosal folds, caused by peristaltic movements, together with the head-directional sliding of the spermatozoa, may provide an opportunity for spermatozoa to pass through the CT.

We also observed unidirectional sperm clustering as a result of spontaneous sperm re-arrangement during sperm beating along the uterine wall (**Figure 4A** and Movie S6A, B). Such unidirectional sperm clustering and their successive beating resulted in synchronised sperm beating on some occasions (sperm self-organising behaviour). We also found such self-organised sperm behaviour on a large scale at the CT in which most of the clustered spermatozoa at the entrance of the intramural UTJ (CT) exhibited synchronised beating (**Figure 4B**; Movie S7). The synchronised sperm beating was observed to generate fluid flows strong enough to prevent other spermatozoa from attaching to the CT or directly push out other spermatozoa, thereby preventing other spermatozoa from entering the UTJ (Movie S6; S7).

## Sperm linkage and their migration kinetics

Unlike the sperm trains found in wood mice (*Apodemus sylvaticus;* Moore et al., 2002) and deer mice (*Peromyscus maniculatus;* Fisher and Hoekstra, 2010), most of the spermatozoa in this study did not form functional sperm trains (linked spermatozoa) (**Figure 5A**; Movie S8). Active sperm linkage or clusters were indeed observed but were rare (only 3 identifiable cases during over100 hours of imaging). For the house mouse, the observed sperm trains did not move faster or slower than unlinked single spermatozoa (**Figure 5B**; Movie S8). Their VCL, VSL, and LIN were not faster nor higher than those of unlinked single spermatozoa as shown in **Figure 5B**.

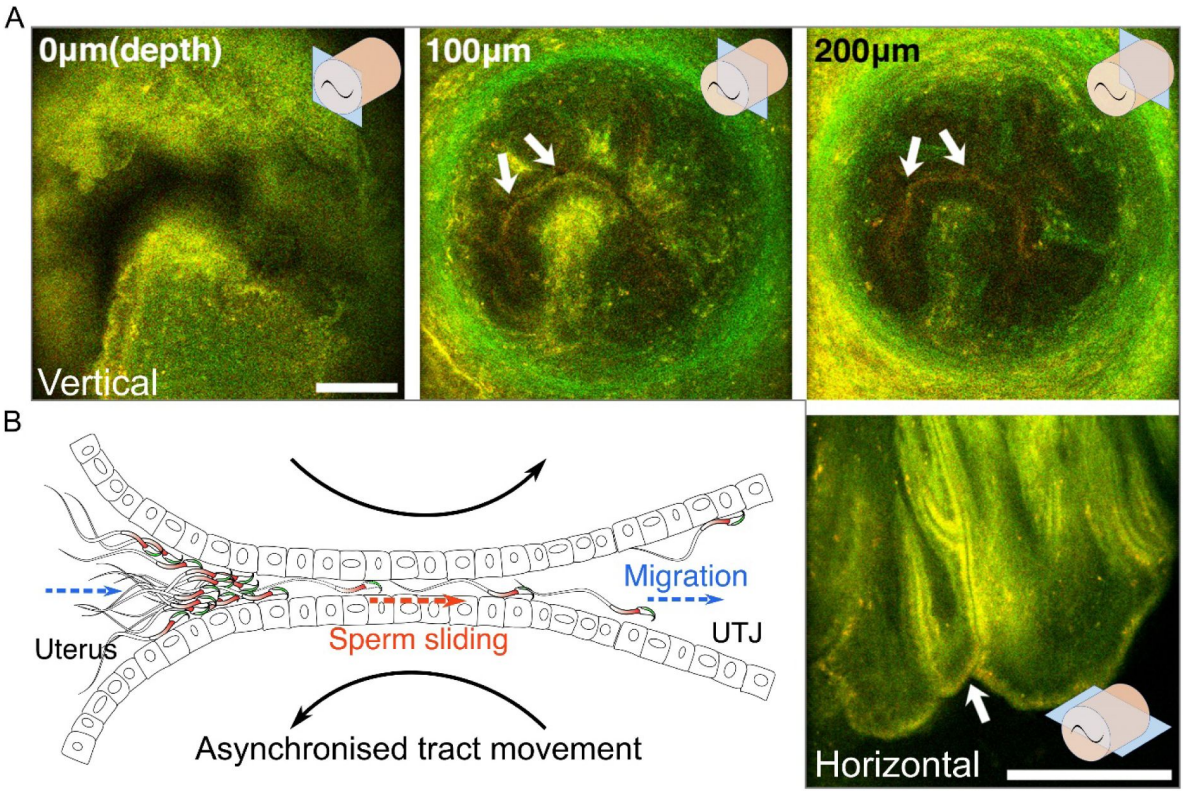


**Figure 3.**

**The structure of the entrance of intramural UTJ and sperm passage.**

(**A**) The upper three images are the vertical view of the CT (entrance to the UTJ) of the intramural UTJ from an unmated female. The bottom right image is a horizontal view of the intramural UTJ. There are only a few small gaps, indicated by arrows, between mucosal folds, which control sperm migration into the UTJ from the uterus (Movie S4). Illustrations at upper and lower right conners indicate imaging planes (blue rectangles) and the intramural UTJ (orange cylindrical structure) with the CT (black tilde). Scale bar: 100 μm, Arrow line thickness: 10 μm for top three images, 5 μm right bottom image. (**B**) Asynchronised movement of mucosal folds (sliding against each other) at the CT due to uterine and UTJ contractions enable spermatozoa to slide into the intramural UTJ from the uterus (Movie S5). Two dashed arrows (in blue) indicate the direction of sperm migration from the uterus to the UTJ, and the dashed arrow in the centre (in orange) indicates the direction of sperm sliding in the intramural UTJ. The two curved black arrows indicate asynchronised (opposite) movement of confronting mucosal folds in the intramural UTJ.

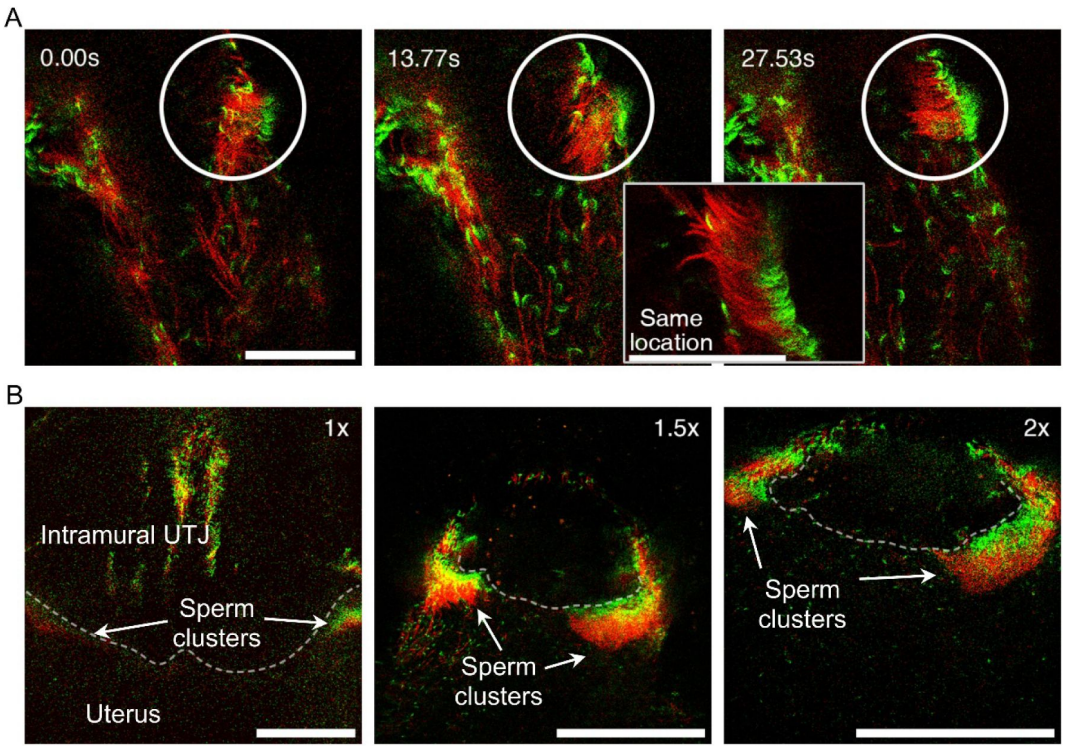


**Figure 4.**

**Self-organised sperm behaviour at CT inside the uterus.**

(**A**) The apical shape of the sperm head, due to the sperm hook, results in head asymmetry. This asymmetrical falciform head shape may facilitate sperm re-arrangement and clustering at uterine wall (Movie S6). The circle highlights spermatozoa undergoing unidirectional re-arrangement over time. The elapsed time after the first frame is shown in the upper left of the images. The right lower zoom-in inset shows an instant of synchronised motion and unidirectional re-arrangement. Scale bar: 50 μm. (**B**) Unidirectional sperm clustering at the entrance of the intramural UTJ (CT, indicated by a dashed line) is marked with arrows. Such large sperm clustering resulted in synchronised sperm beating at the CT (Movie S7). Scale bar: 200 μm. Note that due to a lack of fluorescence, the principal and terminal pieces of sperm tails are not seen in the images.

Although their SWR may be higher than that of unlinked single spermatozoa, due to the rare number of observed events, further experiments will be necessary to clarify whether the sperm train formation is advantageous in the house mouse.

### Sperm migration and accumulation in the oviduct

After entering the UTJ, spermatozoa continued migration through the narrow UTJ lumen. In the UTJ, spermatozoa interact with epithelia with their hook and penetrate their thin head into a narrow space (Movie S9A). Contraction of the UTJ reduced the width of the UTJ lumen and prevented sperm migration (Movie S9B). However, some spermatozoa could pass through the narrow luminal space by putting their thin hook (**Figure 6A, B**; Movie S9B). During UTJ lumen contraction, both swimming and beating of spermatozoa therein were physically suppressed. When the UTJ lumen dilates, the suppressed spermatozoa started beating and swimming again (**Figure 6C**; Movie S9B). We also found sperm accumulations in the oviduct, including UTJ and isthmus (**Figure 6D**). However, as these sperm accumulations were arranged irregularly and consisted of spermatozoa that were mostly acrosome reacted and inactive, they were considered inactive entangled spermatozoa rather than active linked spermatozoa (or sperm trains). These entangled spermatozoa filled the narrow oviductal lumen, creating an obstruction for the migration of other spermatozoa (Movie S9C). If sperm hook facilitates such entanglement of inactive spermatozoa in the UTJ and isthmus, it can play a role in sperm competition by obstructing migration of the other spermatozoa including those from a second male.

Sperm beating rates of the epithelia-attached spermatozoa were observed to change over time according to the changes in widths of the UTJ lumen (Movie S10). Although such a change in beating rates may simply reflect luminal flow speed or luminal width related to physical space for beating, it may also assist in attaching spermatozoa to the epithelium by providing propulsive forces (Kantsler et al., 2014). Fluid flow in the UTJ and isthmus can damage unattached spermatozoa, particularly when they get entangled with inactive entangled spermatozoa that are moving back and forth by the flow (Movie S9C). If such beating prevents epithelium-attached spermatozoa being swept away by the flow, sperm beating corresponding to the fluid flow will be advantageous. The oviductal fluid gradually flows upward while continuously repeating back-and-forth directional changes and such flow is assumed to aid sperm migration from UTJ to ampulla (Hino and Yanagimachi, 2019). However, given our observation of resistant (anchoring) behaviour of spermatozoa to the flow in the UTJ, passive sperm transfer may not always be beneficial for healthy spermatozoa. Additionally, such passive transfer can cause physical damage to the spermatozoa by the collision with other entangled spermatozoa or epithelia of the narrow lumen. Further experiments will be necessary to clarify the role of the rapid upward flow in the oviduct in the fertilization process in mice.

## Discussion

Our real-time deep tissue imaging enabled by two-photon microscopy shows that the mouse sperm hook (i) facilitates sperm interaction with the epithelium for better navigation and (ii) provides an anchor-like role that assists attachment of sperm to the epithelium. These results suggest that the sperm hook in house mice functions to facilitate sperm migration by interacting with the female reproductive tract (Firman and Simmons, 2009; Suarez, 1987; Tourmente et al., 2016). We also showed that when spermatozoa swim along the uterine wall, their apical hook interacts with the epithelia and determines sperm travelling direction upon encountering uterine epithelium. This finding implies that the sperm hook functions as a pivot when spermatozoa reach the uterine epithelium, and help spermatozoa migrate along the uterine wall by assisting in pro-wall-hook sperm orientation. Additionally, the sperm hook plays an important role in resisting endogenous fluid flow in the female reproductive tract by providing an anchor-like function. We did not

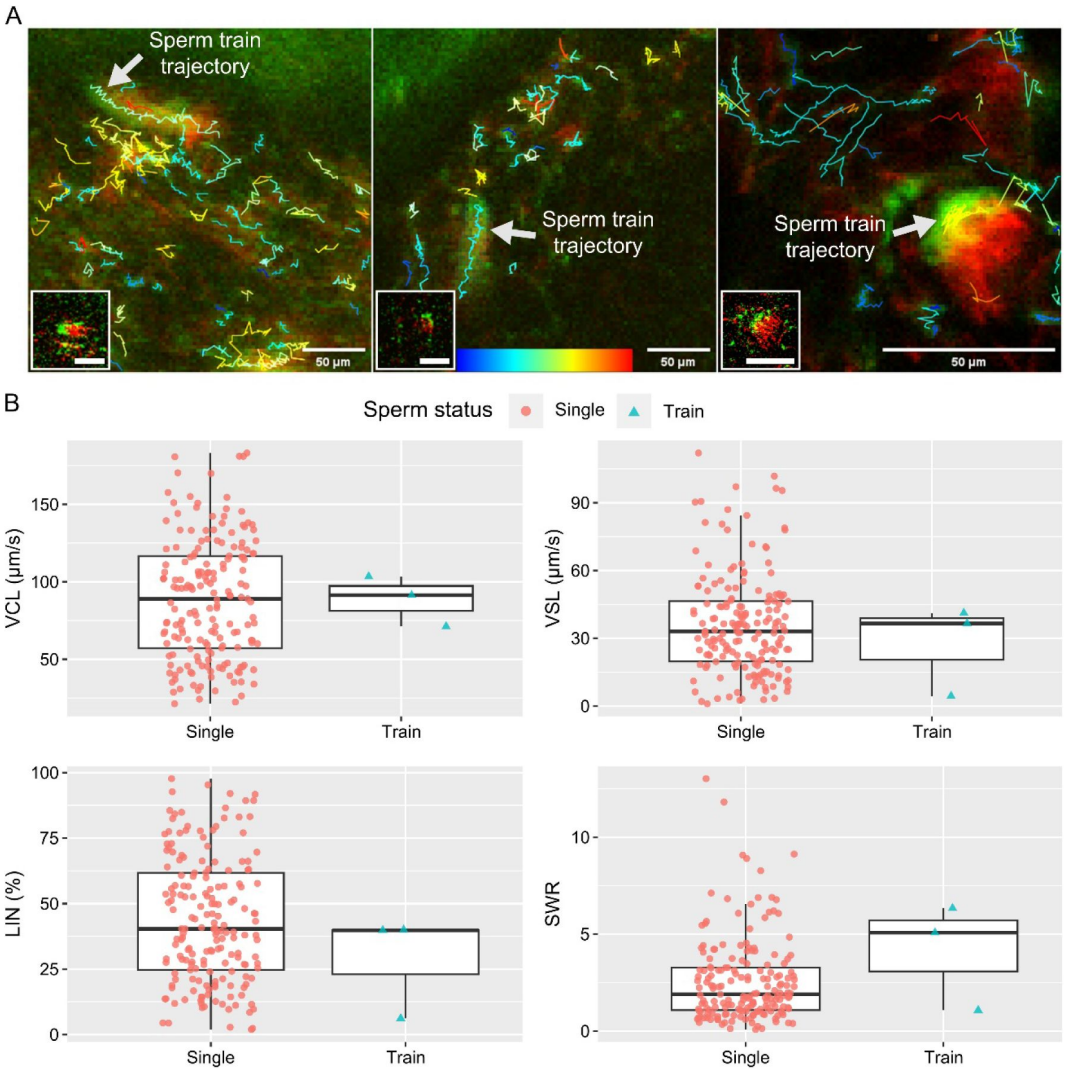


**Figure 5.**

### Comparative trajectories and kinetics of linked spermatozoa (sperm trains) and unlinked single spermatozoa

(**A**) The projected images, comprising 60 frames, depict the trajectories of sperm trains and unlinked single spermatozoa. Each of the images in the lower left corner shows a sperm train that was traced. Movie S8 shows how the three traced sperm trains are moving. The colour bar located at the bottom centre represents the VCL of each sperm trajectory, with blue indicating slower speeds and red indicating faster speeds. (**B**) The boxplots, which include individual data points, represent the kinetic parameters of the sperm trains and unlinked single spermatozoa. The parameters, including curvilinear velocity (VCL), straight-line velocity (VSL), linearity of forward progression (LIN), and straight line-to-sideward movement ratio (SWR), were computed using images of 100×100 pixels that contained a sperm train. The three sperm trains that were traced did not exhibit a faster VCL or VSL, nor a higher LIN. However, it is still possible that the SWR is higher in the sperm train. The lines within the boxes represent the medians, and the whiskers represent 1.5 times the interquartile ranges, and the symbols show the individual data points.

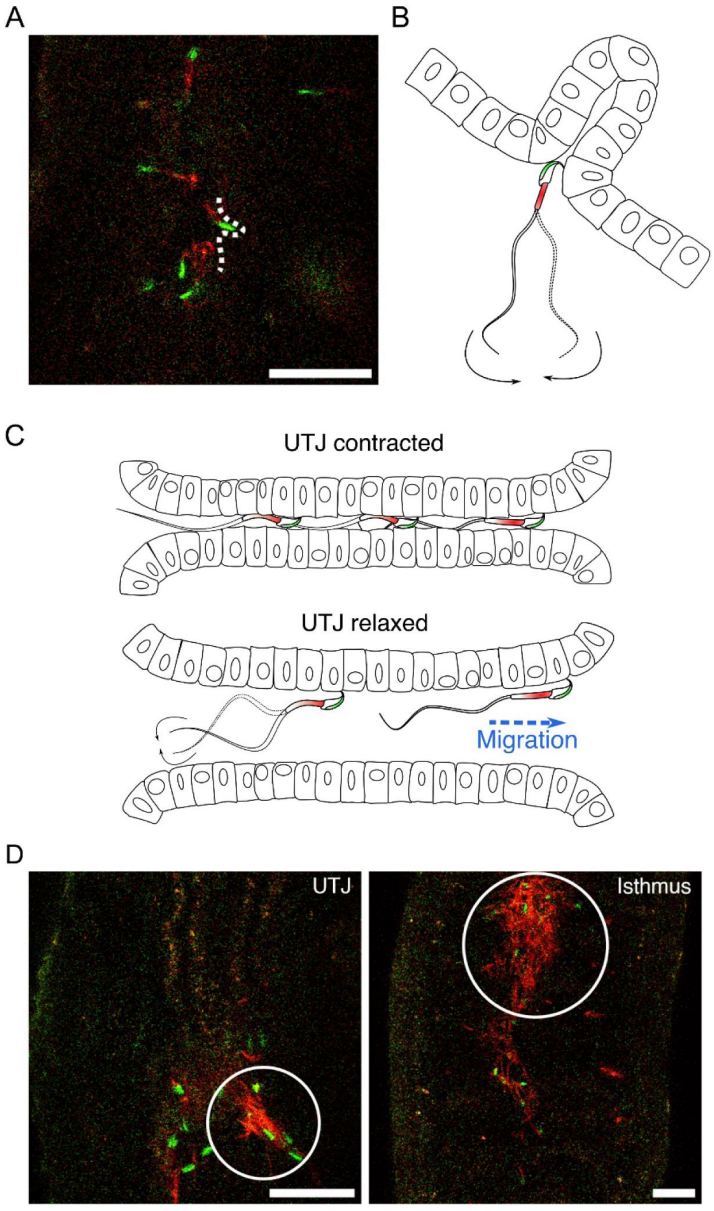


**Figure 6.**

**Sperm migration through narrow luminal space in UTJ and various sizes of accumulated sperm in the oviduct.**

(**A** and **B**) Spermatozoa can penetrate narrow space with their thin head and anchor their hook when they pass through the narrow gaps between mucosal folds during migration in the UTJ (Movie S9A). Scale bar: 50 μm. (**C**) Spermatozoa can only migrate through the UTJ when the luminal space is extended due to oviductal contraction and relaxation in the UTJ. (**D**) Entangled spermatozoa in the oviduct, including the UTJ and isthmus, were predominantly made up of inactive acrosome reacted spermatozoa. These entangled spermatozoa obstruct the migration of other active spermatozoa and can cause damage to live spermatozoa. Scale bar: 50 μm.

observe linkage of spermatozoa (sperm trains) that enables faster and straighter swimming of the sperm train in the uterus or oviduct. Instead, we found instances of the entanglement of inactive spermatozoa in the oviduct. Such aggregates of inactive spermatozoa appear to obstruct the migration of other spermatozoa. However, the role that these entangled spermatozoa play in sperm competition within the oviduct is a subject that necessitates further investigation.

Previous studies using various murine rodents suggest that acquiring the ability to form sperm train is a relatively rare evolutionary events in this taxon (Tourmente et al., 2016; Varea-Sánchez et al., 2016). Instead of finding evidence that the sperm hook aids in sperm train formation, those studies showed that variations in the length of the sperm hook, and asymmetry of sperm head influenced by the sperm hook are two important variables that reflect the degree of the sperm competition in murine rodents. One study even suggested that the sperm train formation (sperm linkage) is independent of the existence of sperm hook (Tourmente et al., 2016). Therefore, it is questionable whether acquiring the sperm hook in the murine rodents has evolved in general for a better sperm linkage. In line with the previous studies, our current study also showed that sperm hook plays a pivotal role in sperm-epithelium interactions by aiding sperm attachment to the uterine and oviductal epithelium and influence sperm orientation during migration inside the female reproductive tract (**Fig 1E, F**; Movie S3A, B). Such a role of the sperm hook suggests that the hook facilitates interactions between sperm and epithelia of the female reproductive tract and supports the migration hypothesis.

As we did not observe any passive sperm transfer by the internal fluid flow from the uterus to intramural UTJ through the CT, it is puzzling how spermatozoa enter the intramural UTJ. Although we proposed a hypothetical model of sperm passage through the CT in which the sperm hook plays an important role by providing an anchor-like role that prevents backward movement but allows head-forward sliding, further investigation with new experimental and observational tools will be necessary. Nevertheless, our findings suggest that the CT is a key female anatomical structure that controls sperm entry to the UTJ and influences sperm competition. If sperm passage through the CT is too easy, associated risks such as polyspermy or pathogen transmission that incur fitness costs for females also increase (Firman and Simmons, 2013; Mahabir et al., 2008). Therefore, the number of sperm passing through the CT should be balanced by the conflict between the two sexes depending on species specific mating systems and the degree of promiscuity. This conflict then gives rise to an evolutionary arms race, with male's sperm fertilization ability on one side, which includes aspects such as sperm swimming speed, and the female's sperm selection process on the other, which encompasses elements like physio-chemical barriers in the female reproductive tract (Firman et al., 2017; Lüpold and Pitnick, 2018; Simmons and Wedell, 2020). Therefore, species variations in the length of the sperm hook and head asymmetry in rodents suggested in the previous studies (Tourmente et al., 2016; Varea-Sánchez et al., 2016) will also reflect the mechanical and structural characteristics of the CT and lumen in the intramural UTJ.

In the current study, we could not confirm evidence of sperm cooperation via sperm train formation that aids faster sperm swimming. Instead, we discovered a new form of potential sperm cooperation – synchronised sperm beating that resulted from spontaneous unidirectional sperm clustering. Based on these observations, we propose that the asymmetry of the mouse sperm head with apical hook plays a crucial role in synchronised sperm beating (sperm cooperation) by facilitating unidirectional sperm re-arrangement at the uterine wall. The asymmetrical head shape in the house mouse, therefore, may have evolved not only to facilitate sperm migration but also to facilitate such sperm self-organised behaviours including unidirectional clustering and following beating synchrony. Under this scenario, sperm cooperation in house mice is not only mediated by sperm train formation as in some rodent species (Fisher and Hoekstra, 2010; Moore et al., 2002), but mediated by the synchronised sperm beating that obstruct migration of other spermatozoa in the uterus. Unidirectional sperm clustering and its potential role in sperm migration were also suggested in a previous study using fixed and tissue-cleared samples with in-

vitro live sperm analysis (Qu et al., 2021). We could not find any supporting evidence that such clustering helps sperm passage through the CT. However, our observations regarding self-organised sperm behaviour provides insights into the evolution of the asymmetrical structure of the sperm head in rodent species. These findings present an opportunity to reconcile two hypotheses: the cooperation hypothesis (Fisher and Hoekstra, 2010; Immler et al., 2007; Moore et al., 2002) and the migration hypothesis (Firman and Simmons, 2009; Smith and Yanagimachi, 1990).

The evolution of sperm characteristics, including the sperm hook, is a complex process influenced by several factors. For instance, sperm competition between ejaculates is a significant driving forces influencing sperm head morphology and behaviour in rodents (Fisher et al., 2014; Immler et al., 2007; Tourmente et al., 2016; Varea-Sánchez et al., 2016). Cryptic female choice also plays a crucial role in the evolution of sperm head shape and sperm kinetic characteristics (Birkhead, 1998; Eberhard and Lehmann, 2019; Firman et al., 2017). Further investigation of sperm behaviour inside the female reproductive tract or tissue mimicking microfluidic devices with real-time deep tissue imaging as in the current study, will provide valuable opportunities for a more comprehensive examination of both sperm-sperm and sperm-epithelium interactions in the female reproductive tract. This will help us better understand not only sperm competition and cryptic female choice in mice, but also those in other animals, including humans. While current assessments of sperm health generally involve measuring sperm count, movement, and shape, the current study suggests that analysing interactions between spermatozoa and the female reproductive tract is important and warrants further exploration. Given the significance of sperm health for fertility, this work not only highlights the importance of interactions between male sperm and female reproductive tract in successful migration, but also opens new avenues for understanding different causes of infertility and possible targets for treatment.

# Materials and Methods

## Custom-built two-photon microscope

To observe sperm behaviour in the female mouse reproductive organ, we built a video rate (30 frames/second at 512 x 512 pixel resolution) Two-Photon Laser Scanning Fluorescence Microscope (2PLSM; Fig. S1). A tunable femtosecond pulse laser (Chameleon, Discovery) was tuned to a choice of wavelengths from 960, 970, 980 and 1000 nm to simultaneously excite GFP and Ds-Red for sperm imaging and for autofluorescence imaging of the reproductive tract. Imaging quality was found to be similar for these wavelength ranges. All images were taken using a water dipping low magnification high NA objective lens (Nikon 16X, 0.8NA). Video-rate imaging was achieved using a resonant Galvo scanning mirror system oscillating at 8 kHz. The laser power was actively controlled using a Pockels cell. Synchronization between the Galvos, sample/objective stages, Pockels cell, photomultiplier tubes (PMTs), and the data acquisition systems were controlled using ScanImage, ver. SI2021.1.0 (Pologruto et al., 2003).

## Mice preparation and mating

We used two male transgenic mice lines for mating experiments. We purchased B6D2-Tg(CAG/Su9-DsRed2, Acr3-EGFP)RBGS002Osb male mice that express DsRed in the mitochondria at sperm midpiece and EGFP in the sperm acrosome from Riken BRC, Japan, depositor: M. Ikawa (Hasuwa et al., 2010). We then conducted in vitro fertilization to produce specific pathogen-free (SPF) F1 mice. The fertilized eggs (2-cell stage embryos) were then artificially inseminated into SPF wild-type C57BL/6J females. After we confirmed successful production of transgenic F1 male mice by PCR, we confirmed the SPF status and formed two breeding colonies with the transgenic F1 males under the SPF condition. One breeding colony comprised two wild-type C57BL/6J females to better reproduce F2 generation. We also made breeding colonies that consisted of a transgenic C57BL/6J female, Cx3cr1tm2.1(cre/ERT2)Litt/WganJ (JAX stock #021160, Cx3cr1 female) that expresses EYFP

in microglia (Parkhurst et al., 2013) to test whether sperm functionality changes in other mice including double-transgenic mice. When F2 mice got older than 6 weeks, they were transferred to another room where mating experiments were conducted. After transfer, each male mouse for mating experiments was single-caged. We used the F2 males that derived from both colonies that had the two genes (CAG/Su9-DsRed2 and Acr3-EGFP; RBGS male) or three genes (CAG/Su9-DsRed2, Acr3-EGFP, and Cx3cr1; RBGS-Cx3cr1 male) for mating experiments. We could not find any phenotypic difference in the sperm of the two strains – sperm from both strains expressed red fluorescence at the midpiece and green fluorescence at the acrosome at the head. We confirmed that both strains of F2 males were fertile, and their sperm also successfully migrated through the female reproductive tract, from the uterus to the ampulla.

In total, we used 3 males that successfully mated with females due to space limits in the experimental room (Table S1). The 3 males were used repeatedly for all mating experiments in the current study except one vasectomized RBGS-Cx3cr1 male that was used only once for comparison of the CT structure for virgin and non-virgin female mice (Table S2). All mice were kept under a housing condition that allows free access to food and water with 12 hours of light and dark cycle (lighting from 6 to 18 o'clock, dark from 18 to 6 o'clock). Mating experiments were done under light conditions from 9 am to 12 pm for three hours. Oestrus was induced by exposing male bedding materials (wood shavings) that consisted of male excretion to females older than 8 weeks. Three to seven days after exposure to the bedding materials, oestrus was checked daily following previously established protocols (Byers et al., 2012). When we found oestrous females, we relocated one or two females to a single-caged male. When males showed no interest in the female (no mounting attempts) or the female rejected the male's mounting attempt for the first 10 minutes, we returned the female to its original cage. The returned females were not exposed to other males until the next mating trial on the next day or one week later. All females in the experiments had no birth records before successful copulatory plug-confirmed mating. However, some of them probably had multiple oestrous cycles given our multiple oestrus-inducing trials. We did not limit the age of females and males for our experiments to minimize the number of sacrificed animals. We observed the male's mating until we could observe ejaculation. To confirm male ejaculation, we checked the copulatory plug from the female genitalia after we observed ejaculatory behaviour – the male stops thrusting and holds the female for about 5 to 10 seconds when it ejaculates. After this ejaculatory behaviour, we waited for 2 minutes and if the male did not exhibit further mounting, we checked the copulatory plug from the female genitalia. When the male ejaculated, we kept them together for up to 3 hours then took out the female for imaging experiments.

## Ex vivo imaging with two-photon microscopy

Female mice were sacrificed by cervical dislocation after anaesthesia using 2% isoflurane inhalation which usually took less than 5 minutes. After euthanasia, the female reproductive tract with the copulatory plug was excised and washed with Dulbecco's modified Eagle's medium (Tayama et al., 2006) (DMEM; GibcoTM, cat. No. 21063029). After washing, the reproductive tract was attached to a tissue culture dish with tissue adhesive (3M Vetbond 1469SB). After attachment, we filled the dish with 37°C preheated medium that contained an equal amount of DMEM and modified human tubal fluid (mHTF; Fujifilm Irvine Scientific, cat. ID. 90126) medium. All media were stored for at least 1 hour in a 37°C preheated incubator with 5% of $CO_2$ concentration before use. The culture dish was then placed on the 37°C preheated metal mount of the two-photon microscope and imaged with varying laser power for different depths. Most of the images were taken with 512 x 512 pixel image size at 30 fps (262,144 pixels per frame). Multicolour imaging was performed where each frame of the image has two colour channels (red and green). If needed, we could increase the frame rate up to 110 fps or higher by reducing the acquired image size to 128 x 128 (16,384 pixels per frame). We conducted observation for about 3 to 6 hours. During our observation, the uterus continued contraction and relaxation cycles.

To image the entire depth of the reproductive tract, we applied tissue clearing to investigate the structure of the UTJ entrance (or colliculus tubarius, CT) using the C-Match solution (RI = 1.46, Crayon technologies, Korea). In brief, the tissue was first washed 3 times in PBS and fixed in 4% paraformaldehyde for 3 hours at 4°C in a refrigerator. After fixation, we washed the tissue 3 times with PBS and the absorbed residual PBS using paper towels. We then added C-Match to the sample and waited overnight and imaged it on the next day with the sample submerged in C-Match. We imaged three cleared samples from three different females. In one sample (**Figure 3A**), only the intramural UTJ part was excised from one unmated transgenic C57BL/6J female mouse – a hybrid female that was delivered from Cx3cr1 female and Thy1 male (JAX stock #030526) called Tg(Thy1-jRGECO1a)GP8.31Dkim (Dana et al., 2018). Another sample was from a female that was mated with a vasectomized RBGS-Cx3cr1 male. In this sample, the whole female reproductive tract was excised with the copulatory plug, so the intramural UTJ was covered by the uterus. This sample was used to compare the UTJ for virgin and non-virgin females. The final sample was from a wild-type female that was mated with an RBGS-Cx3cr1 male. For cleared tissue imaging, we acquired 3D volume images with 2 um Z-axis step size while averaging 20 (first sample) or 30 images (second sample) per slice to increase the signal to noise ratio using autofluorescence of the reproductive tract tissue.

## Sperm tracking and speed measurement

We used Fiji (Schindelin et al., 2012) to process acquired images and its plugin, called TrackMate (Ershov et al., 2022; Tinevez et al., 2017) to track sperm trajectory in the uterus. We extracted trajectories from 60 sequential images (duration 2 seconds) for sperm tracking when uterus movement was the smallest. We also used Turboreg (Thevenaz et al., 1998), an ImageJ plugin, to realign the images when there was a shift between images due to uterine movement. Additionally, out of eight stacked images, we cropped three to obtain a straight view of the uterine wall. After preparing the images, we targeted the sperm head to track sperm as the sperm head expressed EGFP which was easy to track. We used Thresholding Detector to select sperm heads and LAP Tracker to trace sperm trajectories using the TrackMate plugin. We also adjusted parameter values in the plugin to better select sperm trajectories. Our final parameter values are as follows: head radius (> 0.75 μm), frame-to-frame linking (10 ~ 11 μm), track segment gap closing (max distance: 10 ~ 11 μm, max frame gap: 2), number of spots in track (> 6), and max distance travel (2.5 μm). When there were artificial trajectories that were not from sperm, we manually removed the track. If the original parameters could not detect well or had too many false tracks, we adjusted two parameters; frame-to-frame linking (up to 12 μm), and track segment gap closing (only max distance up to 12 μm). We also tracked the trajectories of sperm trains using the same parameters and settings. However, to reduce computation time and prevent mis-tracking of non-sperm cells, we cropped the images and utilized 100×100 pixel images that contain the entire trajectory of each sperm train as well as other unlinked single spermatozoa.

To calculate sperm speed in relation to the distance from the uterine wall, we need to define the uterine wall. To define the uterine wall, we first selected images with straighter uterine wall and projected the extracted 60 sequential images into one plane by taking the maximum intensity projection with some adjustment of brightness and contrast using Fiji. For some images taken at a low magnification level that contained curved uterine walls, we used parts of the field of view that contained straight walls appropriate for our analysis. Next, we used the object selection tool of Adobe Photoshop CC (23.1.0 version) to automatically select the walls from the projected images. We then extracted the uterine wall image layer and pasted it to a blank image with white background. Finally, we extracted the wall coordinates by selecting the non-zero-valued pixels that formed the boundary of the uterine wall (blue coloured area in Fig. S2A). The boundary coordinates were converted to a micrometre scale based on the magnification and image resolution to normalize the units between different images obtained with different magnification factors. We fitted the uterine wall coordinates using linear regression. The fitted linear regression line was considered the uterine wall of each female (① in Fig. S2B) and used to calculate the distance and angle (radian) between sperm and the wall.

We measured the distance between sperm and the uterine wall by calculating the minimum distance between the mid-point of each sperm trajectory and the fitted line of the uterine wall (② in Fig. S2B). The angle between a sperm trajectory and the uterine wall was calculated by measuring the angle between the fitted line and a straight line that passed the first and last spots of the trajectory (③ in Fig. S2B). We then computed sperm progression speed parameters used in CASA (Amann and Waberski, 2014) using our sperm tracking data using the TrackMate plug-in. We first calculated curvilinear velocity, VCL by dividing the total distance travelled (μm) by total track time – the time (second) taken from the first point (spot) to the last point in a sperm trajectory. The straight line velocity, VSL was calculated by dividing the track displacement – the distance between the first and last spots of a sperm trajectory – by the total track time (second). We also calculated the linearity of forward progression, LIN of sperm by dividing VSL by VCL (range 0 to 1). Along with the above CASA-used parameters, we defined a new parameter, the straight line-to-sideward movement ratio (SWR) to estimate sperm migration linearity by comparing forward and sideward moving distances. SWR was calculated by dividing the track displacement (μm) of a sperm trajectory by the maximum sideward movement distance (μm) – the maximum distance between two parallel lines passing each point (spot) that parallel to the track displacement line (④ in Fig. S2B). Further information on the terms and parameters of the TrackMate plug-in used in the current paper is also described in a paper and manual by the developers (Ershov et al., 2022; Tinevez et al., 2017).

### Statistical analysis

All statistical analyses were done using R, version 4.3.0 (R Core Team, 2023). To estimate sperm swimming speed and linearity in relation to the uterine wall, we used data from 8 copulation experiments between 8 females and 3 males. We ran 4 generalized linear mixed models to test whether sperm move faster and straighter when they migrate along the uterine wall. We used log-transformed VCL, VSL, LIN, and SWR as response variables in each model. In all models, we included the angle and distance between the wall and sperm trajectories as explanatory variables. We also included whether we cropped the image or not (O or X) to check the effect of image cropping on the analysis. Male IDs and the date of experiments were included as random effects in all models to control possible individual variations in sperm and reproductive tract properties. All models did not violate assumptions. All full models were also compared with null models that only included random effect and all full models were significantly better than the null models. All variance influencing factors (VIFs) were less than 1.1 which indicates no serious drawback from collinearity.

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## Author Contributions

Conceptualization: HR, JP, Methodology: HR, KN, YJ, SL, JK2 (Jungmo Kim), JP, Investigation: HR, KN, BL, YJ, JP, Visualization: HR, KN, YJ, JP, Funding acquisition: HR, JK1, JP, Project administration: JK1, JP, Supervision: JK1, JP, Writing – original draft: HR, JP, Writing – review & editing: all authors

## Declaration of interests

Authors declare that they have no competing interests.

## Data and materials availability

Raw data on sperm trajectories in the female reproductive track is available at *https://figshare.com/s/82d3f991ba884af73898*. Custom codes for sperm tracking data analysis written with Matlab (version R2019b) are available at *https://github.com/YundonJeong/Sperm_tracking*.

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## Editors

Reviewing Editor
**Erika Bach**
NYU Grossman School of Medicine, New York, United States of America

Senior Editor
**Felix Campelo**
Institute of Photonic Sciences, Barcelona, Spain

**Reviewer #1 (Public Review):**

Summary:

The authors want to determine the role of the sperm hook of the house mouse sperm in movement through the uterus. They use transgenic lines with fluorescent labels to sperm proteins, and they cross these males to C57BL/6 females in pathogen-free conditions. They use 2-photon microscopy on ex vivo uteri within 3 hours of mating and the appearance of a copulation plug. There are a total of 10 post-mating uteri that were imaged with 3 different males. They provide 10 supplementary movies that form the basis for some of the quantitative analysis in the main body figures. Their data suggest that the role of the sperm hook is to facilitate movement along the uterine wall.

Strengths:

Ex vivo live imaging of fluorescently labeled sperm with 2-photon microscopy is a powerful tool for studying the behavior of sperm.

Weaknesses:

The paper is descriptive and the data are correlations.

The authors cannot directly test their proposed function of the sperm hook in sliding and preventing backward slipping.

https://doi.org/10.7554/eLife.96582.2.sa1

**Author response:**

The following is the authors' response to the original reviews.

Thank you for your time and consideration on our submission. We also thank the reviewers for their consideration and helpful comments. We have revised the introduction, results, and discussion sections of the revised manuscript in accordance with the reviewers' suggestions, which have enhanced the clarity of our work. Specifically, we have clarified that the aim of the study is to report newly discovered sperm behaviours inside the uterus via high resolution deep tissue live imaging, and to stimulate further studies and discussion in the field of postcopulatory sexual selection in mice based on our observations. To the best of our knowledge, many of the specific sperm behaviours described in our manuscript are being reported for the first time, proven through direct observation inside the living reproductive tract.

We have also restructured our manuscript and moved our hypothetical interpretations based on our experimental observations to the discussion section. We hope that these revisions have clarified our claims and that our revised manuscript effectively communicates the

importance of our findings and its values in prompting new questions and insight that encourage further studies. We believe that our work clearly demonstrates the importance of sperm/reproductive tract interaction, which cannot be adequately studied in artificial environments, and may become an important guideline for designing future experiments and studies.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors want to determine the role of the sperm hook of the house mouse sperm in movement through the uterus. The authors are trying to distinguish between two hypotheses put forward by others on the role of the sperm hook: (1) the sperm cooperation hypothesis (the sperm hook helps to form sperm trains) vs (2) the migration hypothesis (that the sperm hook is needed for sperm movement through the uterus). They use transgenic lines with fluorescent labels to sperm proteins, and they cross these males to C57BL/6 females in pathogen-free conditions. They use 2-photon microscopy on ex vivo uteri within 3 hours of mating and the appearance of a copulation plug. There are a total of 10 post-mating uteri that were imaged with 3 different males. They provide 10 supplementary movies that form the basis for some of the quantitative analysis in the main body figures. Their data suggest that the role of the sperm hook is to facilitate movement along the uterine wall.*

We thank the reviewer for summarizing our work and the critical review of our paper. As summarized, the sperm hook has been primarily associated with the sperm cooperation (sperm hook) hypothesis and the migration hypothesis. However, we would like to emphasize that the aim of our work is not to cross check between the two hypotheses. Our aim was not to disprove either hypothesis, but rather to develop an experimental platform that enables detailed observation of sperm migration dynamics within the live reproductive tract.

Through live imaging, we observed both the formation of sperm trains as well as interaction between the sperm and female reproductive tract epithelium. However, in our observations, we could not find advantage in terms of faster movement for the rarely observed sperm trains. While these events were infrequent in our experiments, we are not asserting that the sperm train hypothesis is invalid but rather reporting our observations as is.

The main findings of our work lie in the newly observed dynamic behaviours of mouse sperm interacting with the female reproductive tract epithelium. Specifically, tapping and associated guided movement along the uterus wall, anchoring and related resistance to internal fluid flow and migration through the utero-tubal junction, and self-organized behaviour while clinging onto the colliculus tubarius. We have extensively revised the manuscript structure to clarify our findings.

> *Strengths:*
>
> *Ex vivo live imaging of fluorescently labeled sperm with 2-photon microscopy is a powerful tool for studying the behavior of sperm.*
>
> *Weaknesses:*
>
> *The paper is descriptive and the data are correlations.*
>
> *The data are not properly described in the figure legends.*

> *When statistical analyses are performed, the authors do not comment on the trend that sperm from the three males behave differently from each other. This weakens confidence in the results. For example, in Figure 1 the sperm from male 3613 (blue squares) look different from male 838 (red circles), but all of these data are considered together. The authors should comment on why sperm across males are considered together when the individual data points appear to be different across males.*

Thank you for your comments and suggestions. We have revisited all figure legends and made the necessary amendments (shown in the red-lined manuscript). Please note that, for a better flow of the paper, the previous Figure 1 has been changed to Figure 2 in the revised manuscript.

Regarding the analysis using different males, we would like to explain the statistics used. We used generalized linear mixed models to test the effect of the Angle and Distance to the wall on the migration kinetic parameters. The advantage of the generalized linear mixed models is that they consider individual variations in the data as an error term, thereby controlling such individual variations.

There are two main factors contributing to individual variations. One is, as you pointed out, the difference in sperm from different males. However, we used genetically similar mice, so genetical variations must be minimal. Nonetheless, there must be individual differences that caused variations including age, stress level as well as body conditions. As these factors cannot be controlled, we used the mixed model approach where individual variations are grouped within the individual. This approach enabled us to test the effect of each explanatory variable (Angle and Distance) within an individual.

The second factor that could cause variations is the female oestrous status. To avoid artifacts that could influence sperm behaviour, we did not use any invasive methods, such as hormone injections, to control or induce female oestrus. We controlled for this possible effect by including the mating date as a random effect. Since each female was used only once, the mating date reflects the variation caused by each female.

To provide further verification that the variation between individual males do not affect our results, we conducted analysis per individual male and mating dates (per each female). As clearly shown, sperm data points from individual males or female also show consistent clear correlations with the distance from the uterus wall. As pointed out, while the mean sperm speed could be different between individuals, they are not the topic we are interested in here. Our interest here is the effect of the distance between sperm and the uterine wall. Additionally, the variation between males is not always larger than those effect of the day (female), which in total suggest that integrating male variation is not essential. We have added this information to Supplementary Figure (Fig. S3) of the revised supplementary materials.

Moving forward, we can also consider the same analysis for the effects of the distance from wall on sperm SWR and LIN (linearity of forward progression) where no statistical significance was found. As see in the following figures, no statistically significant effect of the distance to wall on SWR and LIN are seen in that the regression lines drawn for each male and mating dates.

In summary, the statistical approach we used here has successfully reflected variations in sperm kinetics from different males as well as the variance from different females. We hope that our explanations and additional analysis answer your concerns.

> *Movies S8-S10 are single data points and no statistical analyses are performed. Therefore, it is unclear how penetrant the sperm movements are.*

With respect to Movie S8, Figure 4A and B (Figure 5A and B in the current revised manuscript) depict the trajectories of accumulated spermatozoa (sperm trains) in the female uterus, as shown in Movie S8. We have added this information to the revised figure legend (L 293) for clarity. We could not observe sperm trains that moved faster than single sperms during over 100 hours of observation and collection of over 10TB of images. The three sperm trains presented in Fig. 5B were the sperm trains that moved in the head-forward direction. Most other identifiable trains, or clusters, did not move or could not move forward as their heads were entangled randomly. Although we of course agree that a statistical test for Movie S8 (also Fig. 5B) would be great, due to the small number of sperm trains we found, we could not perform meaningful statistical tests. Instead, we provided all data in the box plots in Fig. 5C so that readers can evaluate and understand our points. We believe that this is a more neutral way of presenting our data rather than providing statistical significance.

Regarding Movies S9 and S10, we are not entirely sure whether we understood your comments clearly. It would be very helpful if you could point out more specifically to the manuscript with line numbers as we would like to address your concerns and suggestions, and we believe that your input will improve our manuscript. We did not describe the penetration of sperm in these movies. Movies S9 and S10 are newly found sperm behaviours inside the UTJ and Isthmus. We observed that sperm beating is influenced by the width of luminal space as well as internal flow as see in Movies S9 and S10. As our animal model only expresses red fluorescence in the midpiece, accurate beating frequency measurement cannot be performed. However, we can clearly observe that beating is not continuous and almost results in a halt with respect to reproductive tract variations. We revised our description about the findings about beating speed changes in the revised manuscript (LL 305-335).

> *Movies S1B - did the authors also track the movement of sperm located in the middle of the uterus (not close to the wall)? Without this measurement, they can't be certain that sperm close to the uterus wall travels faster.*

We revised the new Movie S1B to include videos that were used for the sperm migration kinetics analysis in Figure 2 (previously Figure 1). As you can see in the movies, the graph, and statistical analysis, there is a clear trend showing spermatozoa migration is slower as a function of distance from the uterus wall. Regarding your comment with respect to the middle of the uterus (not close to the wall), we have added another movie (Movie S1C) that was acquired at different depths from the wall (going towards the centre of the uterus). As clearly seen in Movie S1c, when imaging deeper into the uterus, there are an increasing number of inactive or slow-moving spermatozoa. Since the diameter of the uterus is easily over 2mm, we currently do not have optical access to exactly the centre of the uterus, but for all depths that are observable, spermatozoa near the wall were clearly faster.

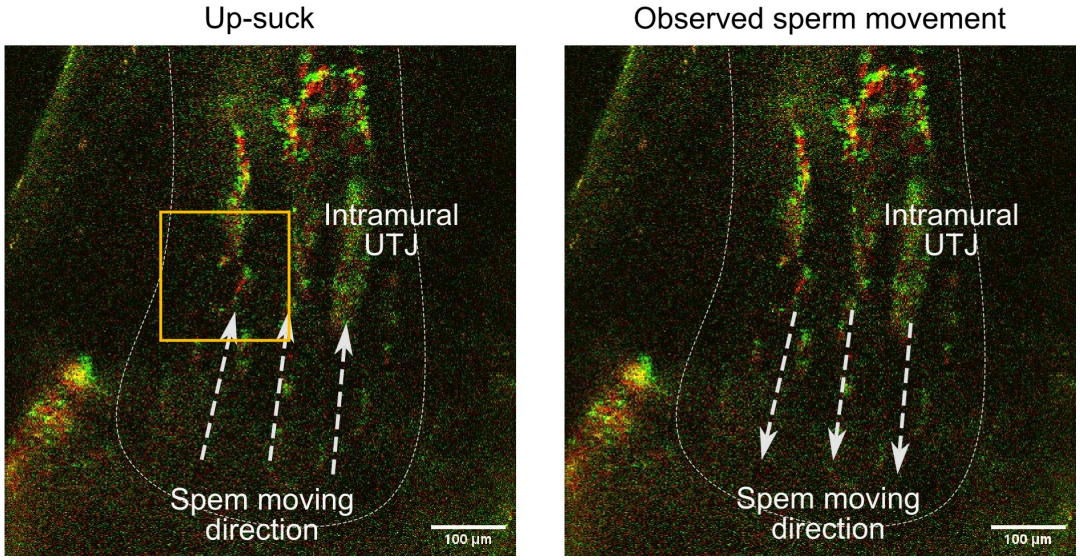
> *Movie S5A - is of lower magnitude (200 um scale bar) while the others have 50 and 20 uM scale bars. Individual sperm movement can be observed in the 20 uM (Movie 5SC). If the authors went to prove that there is no upsucking movement of sperm by the uterine contractions, they need to provide a high magnification image.*

The main focus of video S5A, is the intramural UTJ where spermatozoa are located in rows within narrow luminal space (see Author response image 1). When there is up-suck like sperm passive carriage, there must be sperm movement from the uterus to intramural UTJ as in Author response image 1 left. However, there is no such sperm movement could be seen in our observations, as shown in Movie 5A. Importantly, as you can see in Movie 5A, indicated by an arrow from 5 sec to 6 sec, some spermatozoa are moving downward (see also Author response image 1 right). This is the opposite direction of movement with respect to possible up-suck like sperm carriage.

Genetical evidence also support up-suck like passive sperm carriage is not the case for sperm migration from the uterus to UTJ. If environmental up-suck like passive transfer plays an important role, it is unlikely that genetically modified spermatozoa cannot pass the entrance of the intramural UTJ (Nakanishi et al., 2004, *Biol. Reprod.*; Li et al., 2013, *J. Mol. Cell Biol.*; Larasati et al., 2020, *Biol. Reprod.*; Qu et al., 2021, *Protein Cell*).

**Author response image 1.**

The left image represents what is expected when up-suck like passive sperm carriage occurs. The right image represents what is actually experimentally observed in the intramural UTJ (see Movie S5A). The direction of the arrowheads indicates the direction of sperm movement.




> *Movie S8 - if the authors want to make the case that clustered sperm do not move faster than unclustered sperm, then they need to show Movie S8 at higher magnification. They also need to quantify these data.*

We understand your concern. As shown in Figure 5B, we included all sperm kinetics data of each sperm train and unlinked spermatozoon around the trains as individual dots. The only analysis we did not conduct was a statistical test with the data as it could be erroneous due to the large sample size difference (3 trains vs 181 unlinked spermatozoa). As the medians of the four sperm kinetic parameters are similar except SWR, we concluded that they are not necessarily faster than unlinked single spermatozoa. Since there is no known advantage to spermatozoa (including sperm trains) with intermediate moving speeds for sperm competition – for example in IVF, success fertilization rate is high when faster and active spermatozoa with normal shape are selected (Vaughan & Sakkas, 2019, *Biol. Reprod.*) – it is questionable whether there can be an advantage to the formation of sperm trains whose speed is not faster than unlinked spermatozoa in our data.

However, we do not agree with your comment regarding the need for higher magnification. Measurement of the sperm migration speeds (kinetic parameters) does not require measurement of exact tail movements in this study. Only sperm heads were tracked to measure their trajectory and such tracking was better done at low mag. For example, measuring the speed of a car does not need higher magnifications to visualize the rotation of the wheels. Additionally, including the effect of observation magnification on the sperm kinetic parameters for all 4 GLMM models for Figure 2 (Table S3) does not change the result, which shows that magnification is not a factor that influences our analysis.

> *Movie S9C - what is the evidence that these sperm are dead or damaged?*

Thank you for your valid comment. We tracked sperm movements for at least 10 minutes and such entangled spermatozoa in the UTJ never became re-active. As you can see in the new Movie S9b, entangled spermatozoa were also acrosome re-acted (green acrosome head is gone) while active spermatozoa are responding to peristaltic movement by exhibiting movements within the same video. However, as you pointed out, we did not measure their viability with appropriate dyes. Although we also considered about extracting these spermatozoa and performing viability tests, we could not come up with a way to specifically extract the exact spermatozoa that were imaged. Considering your comments, we changed the term damaged or dead to inactive in the revised manuscript (LL 313-316, Legend Figure 6D. LL 380-384).

> *Movie S10 - both slow- and fast-moving sperm are seen throughout the course of the movie, which does not support the authors' conclusion that sperm tails beat faster over time.*

There must have been a misunderstanding. We did not indicate that sperm beating got faster over time anywhere in the main manuscript, including the figure legend and related movie captions. As correctly pointed out, the sperm beating speed changes over time (not getting faster over time) and shows a correlation with internal fluid flow and width of luminal space (LL 320-332). Please let us know if you meant something else.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The specific objective of this study was to determine the role of the large apical hook on the head of mouse sperm (Mus musculus) in sperm migration through the female reproductive tract. The authors used a custom-built two-photon microscope system to obtain digital videos of sperm moving within the female reproductive tract. They used sperm from genetically modified male mice that produce fluorescence in the sperm head and flagellar midpiece to enable visualization of sperm moving within the tract. Based on various observations, the authors concluded that the hook serves to facilitate sperm migration by hooking sperm onto the lining of the female reproductive tract, rather than by hooking sperm together to form a sperm train that would move them more quickly through the tract. The images and videos are excellent and inspirational to researchers in the field of mammalian sperm migration, but interpretations of the behaviors are highly speculative and not supported by controlled experimentation.*

Thank you for your critical review and valuable comments on our manuscript. As pointed out, some of our findings and suggestions were largely observation based. However, to the best of our knowledge, many of our observations are novel, particularly in the context of live imaging inside the female uterus and reproductive tract. We believe these observations open doors to many questions and follow up studies that can be envisioned based on our findings, which is what drives science forward.

That being said, we entirely agree that many follow up experiments need to be designed and performed, especially to validate the exact molecular mechanisms of the observed dynamics. We acknowledge that it is unfortunate we currently lack the proper molecular experimental toolsets to perform further tests. We have removed much of the hypothetical discussions from the results section and moved them to the discussion section. We hope that our revision more clearly defines the observed experimental data and our interpretations.

> *Strengths:*
>
> *The microscope system developed by the authors could be of interest to others investigating sperm migration.*
>
> *The new behaviors shown in the images and videos could be of interest to others in the field, in terms of stimulating the development of new hypotheses to investigate.*
>
> *Weaknesses:*
>
> *The authors stated several hypotheses about the functions of the sperm behaviors they saw, but the hypotheses were not clearly stated or tested experimentally.*
>
> *The hypothesis statements were weakened by the use of hedge words, such as "may".*

We appreciate your helpful comments and have revised our hypotheses and suggestions accordingly. We have removed instances of “may” or revised it to be more direct. We have also moved most of our interpretations and hypotheses from the results to the discussion section.

It is important to note that experimental approaches to test what we suggested from our findings in the current ex-vivo observation platform are not trivial and require extensive investigation of several unknown factors of the female reproductive tract. For instance, obtaining detailed information on the chemical characteristics and fluid dynamics in the female reproductive tract is essential to build a microfluidic channel that accurately resembles the uterus and oviduct, replicating what we found in an extracted living entire organ. This poses a significant challenge and requires collaborative expertise from many labs, which we hope to build in the near future.

Furthermore, our biggest concern is that, even if we were to construct the appropriate microfluidic channel to test sperm migration, it is very likely that the sperm behaviours that we observed under natural conditions may not be replicated in artificial environments. This raises questions about whether in-silico or in-vitro findings can truly resemble what we reported here using the ex-vivo observation inside a living organ.

To share our experience related to this difficulty, at the initial stage of our study, we attempted sperm injection combined with fluorescent beads to visualize the fluid flow, as well as dyeing the female reproductive tract and spermatozoa after mating. However, none of these resulted in meaningful results. Another potential approach to perform similar research regarding our claims is using genetical engineering to indirectly confirm the influence of the sperm hook morphology on sperm behaviour. However, such an approach lacks a mechanical demonstration about how the sperm hook interacts with the female reproductive tract.

It is unfortunate that the sperm behaviours that we found and reported here are considered as highly speculative. The main findings of our work lie in the newly observed dynamic behaviours of mouse sperm interacting with the female reproductive tract epithelium. Specifically, these behaviours include tapping and associated guided movement along the uterus wall, anchoring and related resistance to internal fluid flow and migration through the utero-tubal junction, and self-organized behaviour while clinging onto the colliculus tubarius.

We have extensively revised the manuscript structure to clarify our findings and integrated our points in the introduction. Although we understand our following hypotheses may be considered speculative and the causative relationship between the sperm hook and its role in sperm migration requires further experimental approaches, we believe that the image-based

observation of dynamic behaviours of spermatozoa are solid. We believe our findings will facilitate further studies and discussion in the field of studies on postcopulatory sexual selection in rodents.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *The manuscript is written for an expert in a fairly small field. I recommend that the authors rewrite the manuscript to make it more accessible to people outside of the field. These suggestions include*
>
> *(1) Provide a diagram of the female reproductive tract in Figure 1.*
>
> *a. Indicate where sperm enter the tract and the location of the oocyte they are trying to reach.*
>
> *b. Label all areas of the uterus that are mentioned in this study and be consistent about the label.*
>
> *(2) All movies should have a diagram of the location of the uterus that is being imaged.*

Thank you for the great suggestion. We have added a diagram of the female reproductive tract in the revised Figure 1A. In response to your comments 1a and b, we have indicated such information by including eggs in the ampulla and arrows that indicate sperm migration direction. We have also labelled the name of the specific areas that were studied in the manuscript.

We are unsure how to integrate the diagram in all movies without reframing the videos, which could cause serious corruption of the files. More importantly, we think that adding the same diagram to all movies may complicate the visuals and disrupt indications and subject in the movie. Instead, we have referred to the common diagram (Figure 1A) in each movie caption, specifying where the video was taken. Thank you for the suggestion. With this information, we hope readers can now more easily understand where we made the observations.

> *(3) The major questions in the field need to be better described in the introduction.*

Thank you for your valuable suggestions and specific comments which have greatly helped improve our manuscript. We have revised our introduction and discussion sections by adding more literature reviews and integrating studies across a wider range of the postcopulatory sexual selection, as per your suggestion (LL 34-57, LL 385-398).

> *(4) The major question that the authors are trying to address should be described in the introduction.*

Thank you for the helpful suggestion. We have clarified in the introduction that our aim was to contribute to the field of postcopulatory sexual selection in rodents by advancing methodological progress and to stimulate discussion and future research on the function of the sperm hook in murine rodents (LL 76-94) based on our observations.

> *(5) A discussion of the sperm hook should be provided. How many species have this structure (or similar structure)?*

We have integrated your point into the revised discussion section. Essentially, most murine rodent species have sperm hooks (while their exact shapes differ). However, as there are over

500 species and not all of them have been tested, we do not know exactly how many of them have this structure. Therefore, we included paper references that examined species variations in sperm hook characteristics and their possible correlation with sperm competition (LL 385417) in the discussion. Additionally, we also included papers by Breed (2004) and by Roldan et al (1992) that investigated murine rodents with a sperm hook in the introduction section as well (LL 58-61).

> *(6) The figure legends must describe everything in the figure or movie.*

Thank you for the helpful suggestion. We previously thought that our figure legends may be too long. We have included further information in the figure legends and movie captions. We have also revised the movies by adding some clips following our revision (Movie S1).

> *Reviewer #2 (Recommendations For The Authors):*
>
> *Here are some specific concerns I had about the clarity of approach to experiments and interpretations of results.*
>
> *In the Introduction, the authors stated that the study was intended to determine the function of the hooks on the mouse sperm heads. However, in the Results section, the authors did not explain the rationale for the first set of experiments with respect to the overall objective of the study. In this experiment, the authors measured the velocities of sperm swimming in the uterus and found that the sperm moved faster when closer to the uterine wall (VCL, VSL). They concluded that migration along the uterine wall "may" be an efficient strategy for reaching the entrance to the uterotubal junction (UTJ) and did not explain how this related to the function of the hooks.*

Thank you for your critical comment and guidance. We have changed the order of Figure 1 and Figure 2 and revised the result section to integrate your points. At the initial stage of the study, we expected to find evidence of the function of sperm trains in aiding sperm migration in the female uterus (which has not been observed in the live uterus; previous works were done invitro with extracted sperm from epididymis or uterus after mating). However, what we found was something unexpected: dynamic sperm hook related movements facilitating sperm migration inside the female uterus by playing a mechanical role in sperm interaction with the uterine wall. These results that were presented in the previous Figure 2 has been reorganized as the new Figure 1.

Based on this observation, our research later moved to clarify whether such sperm-epithelium interaction indeed helps sperm migration. This led us to measure sperm kinetics in relation to their distance and angle to the uterine wall. We have revised our introduction and result parts by integrating these points. We hope that our revision will answer your questions. We have also reduced the use of ‘may’ or ‘can’ in the results section. In the revised manuscript, we have moved such hypotheses to the discussion section and focused on what we observed in the results section.

> *The authors proposed that the sperm hook "may" play a crucial role in determining the direction of migration. When sperm encountered a uterine wall, significantly more changed migration direction toward the pro-hook direction than toward the anti-hook direction. In Figure 2B, sperm behavior is not visually understandable nor clearly explained.*

Thank you for the helpful comments. We have removed “may” and “might” to make our claim clearer and more concise. We have also revised the previous Figure 2B by combining it with the previous Figure 2C (they have been combined into Figure 1C now). We have also revised Figure 1B by increasing the line thickness of the sperm trajectory of the pro-wall-hook

direction and added the anti-wall-hook trajectory. We hope that these revisions make the figure easier to understand.

> *In Figure 2E, are the authors showing that the tip of the hook is caught between two epithelial cells? Please clarify the meaning of this figure.*
>
> *Please clarify the difference between "tapping" and "anchoring".*

Thank you for the detailed comments. As you pointed out, we currently have no evidence whether sperm can be caught in epithelia inter-cellular gaps. We have revised this source of confusion by removing the gap in the revised figure (Figure 1E). We have also included the definition of anchoring (LL 142-143) and tapping (LL 128-130). Anchoring facilitates the attachment of sperm to the uterine epithelia. Such anchoring also involves the catching of the sperm head in the inter-mucosal fold or gap, particularly at the entrance of the intramural UTJ at the end of the uterus. Tapping is the interaction between the head hook and epithelia in which the sperm hook is tapping (or patting) on the surface. Sperm tapping can be a byproduct that results from flagella beating when spermatozoa migrate toward the pro-wall-hook direction along the uterine wall (epithelia) or can play some role in sperm migration. As we currently cannot draw a conclusion, we did not integrate the possible function of the tapping in the manuscript.

> *The authors proposed that opposite sliding of neighboring mucosal folds lining the UTJ would cause small openings to form, through which only perhaps one sperm at a time could enter and pass through the UTJ into the uterus. This hypothesis was not actually tested.*

Imaging inside deep tissue is challenging due to light scattering as it penetrates through biological tissue. While this is also true for the uterus, the intramural UTJ is especially difficult to image because the UTJ consists of several thick muscle and cell layers (see Movie S5A). Another challenge is that the peristaltic movement of the UTJ results in constant movement, making continuous tracking of single sperms while passing through the entirety of the UTJ impossible in our current experiments. We have moved this hypothesis to the discussion section and restated that this is a pure hypothetical model (LL 399-406). We hope that our model encourages the community in designing or establishing an improved ex-vivo observation system that may be able to test this hypothetical model in the near future.

> *Next, the authors hypothesized that sperm that encounter the small openings in the UTJ may then be guided onward and the hooks could prevent backward slipping. This was also not tested.*

As you've noted, the function of the sperm hook that aids in sliding and preventing backward slipping could not be tested directly in our ex-vivo observation platform that relies on natural movement of the living organ. However, we believe that these limitations also highlight the importance of continued research and the development of more advanced methodologies in this field.

We would also like to note that we provide direct observations of spermatozoa resisting internal flow due to reproductive tract contractions in Movie S3A, B as well as Movie S5B. We referred to these movies and pointed out the role of anchoring (sperm attachment) in preventing sperm from being squeezing out (LL 140-149, LL 224-241). Unfortunately, we cannot conceive of how this behaviour can be tested additionally in any uterus-resembling microfluidic device or ex-vivo systems. In line with your suggestion, we have rewritten the related result section and moved our related discussions in the result part to the discussion section (LL 224-241, LL 399-417).

> *The authors observed that large numbers of uterine sperm are attached to the entrance of the UTJ. Some sperm clustered and synchronized their flagellar beating. The authors speculated that this behavior served to push sperm in clusters onward through the UTJ.*

We would like to note that we did not speculate that sperm clustering and their synchronization could serve to push spermatozoa in a cluster to move onward through the UTJ. We only pointed out our observation in recorded videos, that generative flow from the clustered spermatozoa pushed away other spermatozoa as seen in Movie S7 (LL 261-264). Although such sperm cooperation is possible (blocking passage of later sperm), we cannot draw that conclusion from our observation. The possibility you pointed out (pushing sperm onward through the UTJ) was suggested by Qu et al in 2021 [Cooperation-based sperm clusters mediate sperm oviduct entry and fertilization, *Protein & Cell*] based on their observations on cleared dead reproductive tracts.

> *The authors found only a few sperm trains in the uterus, UTJ, and oviduct, so they could not measure sufficient numbers of samples to test whether sperm trains swim faster than single sperm. Without sufficient data, they concluded that the "sperm trains did not move faster than unlinked single spermatozoa."*

We would like to take this opportunity to clarify our claims. We do not claim that our current experiments can give the final verdict on whether the sperm train hypothesis for faster swimming is correct or not. The phrase “sperm trains did not move faster” was not intended to mean that the sperm train hypothesis is invalid. We did not draw a conclusion but dryly described the experimental data that we observed (LL 279-286). We would once again like to emphasize that the main claim of our manuscript is not to rule out the sperm train hypothesis, but to present the various dynamic interactions of the sperm head with the female reproductive tract. To make the statement more balanced, we revised the sentence as “observed sperm trains did not move faster or slower than unlinked single spermatozoa” (LL 281-282).

> *The authors hypothesized that the dense sperm clusters at the entrance into the UTJ could prevent the rival's sperm from entering the UTJ (due to plugging entrance and/or creating an outward flow to sweep back the rival's sperm), but they did not test it.*

We agree that we were not able to test such possible function of the sperm cluster at UTJ entrance. Following your concerns, we revised the result part (LL 256-264) by removing most of our discussions related to the observed phenomena. We also integrated some interpretation rather to the discussion section (LL 421-437) and suggested that future works using appropriate microfluidic channel designs or sequential double mating experiments may be performed for additional tests (LL 443-447). However, we would like to point out that Movie S7C clearly shows surrounding sperms that are swept away from the sperm clusters. Since the sperm density is high, this is almost equivalent to a particle image velocimetry experiment, and we can clearly see the effect of the outward flow generated by the sperm clusters.

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