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Sperm activation by heat shock protein 70 supports migration of sperm released from sperm storage tubules in Japanese quail (*Coturnix japonica*).

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Short title: Sperm activation by heat shock protein 70

Abstract

Systems for maintaining the viability of ejaculated sperm in the female reproductive tract are widespread among vertebrates and invertebrates. In birds, this sperm storage function is performed by specialized simple tubular invaginations called sperm storage tubules (SST) in the utero-vaginal junction (UVJ) of the oviduct. Although the incidence and physiological reasons for sperm storage in birds have been reported extensively, the mechanism of sperm uptake into the SSTs, sperm maintenance within and control of release from the SSTs are poorly understood. We showed that the highly conserved heat shock protein 70 (HSP70) stimulates sperm motility *in vitro*, and also that HSP70 expressed in the UVJ appears to facilitate the migration of sperm released from the SST. Quantitative RT-PCR analysis demonstrated that expression of *HSP70* mRNA in the UVJ increases before ovulation/oviposition. Gene-specific *in situ* hybridization and immunohistochemical analysis with a specific antibody to HSP70 demonstrated that HSP70 localized in the surface epithelium of the UVJ. Further, injection of anti-HSP70 antibody into the vagina significantly inhibited fertilization *in vivo*. In addition, we found that recombinant HSP70 activates flagellar movement in the sperm and that binding of recombinant HSP70 to the surface of sperm are mediated by interacting with voltage-dependent anion channel protein 2 (VDAC2). Our results suggest that HSP70 binds to the sperm surface by interacting with VDAC2 and activating sperm motility. This binding appears to play an important role in sperm migration within the oviduct.

Introduction

The ability to store sperm in the female reproductive tract is widespread among insects, fish, amphibians, reptiles, birds, and mammals (Birkhead & Moller, 1993; Holt & Lloyd, 2010; Holt, 2011). The females of these taxa employ a variety of specialized structures in their genital tracts to store sperm, including sperm reservoirs in mammals (Suarez, 2010), spermathecae in amphibians (Kuehnel & Kupfer, 2012), and spermathecae and seminal receptacles in insects (Wolfner, 2011). These structures store the sperm until ovulation or when the ovum is transported to the site of fertilization. Birds employ specialized simple tubular invaginations referred to as sperm storage tubules (SST) which are found in the oviduct (Bobr *et al*., 1964; Shindler *et al*., 1967; Frieß *et al*., 1978; Brillard, 1993). In domestic birds, such as chickens, turkeys, quails and ducks, these SSTs can store sperm that have entered the female reproductive tract for 2-15 weeks; in other bird species, sperm can be stored for varying periods (Bakst *et al*., 1994; Bakst, 2011). This extended viability in avian sperm contrasts markedly with relatively short life span of mammalian sperm, which only remain viable for several days, though the phenomenon of sperm storage had been demonstrated in some species of bat (Roy & Krishna, 2010).

Although SSTs of birds are located in the lamina propria of the mucosal folds in the utero-vaginal junction (UVJ) and in the infundibulum, the primary site of sperm storage are the SSTs in the UVJ (Burke & Ogasawara, 1969; Brillard, 1993). The spermatozoa are transported to the infundibulum, which is the site of fertilization and which also serves as a secondary sperm storage site (Shindler *et al*., 1967; Bakst, 1981). The precise mechanism of sperm storage in the avian oviduct has not been fully resolved since their discovery in the 1960s, primarily because direct observation of sperm movements in the oviduct is difficult due to the thickness and opacity of the oviduct wall. In addition, isolation and analysis of the fluids in the SST lumen that may be involved in sperm maintenance are difficult due to small luminal diameter of the SSTs (ca. 10-20 μm) and location of the SSTs (*i. e.* embedded in the UVJ mucosa). As a

result, only indirect methods, such as counting sperm recovered from different oviduct sections after insemination, or determining the sperm filling rate of the SST can be used to elucidate the mechanism of sperm storage and sperm migration in the oviduct (Burke & Ogasawara, 1969; Compton *et al*., 1978; Brillard, 1993). We previously employed similar techniques to show that the release of sperm from the SST is a regulated event during the ovulatory cycle, and that progesterone acts as a sperm-release factor in birds (Ito *et al*., 2011). Our observations indicated that the sperm release from the SST is orchestrated with the ovulations by the stimulation of progesterone in order to increase the chance of the fertilization. Nonetheless, our knowledge of the mechanism of the related events including sperm uptake into the SSTs, sperm maintenance within and control of release from the SSTs remains insufficient.

Recently, Holt and coworkers reported that the soluble fraction of the oviductal apical plasma membrane (sAPM) can both reduce sperm motility and enhance sperm viability in pigs (Satake *et al*., 2006). The same research group found that heat shock 70 kDa protein 8 (HSPA8), also referred to as Hsc70 or Hsp73, was present in the sAPM, and that recombinant HSPA8 exerts similar effects on sperm (Elliott *et al*., 2009). The heat shock protein 70 (HSP70) family contains multiple homologs, ranging in size from 66-78 kDa; these homologs are the eukaryotic equivalents of bacterial DnaK (Duagaard *et al*., 2007). Members of the HSP70 family are characterized by having highly conserved N-terminal ATPase and C-terminal protein binding domains. Binding of peptides to HSP70 stimulates the inherent ATPase activity of HSP70, and facilitates ATP hydrolysis and enhanced peptide binding (Duagaard *et al*., 2007). The nucleotide exchange and the substrate binding by HSP70 coordinates the folding of newly synthesized proteins, re-folding of misfolded or denatured proteins, the trafficking of proteins across cellular membranes, the inhibition of a protein aggregation, and the targeting of the protein degradation via the proteasomal pathway in the cells (Duagaard *et al*., 2007). Our preliminary proteomic analyses have shown that the soluble extracts of the UVJ mucosa contained HSP70, but we do not know whether HSP70 plays a role

in sperm storage or the migration of sperm in the oviduct of birds.

In this report, we present the first evidence demonstrating that HSP70 stimulated the sperm motility *in vitro*, and that the HSP70 expressed in the UVJ might support the migration of the sperm that are released from the SST in Japanese quail.

Materials and Methods

Animals and tissue preparation

Male and female Japanese quail, *Coturnix japonica*, 8-20 weeks of age (Motoki Corporation, Tokorozawa, Japan), were maintained individually under a photoperiod of 14L: 10D (with the light on at 05:00) and provided with water and a commercial diet (Motoki Corporation) *ad libitum*. The time of oviposition in each bird was recorded everyday in order to estimate the time of ovulation (ovulation occurs approximately 30 min after oviposition) (Etches and Schoch, 1984). We selected the birds regularly laid egg. Females were decapitated at 8, 14, 20 or 25 h after oviposition, and the funnel part of the infundibulum or the mid portion of the magnum, the isthmus, the uterus or the vagina was dissected (approximately 0.1 g wet weight each). The UVJ mucosa around the junction of the uterus and the vagina were dissected out and placed in physiological saline. The UVJ containing the SSTs were localized by stereomicroscopy and isolated with forceps and scissors under a stereomicroscopy according to the method of Ito *et al.* (2011).

All experimental procedures for the care and use of animals in the present study were approved by the Animal Care Committee of Shizuoka University (Approval number: 24-12).

Real-time PCR analysis

Total RNA was isolated from the dissected tissue using RNAiso (Takara Biomedical, Otsu, Japan) according to the manufacturer's instructions. Aliquots (0.5 μg) were reverse transcribed at 37˚C for 15 min with ReverTra Ace qPCR kit (Toyobo, Osaka,

Japan). The reaction product was subjected to real-time PCR according to the instructions for the Light Cycler Nano System with the FastStart Essential DNA Green Master (Roche Applied Science, IN, USA). Briefly, following a denaturing step at 95˚C for 10 s, PCR was performed using a thermal protocol consisting of 95˚C for 20 s, 55.4˚C for 20 s and 72˚C for 20 s in 20 μl buffer containing 0.2 μM of each primer. The sense and antisense primers used for *HSP70* amplification were 5'-GCTATTGCCTATGGCTTGGA-3' and 5'- CAGCTGTGGACTTCACCTCA-3', respectively (GenBank Accession number: EU622852). For normalization of the data, we amplified the *S17* gene (GenBank Accession number: AY232491, sense primer; 5'-CCAGACACCAAGGAGATGCT-3', antisense primer; 5'-GCCTCGTGGTGTTTTGAAGT-3') using same cycling conditions as *HSP70*. To normalize the data, ΔCT was calculated for each sample by subtracting the CT value of *S17* from the CT value of HSP70. For relative quantitation, ΔCT for the defined control group was subtracted from the ΔCT of each experimental sample to generate ΔΔCT. The ΔΔCT values were then used to calculate the approximate fold difference, $2^{-\Delta\Delta CT}$. The results were expressed as the *HSP70* mRNA/*S17* mRNA ratio.

Production of anti-HSP70 antiserum

Rabbit polyclonal anti-HSP70 antiserum was raised against bacterially expressed His-tagged HSP70 (from Met^{1} to Asp^{634}). Quail *HSP70* cDNA was amplified by PCR (cycling conditions: 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min for 30 cycles) in order to introduce *Bam*HI and *Sal*I sites upstream of the Met^{1} and downstream of the Asp^{634}, respectively. The sense and antisense primers used were 5'-GAGGGATCCATGTCAGGCAAAGGGCCGGC-3' and 5'-CAGGTCGACATCTACTTCTTCAATGGTTG-3', respectively. The PCR product containing the *HSP70* cDNA was digested with *Bam*HI and *Sal*I, and ligated into pCold TF DNA vector (Takara Biomedical) treated with the same restriction enzymes. The resulting construct was transformed into competent *Escherichia coli*, strain *BL21*

(Takara Biomedical), and an ampicillin-resistant clone was selected after the nucleotide sequence analysis was performed. Recombinant HSP70 or tag protein alone was expressed in the presence of 1 mM isopropyl-β-thiogalactopyranoside at 15°C for 24 h, and the protein was purified from the cell lysate using nickel resin (Novagen, Madison, WI, USA) according to the manufacturer's instructions. The purity of the recombinant HSP70 or tag protein was verified by SDS-PAGE followed by CBB staining.

A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized with the recombinant HSP70 as described previously (Kuroki and Mori, 1997). Briefly, the rabbit was injected subcutaneously at multiple sites along the back with a total of 1ml of an emulsion made by mixing equal volumes of Freund's complete adjuvant (Sigma-Aldrich, St Louis, MO, USA) and recombinant HSP70 (300 μg/ml). Booster injections with the same amount of antigen in Freund's incomplete adjuvant (Sigma-Aldrich) were made 6 and 8 weeks after the first immunization. Two weeks after the final injection, blood was collected and serum aliquots were stored at 4°C.

Effects of intra-vaginal injection of anti-HSP70 antibody

IgG of anti-HSP70 antiserum or normal rabbit serum was purified with rProtein A Fast Flow media (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. The recovered IgG was extensively dialyzed against PBS and diluted at a concentration of 2 mg/ml with PBS. IgG was intra-vaginally injected (50 μl/bird, n=6) in the morning and evening (evening corresponds to the time after oviposition). The next morning, the female was housed with males (one female with 2 males) and allowed to copulate for 8 h. Oviposited eggs were collected everyday and the presence or absence of fertilization was observed visually by confirming the presence of an area pellucida and or area opaca in the blastoderm without additional incubation.

Gel electrophoresis and Western blotting

UVJ mucosa (approximately 0.1 g) isolated from two birds at 8, 14, 20 or 25 h after

oviposition was minced in 0.2 ml ice-cold PBS supplemented with 0.1 m mol/l phenylmethylsulfonyl fluoride (Sigma-Aldrich), 40 μg/ml bestatin (Sigma-Aldrich), 0.5 μg/ml leupeptin (Sigma-Aldrich), and 10 μg/ml soybean trypsin inhibitor (Sigma-Aldrich), and extracted on ice for 3 h with occasional vortexing. Debris was removed by centrifugation at 800 x *g* for 5 min and the supernatants were centrifuged at 20,000 x *g* for 10 min at 4°C. The supernatant of the sample was used as the UVJ extract. The protein concentration of the sample was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

SDS-PAGE was carried out under reducing conditions as described previously (Laemmli, 1970), using 12% (w/v) and 5% (w/v) polyacrylamide for the resolving and stacking gels, respectively. For Western blotting, proteins separated by SDS-PAGE were transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) (Matsudaira, 1987). The membrane was then incubated with blocking buffer containing 5% (w/v) skim milk in PBS supplemented with 0.1% (w/v) Tween 20 for 30 min. The membrane was reacted with anti-HSP 70 antiserum (1:1,000) or anti-HSP70 antiserum preincubated with antigen protein (1:1,000) diluted with blocking buffer (Sasanami *et al*., 2002) and visualized using a chemiluminescent technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit IgG (Cappel, Durham, NC, USA). Chemiluminescence was detected using an ImageQuant^{TM} LAS 500 (GE Healthcare). Visualized blots were digitized using a Macintosh image analysis system (ImageJ v. 1.440, http://imagej.nih.gov/ij).

Semen collection and preparation

Semen was obtained from male quail during mating prior to ejaculation according to the procedure of Kuroki and Mori (1997). Semen obtained from two to three males was suspended in sperm extender (136 m mol/l NaCl, 5.4 m mol/l, KCl, 0.8 m mol/l $MgSO_4$, 1.26 m mol/l $CaCl_2$, 4.2 m mol/l $NaHCO_3$, 5.6 m mol/l glucose buffered at pH 7.4 with 10 m mol/l HEPES). The concentrations of sperm were measured with a

hemocytometer and sperm viability was assessed using a LIVE/DEAD sperm viability kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Sperm were incubated at 39 °C in all of the experiments.

To obtain the sperm membrane (SPML), the ejaculates were washed three times with ice-cold PBS with repeated centrifugation at 800 x g for 3 min at 4°C, and the final pellet was suspended in cavitation buffer containing 150 m mol/l NaCl, 20 m mol/l HEPES (pH 7.4). The suspension was cavitated with a cell disruptor (Parr Instrument Company, Moline, IL, USA) at 400 psig, and the cellular debris was removed by centrifugation at 10,000 x g for 10 min. The supernatants were further centrifuged at 158,000 x g for 30 min, and the precipitates were suspended in cavitation buffer containing 0.1% (w/v) Triton X-100, 0.1 m mol/l PMSF, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. After determining the protein concentration, the suspension was mixed with Laemmli's sample preparation buffer (Laemmli, 1970) and used as SPML. The SPML samples were stored at -80°C for far-Western blotting.

Far-Western blotting

For far-Western blotting, the SPML (5 μg per lane) samples separated by SDS-PAGE were electrotransferred to a PVDF membrane as described above. The strips were blocked with N101 blocker (NOF Corporation, Japan) for 30 min with shaking, rinsed with PBS and incubated with or without recombinant HSP70 (0.1 μg/ml) for 30 min at room temperature. After the incubation, the strips were washed with PBS and blocked again with the same blocker. After rinsing with PBS, the bound HSP70 on the membrane was visualized using the same procedure employed for Western blotting. After the detection, the PVDF membrane was washed with three changes of PBS, and stained with Coomassie Brilliant Blue (CBB). After staining, the image visualized by CBB was merged with the chemiluminescent image by means of imaging software (ImageQuant™, GE Healthcare) to identify the band that interacted with HSP70.

Detection of bound HSP70 on sperm surface

Ejaculated sperm isolated from 2-3 males were suspended in same semen extender as described above and washed three times with repeated centrifugation at 800 x *g* for 3 min. The washed sperm were then incubated with semen extender containing 30 μg/ml recombinant HSP70 for 30 min. After washing with three changes of sperm extender, sperm were fixed in formaldehyde (final concentration, 3.7% (v/v)) for 10 min at room temperature. After air drying, the slides were washed with PBS for 5 min, and the cells were incubated with PBS containing 1% BSA and 10% normal goat serum for 1 h for blocking. The cells were then incubated with anti-HSP 70 serum (1:100) or anti-HSP70 serum preincubated with antigen protein (1:100) for 2 h at 4 °C. After washing with PBS, they were incubated with Texas red-conjugated sheep anti-rabbit IgG (1:200, Cappel) for 1 h at 4 °C. After washing with PBS they were embedded in glycerol and examined under a fluorescence microscope equipped with an interference-contrast apparatus with a 40 x objective (BX 51, Olympus Optics, Tokyo, Japan).

LC-MS/MS analysis

The SDS-PAGE gel strip containing the SPML (20 μg) was stained with CBB. The piece of the gel (approximately 1 mm^2), containing the HSP70 binding protein identified as described above was excised. The proteins in the gel were processed for in-gel digestion using sequence grade trypsin as suggested by the manufacturer (Promega Corporation, Madison, WI, USA). The peptides recovered from the gel were analyzed by tandem mass spectrometry (MS/MS) (NanoFrontier eLD, Hitachi High-Technologies Corporation, Tokyo, Japan) according to the manufacturer's instructions. In order to identify the protein(s) obtained from the MS/MS data, a *de novo* sequencing software package, PEAKS, was used as described previously (Ma *et al*., 2003). To confirm the reliability of the *de novo* sequencing data, we used two other proprietary identification programs, Mascot (http://www.matrixscience.com/) and SPIDER (http://www.bioinfor.com/products/peaks/spider.php) as described previously (Perkins

et al., 1999).

Sperm motility analysis and intracellular Ca^{2+} imaging

Ejaculated sperm were incubated with sperm extender containing 30 μg/ml recombinant HSP70 or 10 μ mol/l erastin. Erastin is an anti-tumor agent binds to VDACs, more specifically to VDAC2 and alters its gating (Yagoda *et al*., 2007; Simamura *et al*., 2008). The concentration of erastin was adopted from Yagoda *et al*., 2007. Appropriate vehicle (dimethylsulfoxide for erastin and PBS for HSP70) was included in the incubation mixture for the control experiments. Motility was evaluated by observing by sperm in several areas of the petri dish directly using a stereomicrosope and their motility was scored on the 0-5 system of Wheeler and Andrews (1943). Sperm movements and waveforms of flagellar beating were observed using a phase-contrast microscope (BX51; Olympus Optics, Tokyo, Japan) with a 20x objective and recorded with a high-speed CCD camera (HAS-220; Ditect, Tokyo, Japan). Images were captured using a frame rate of 200 frames per second (fps). The linear velocities of the sperm and the amplitudes of the flagella were analyzed using Bohboh software (Bohboh Soft, Tokyo, Japan). Briefly, the flagellar movements of the sperm were recorded by a high-speed camera and 10 images taken at every 1/20 seconds were overlaid. The linear velocities of the sperm (μm/sec) and the maximum amplitudes of the flagellar bending (μm) were calculated from the overlaying images using Bohboh software.

For Ca^{2+}-imaging analysis, the ejaculated sperm (2×10^7 cells /ml) were incubated with 1 μ mol/l Fluo8H-AM, a fluorescent calcium indicator (Nacalai Tesque, Kyoto, Japan) for 10 min at 39 ˚C. After the incubation, the sperm suspension was added to sperm extender that was supplemented with or without recombinant HSP70 (30 μg/ml) or tag protein (30 μg/ml) and incubated for an additional 30 min. For control experiment, Fluo8H-AM loaded sperm were incubated in the extender containing vehicle alone (PBS). Ca^{2+} imaging was performed as described previously (Mizuno *et*

al., 2012) using an Olympus filter set (excitation filter, BP490-500; dichromatic mirror, DM505; emission filter, BA510-550) with a x40 objective and recorded on a personal computer connected to a digital CCD camera (ImagEM, C9100-13; Hamamatsu Photonics) at 50 fps using the imaging application Aquacosmos (Hamamatsu Photonics, Hamamatsu, Japan). The maximum fluorescent intensity of flagella from randomly selected 10 spermatozoa after subtraction of a background value was quantitated using ImageJ (v. 1.440, http://imagej.nih.gov/ij). The measurement was performed at least 5 points in one sperm. The experiment was repeated 6 times.

ATP assay

For measurements of intracellular ATP, the ejaculated sperm (2 x 10^7 cells /ml) were incubated with or without recombinant HSP70 (30 μg/ml) for 10 or 30 min before centrifugation at 800 x g for 3 min. After washing with sperm extender, the sperm pellet was dissolved in ATP assay reagent ("Cellno" ATP Assay reagent, TOYO B-Net Co, Tokyo, Japan) and the fluorescent signal was measured using an ImageQuant™ LAS 500 (GE Healthcare).

***In situ* hybridization**

The birds were decapitated, the UVJ was removed, and frozen sections were prepared for i*n situ* hybridization, which was performed as described previously (Yoshimura *et al*., 2008). The antisense 45-mer oligonucleotide probes for *HSP70* (5'-ACGTTTCAAGAATGGTCACCACGCAGCAACAGGCAGCAACAGGGC-3', 5'-GCACCACGTATTCCTGTGTGGGTGTCTTCCAGCATGGCAAAGTGG-3', 5'-CTGAACGTCAGTGCCGTGGACAAGAGTACAGGAAAGGAGAACAAG-3', 5'-CACCTCCATCACTCGTGCCCGCTTTGAGGAGCTCAATGCTGATCT-3') were labeled with [^{33}P] dATP (NEN Life Science Products, Boston, MA, USA) using terminal deoxyribonucleotidyl transferase (Gibco, Frederick, MD, USA). The probes were then mixed in equal amounts and used for hybridization. The sense probe was

also labeled as described above and hybridization was performed overnight at 42˚C. Washing was performed twice at room temperature for 30 min and at 55˚C for 40 min. After washing, the slides were coated with Kodak NTB-2 emulsion (Kodak, New Haven, CT, USA) and exposed for 7 days at 4˚C in a dark box. After exposure, the slides were developed in Kodak Dektol and mounted in water. The specimens were examined under a microscope equipped with an interference-contrast apparatus (BX 51, Olympus Optics).

Immunohistochemistry

To detect HSP70 protein in the UVJ, the UVJ mucosa was fixed in Bouin's fixative and embedded in Paraplast (Oxford Labware, St. Louis, MO, USA). The sections (4 μm thickness) were air dried then incubated with ethanol containing 1% H_2O_2 for 20 min in order to inactivate endogenous peroxidase after deparaffinization. After washing with PBS, the sections were blocked with PBS containing 1% BSA and 10% normal goat serum for 1 h. Immunohistochemical techniques, which were the same as those described previously (Sasanami *et al*., 2002), used anti-HSP 70 antiserum (1:100) or anti-HSP70 antiserum preincubated with antigenprotein (1:100) and HRP-conjugated goat anti-rabbit IgG (Cappel). The samples were lightly stained with hematoxylin. The immunolabeled sections were examined under a light microscope (BX51, Olympus Optics) using a Nomarsky filter.

Data analysis

Data were expressed as means ± SD and were analyzed for significant differences by ANOVA. Means were compared using Tukey's test. For percentage data, an arcsine square-root transformation was performed and the transformed data was compared by Student's-t test. The motility score comparisons between groups were made using Mann-Whitney U test. Differences were considered statistically significant when $P<0.05$.

Results

Expression of HSP70 is regulated during the ovulatory cycle

As shown in Fig. 1A, the levels of *HSP70* mRNA were high in the infundibulum, UVJ, and vagina, and low in the magnum, ithmus, and the uterus. We also examined the changes in the abundance of *HSP70* transcripts during the ovulatory cycle in the UVJ (n=3) (Fig. 1B) and found that *HSP70* expression levels increased dramatically 8 to 14 h after oviposition in the sample from the UVJ, with levels remaining high until 25 h after oviposition.

Western blot analysis showed that the anti-HSP70 antiserum reacted strongly with a 70 kDa band in the UVJ extracts under reducing conditions (Fig. 1C, lane 1). Conversely, no band was detected when the blot was reacted with anti-HSP70 antiserum pre-incubated with antigen proteins (Fig. 1C, lane 2), suggesting that our anti-HSP70 antiserum specifically binds to 70-kDa quail HSP70 proteins. We also determined HSP70 protein expression levels during the ovulatory cycle (Fig. 1D). In accordance with the mRNA expression pattern observed during the ovulatory cycle, the intensity of the HSP70 band was low at 8 h after oviposition. It increased in the sample 14 h after oviposition and was markedly higher at 20 and 25 h after oviposition.

In situ hybridization revealed that intense signals were observed on the surface epithelium of the UVJ (Fig. 2A, arrowheads) when the specimen was hybridized with an antisense probe, suggesting that the *HSP70* mRNA is transcribed in the UVJ. Although the signal is very weak, silver grains indicating the presence of mRNA were observed in the epithelial cells of the SST (arrows in Fig. 2A). These radiolabeled signals were not observed in specimens that had been hybridized with the sense probe (Fig. 2B). The immunohistochemical analysis shown in Fig. 2C revealed that immunoreactive HSP70 proteins were mainly localized in the surface epithelium of the UVJ. Positive signals were not detected in the epithelial cells of the SST. No such intense signals were observed when the specimens were incubated with anti-HSP70

antiserum pre-incubated with antigen proteins (Fig. 2D).

Effect of HSP70 on sperm motility in vitro

First, we injected anti-HSP70 antibody into the vagina, and then observed the fertility of the birds after natural mating. When the 6 birds were treated with nonspecific rabbit IgG, fertility was >70%, however, fertility decreased to <10% when birds were injected with anti-HSP70 antibody (Fig. 3A). Conversely, the anti-HSP70 antibody did not affect sperm uptake by the SST because, as in the case of normal IgG-treated birds (Fig. 3B), sperm was observed in the SSTs of anti-HSP70 antibody-treated birds (Fig. 3C). The percentage of SST in IgG-treated birds containing sperm 24 h after mating was 33.3 ± 17.4% (n=4, mean ± SD), which was not markedly different from that observed in anti-HSP70 antibody-treated birds (27.6 ± 7.3%; n=4, mean ± SD). These results indicate that the anti-HSP70 antibody neither interfere the sperm transport to the UVJ nor the entrance into the SST, but that it might affect sperm migration from the SST to the site of fertilization.

These findings prompted us to examine whether HSP70 affects sperm motility, directly. In order to confirm this hypothesis, we incubated the ejaculated sperm with recombinant HSP70 *in vitro*. After 60 min of incubation in the presence of recombinant HSP70 or tag protein alone, the motility score of HSP70 group was significantly higher than that of tag protein group (n=9, p value =0.015). The linear velocity of sperm incubated with tag protein alone remained high (approximately 80 μm/sec) for 30 min of incubation, before decreasing significantly at 60 min (Fig. 4A, gray bars). Conversely, in the presence of recombinant HSP70, the linear velocity of the sperm remained equivalent to that at the start of the incubation, even after 60 min (Fig. 4A, solid bars). In the absence of recombinant HSP70, wave amplitude decreased gradually, but significantly, reaching approximately half that at the start after 60 min (Fig. 4B, gray bars). On the other hand, the wave amplitude of sperm remained high until 60 min of incubation when recombinant HSP70 was included in the incubation mixture (Fig. 4B, solid bars). The representative waveform of the flagellum after 30 min of

incubation in the presence (Fig. 4C) or absence (Fig. 4D) of recombinant HSP70 is shown. As shown in the figure, the flagellar movements of the sperm were weak and vigorous in the absence and presence of recombinant HSP70, respectively. Moreover, the average maximum intensity of Fluo-8H fluorescence, a fluorescent calcium indicator significantly increase in the presence of recombinant HSP70 compared to that of control (Fig. 5A). The fluorescent level of the tag protein group was comparable with the control sperm. In addition, the ATP content of cells during incubation was high in the presence of HSP70 compared to the concentrations of the control (Fig. 5B). Taken together, these results indicate that HSP70 has the effect of maintaining vigorous sperm motility *in vitro*.

Binding of HSP70 to sperm surface

In order to understand how HSP70 affects sperm motility, we investigated whether recombinant HSP70 can interact with sperm. As a result, immunoreactive signals were clearly observed on the entire surface of the sperm (Fig. 6, panels A and B). These signals were considered specific as no such signal was observed when the same preparations were incubated with anti-HSP70 antiserum pre-incubated with antigen proteins (panels, C and D). To elucidate the nature of HSP70 binding to the sperm, we performed far-Western blot analysis. The SDS-PAGE results showed that HSP70 did bind to the approximately 35 kDa protein of the sperm membrane lysate (SPML) (Fig. 7A, lane +), indicating that HSP70 bound to the sperm through the interaction with the 35 kDa protein on the plasma membrane. By overlaying the chemiluminescent signal on the image of the polyvinylidene difluoride (PVDF) membrane stained with CBB, we successfully identified the 35-kDa immunoreactive band in the gel (arrow in lane CBB). We then excised this band from the gel, and performed MS/MS analysis of the protein. Using the *de novo* sequencing software, PEAKS, the amino acid sequences detected by MS/MS analysis were identified as being voltage-dependent anion selective channel protein 2 (VDAC2) (Fig. 7B and Table 1). The coverage score of the sequences obtained from MS/MS data was 42%, and two other software packages, Mascott and

SPIDER also found that the 35-kDa protein was VDAC2 with high reliability (Table 1). These results demonstrated that the SPML protein that interacts with HSP70 is VDAC2. To gain further support of the idea that VDAC2 involves in the process of the HSP70-mediated sperm activation, we incubated the ejaculated sperm with recombinant HSP70 in the presence or absence of erastin, a selective inhibitor for VDAC. As expected, both the linear velocity (Fig. 7C) and wave amplitude ((Fig. 7D) potentiated by recombinant HSP70 was returned to the control level in the presence of erastin.

Discussion

In this study, we demonstrated that the highly conserved protein HSP70 can activate quail sperm, and also that HSP70 maintains sperm motility for an extended period *in vitro.* Specifically, recombinant HSP70 potentiated sperm flagellar movements by increasing the wave amplitude, which they retained for at least 1 h of incubation. In addition, supplementation of recombinant HSP70 in the sperm extender increased both intracellular calcium and ATP concentration during incubation. Finally, recombinant HSP70 was found to bind on the sperm surface. This is the first demonstration of HSP70 affecting sperm motility in an avian species. Whether HSP70 impacts the beat frequency of the cilia lining the oviductal lumen is not known.

HSP70 is one of the most widespread molecular chaperone molecules and is central to maintaining cellular homeostasis in response to stressful cellular conditions (Duagaard *et al*., 2007). In general, intra-cellular localization of HSP70 within the cytosol and on organelles such as the nucleus, mitochondria, and the endoplasmic reticulum has been reported as this protein lacks a N-terminal signal sequence (Duagaard *et al*., 2007). However, Lancaster and Febbraio recently reported the presence of exosome-mediated release of HSP70 in human peripheral blood mononuclear cells under both basal and heat-stressed conditions (Lancaster & Febbraio, 2005). However, this process of protein export differs markedly from conventional protein secretory pathways because the release of the exosome is not interfered with brefeldin A, a classical inhibitor of membrane trafficking pathways in cells (Lancaster & Febbraio, 2005). While it is not

known whether the surface epithelium of the UVJ mucosa releases exosomes containing HSP70 into the lumen, ultrastructural analysis of turkey SSTs revealed the existence of small vesicular components in the lumen (Schuppin *et al*., 1984). In HeLa cells, overexpression of HSP70 has been reported to result in downregulation of oxidative phosphorylation in mitochondria; consequently, continuous upregulation of glycolysis compensates for any fluctuations in intracellular ATP (Wang *et al*., 2012). The ATP that is responsible for the flagellar movements in mouse sperm is mainly produced by glycolysis (Mukai & Okuno, 2004). Similarly, data from our preliminary experiments appear to show that glycolysis may play a key role in providing ATP for flagellum mobility in Japanese quail. Our data showed that deoxyglucose, an inhibitor of glycolysis, strongly blocked sperm motility (data not shown). Based on these findings it is possible that the enhancement of glycolysis by HSP70 in the sperm could possibly explain the activation of sperm motility observed in the present study. On the other hand, Froman and his colleague reported that rooster sperm motility is dependent on mitochondrial calcium cycling in the presence of extracellular sodium because the sperm rendered immotile by depletion of the extracellular Na^+ or by the treatment of CGP 37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger in the presence of Ca^{2+} (Froman &Feltmann, 2005). Since the mitochondrial Ca^{2+} cycle constitutes a control point for mitochondrial ATP production, these observations indicate fowl sperm obtain energy for flagellum movement, in part, from mitochondria. The additional proteome analysis reported by the same group using indicated that the glycolytic enzymes and related proteins such as glucose transporter 3, lactate dehydrogenase and phosphoglucokinase 1 were more abundant in the high sperm mobility line compared to those of the low sperm motility line (Froman *et al.,* 2011). Although we currently do not know the contribution of the mitochondrial respiration as an energy source for the sperm motility in Japanese quail. However, an increase of intracellular Ca^{2+} appears to be important for maintaining sperm motility. This assumption is supported by the finding in the chicken that sperm cell Ca^{2+} content were significantly greater in high

sperm mobility line than that of low sperm mobility line (Froman *et al.*, 2011). Further experiments will be needed to uncover the machinery of how HSP70 activates sperm motility. In addition, we are currently focusing on the isolation and analysis of exosomes from the oviduct in order to clarify the specific roles of the putative HSP70-containing exosomes in sperm migration within the oviduct.

Given that the vaginal and UVJ are in a spiral configuration bound by thick layers of connective tissue, it is not possible to make direct observations of sperm entering and exiting the SSTs. After natural mating, the ejaculated sperm are deposited into the vagina. However, it is has been reported that, in the turkey, more than 80% of the sperm are rejected from the vagina soon after mating (Howarth, 1971). In addition, less than 1% of the sperm that are inseminated into the vagina enter the SST (Bakst *et al*., 1994). To clarify sperm migration in oviducts, Das *et al*. observed the rate of SST filling after a single insemination event in chicken. Sperm artificially introduced into the vagina of a chicken reached the SST within an hour, and the rate of SST filling tended to increase until 24 h after insemination (Das *et al*., 2006). Although the mechanisms underlying sperm selection in this process are not known, the intrinsic mobility of sperm may be an important factor in the adovarian transport of sperm in the vagina and the uptake of sperm into the SST (Froman, 2003). Bakst (2011) speculated in his review that not only intrinsic sperm motility but also a fluid transport mechanism in the vagina might be responsible for rapid sperm transport to the UVJ. He also found that the luminal pH of hen's mid-vagina within 20 min after oviposition raised from pH 7.15 to 7.51 when the time reached to 8-12 h post-oviposition in the chicken and these variation in environmental pH could impact on the sperm motility (Bakst, 1980).

In our study, the intra-vaginal injection of anti-HSP70 antibody dramatically decreased the fertility of the birds, indicating the physiological importance of HSP70 on sperm migration in the oviduct. We expected that the injected IgG reached to the UVJ because our preliminary experiment of injecting Hoechst 33342 DNA dye into the vagina with same protocol with the experiment of IgG injection was able to stain the

nucleus of the surface epithelium of the UVJ (data not shown). Based on the expression pattern of *HSP 70* mRNA in the UVJ, luminal abundance of the HSP70 might become peak around 20-25 h after oviposition and the injected antibody could efficiently neutralize this protein released from the surface epithelium of the UVJ. However, the rate of SST filling in hens that were intravaginally injected with anti-HSP70 antibody did not differ from the control hens in the rate of SST filling. Considering these reports and the results of our current observations, HSP70 does not appear to affect the sperm movement during the passage in the vagina, which links to sperm uptake by the SSTs.

Our results show that *HSP70* mRNA is highly expressed in the infundibulum and in the UVJ, which is the site of sperm storage in the avian oviduct. In addition, we found *HSP70* mRNA is also actively transcribed in the vagina, which is thought to be the site of sperm selection in birds (Howarth, 1971). We observed that the expression of HSP70 in the UVJ increases before oviposition, indicating that HSP70 expression may be regulated during the ovulatory cycle. It is considered that the observed HSP70 expression pattern would facilitate fertilization as, based on our previous findings in which we demonstrated that sperm release from the SST occurred approximately 20 h after oviposition, the free swimming sperm would move up the oviduct after being released from the SST (Ito *et al*., 2011). The sperm released from the SST would likely be exposed to HSP70 in the lumen of the oviduct, activating sperm migration toward infundibulum, which is the site of fertilization. This assumption would also be supported by our findings as both *HSP70* mRNA and the HSP70 protein were strongly expressed in the surface epithelium of the UVJ where the sperm were initially released from the SST. In our previous results, sperm release from the SST is regulated under the stimulation of progesterone (Ito *et al*., 2011). Although we did not confirm the hypothesis that progesterone stimulates HSP70 expression and release from the surface epithelium of the UVJ, it is reasonable to suppose that progesterone stimulates not only the sperm release from the SST, but also enhances the HSP70 expression in the UVJ in

order to facilitate the fertilization in birds.

More importantly, we found that recombinant HSP70 specifically binds to the entire sperm surface through interacting with VDAC2. VDAC2 was originally characterized as a mitochondrial porin, however, 31 kDa VDAC2 proteins were also enriched from the plasma membrane fraction of human B-lymphocytes (Pinto *et al*., 2010; Sabirov & Merzlyak, 2012). Based on a recent proteomic study on human mesenchymal stromal cell surfaces, the presence of VDAC2 in the plasma membrane has also been suggested (Niehage *et al*., 2011). In addition, VDAC2 has recently been localized in the acrosomal or plasma membrane of human spermatozoa (Liu *et al*., 2011). These authors suggested that the plasmalemmal VDAC was involved in induction of the acrosome reaction through mediating Ca^{2+} transport; specifically, they showed how an ionophore A23187-induced increase in intracellular Ca^{2+} was inhibited in the presence of antibody against VDAC2 (Liu *et al*., 2011). Although we do not currently know how HSP70 modulates VDAC2 functioning by binding, or what form of intracellular signaling potentiates sperm motility, we did find that intracellular Ca^{2+} significantly increase in the presence of recombinant HSP70. Identifying the specific mechanism of how cell-surface VADC modulates sperm motility remains to be elucidated in the future.

In conclusion, we showed that the highly conserved protein, HSP70, activates sperm motility *in vitro*. Analysis of HSP70 expression revealed that *HSP70* mRNA and the HSP70 protein were expressed in the UVJ, where the protein was primarily localized in the surface epithelium. An increase in the expression level of HSP70 in the UVJ before oviposition implied that HSP70 expression was hormonally regulated during the ovulatory cycle. Moreover, HSP70 binding was detected on the entire surface of the sperm and the binding partner for HSP70 was found to be VDAC2. Since the timing of sperm release from the SSTs temporally coincides with a peak in HSP70 expression, it is possible that HSP70 activates sperm in the lumen of the UVJ, facilitating sperm migration toward the infundibulum, the site of fertilization in birds. The findings presented here may clarify the role of the oviducts in sperm migration from the SST to

the infundibulum in birds.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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12

Figure Legends

Fig. 1 **Expression of HSP70 in the oviduct.**

(A) Total RNA extracted from the infundibulum (Inf), magnum (Magn), isthmus (Ithm), uterus (Utr), utero-vaginal junction (UVJ) or vagina (Vag) was reverse transcribed with random primer, and aliquots were used in real-time PCR. Data were normalized with S17 and expressed as means ± SD of three independent experiments. (B) The UVJ isolated from the female birds at 8, 14, 20 or 25 h after egg-laying were processed for real time-PCR analysis using *HSP70* primers. Data were normalized with S17 and expressed as means ± SD of three independent experiments. Values with different letters are significantly different ($P < 0.05$). (C) The UVJ extracts (10 μg protein/lane) were separated by SDS-PAGE under reducing conditions, transblotted onto PVDF membrane, and detected with anti-HSP70 antiserum (lane 1, 1:1,000) or anti-HSP70 antiserum preabsorbed with antigen protein (lane 2, 1:1,000). (D) The UVJ isolated from the female birds at 8, 14, 20 or 25 h after egg-laying were extracted, and subjected to Western blot analysis using anti-HSP70 antiserum. Band intensities were quantified and expressed as the mean ± SD of three independent experiments. Values with different letters are significantly different ($P < 0.05$).

Fig. 2 **Localization of HSP70 in the UVJ.**

Autoradiograms of the UVJ sections after hybridization with ^{33}P-labeled antisense probe specific for *HSP70* (A) or sense probe (B) are shown. Inset in panel A shows the enlarged view of the hybridization site in panel A. Arrowheads and arrows in A and B indicate the surface epithelium of the UVJ and the SST, respectively. Representative results of two experiments are shown (n=2). Bar = 25 μm.

Immunohistochemical observations using anti-HSP70 antiserum (C) or anti-HSP70 antiseurm preabsorbed with antigen protein (D). Nuclei were counterstained with hematoxylin. Representative results of three experiments are shown. Bar = 50 μm.

Fig. 3 **Effect of intra-vaginal injection of anti-HSP70 antibody on fertilization in Japanese quail.**

Female animals injected with nonspecific rabbit IgG (2 mg/ml, 50 μl) or anti-HSP70 antibody (2 mg/ml, 50 μl) were mated, and the oviposited eggs were collected daily for 7 days. The eggs were cracked to observe whether embryo development had occurred in order to confirm fertilization. Fertility was calculated and expressed as mean ± SD. 6 birds were injected within each treatment. Asterisks indicate significant difference, $P < 0.001$. (B-C) UVJ mucosa of IgG-injected (B) or anti-HSP70 antibody-injected (C) animals were processed for histochemical observation by hematoxylin-eosin staining staining. Arrows in panel B and C indicate SSTs with sperm in the lumen. Representative results of three experiments are shown. Bar = 50 μm.

Fig. 4 **Activation of sperm motility by HSP70 in vitro.**

(A-B) Linear velocity of the sperm (A) and maximum amplitude of flagellar bending (B) during incubation (0, 30 or 60 min) in the presence of tag protein (gray bars, 30 μg/ml) or recombinant HSP70 (solid bars, 30 μg/ml) were measured. Data were expressed as mean ± SD of three independent experiments with 10 sperm. Values with different letters are significantly different ($P < 0.05$). (C-D) Sperm flagellar bending patterns at 30 min of incubation in the presence of recombinant HSP70 (C, 30 μg/ml) or tag protein (D, 30 μg/ml). Trajectories of three representative sperm for 1/40 sec are shown.

Fig. 5 **Effects of HSP70 on the $[Ca^{2+}]_i$ and $[ATP]_i$ in sperm during incubation.**

(A) Maximum Fluo-8H fluorescent intensity of the flagellar region in the presence of HSP70 (30 μg/ml) or tag protein (30 μg/ml) are shown. Control sperm were incubated with vehicle (PBS) alone. The data in figure were shown as a percentage of the mean of control values. Data were expressed as mean ± SD of 6 independent experiments with 10 sperm. Values with different letters are significantly different ($P < 0.01$). (B)

Intracellular ATP levels during incubation (10 or 30 min) in the presence of tag protein (gray bars, 30 μg/ml) or recombinant HSP70 (solid bars, 30μg/ml). Data were expressed as mean ± SD of three independent experiments. Values with different letters are significantly different ($P < 0.05$).

Fig. 6 **Binding of HSP70 on the surface of sperm.**

Ejaculated sperm were incubated with 30 μg/ml HSP70 for 30 min, fixed, smeared on a slide and detected with anti-HSP70 antiserum (Panels A and B, 1:300) or anti-HSP70 antiseurm preabsorbed with antigen protein (Panels C and D, 1:300). Sperm nuclei were counterstained with DAPI and merged with anti-HSP70-reactive signals (Panels B and D). Representative images of repeated experiments are shown. Bar = 50 μm.

Fig. 7 **Identification of binding partner for HSP70 in the sperm.**

(A) Sperm membrane (5 μg/lane) was separated by SDS-PAGE and electrotransferred to a PVDF strip. One strip was subjected to Coomassie Brilliant Blue staining (CBB). Intact strips were blocked with N101 and incubated with (+) or without (-) HSP70 (0.1 μg/ml). Washed strips were blocked again with the same blocker, incubated with anti-HSP 70 antiserum (1: 1,000), and then incubated with a horseradish peroxidase-labeled secondary antibody (1: 10,000). The 35 kDa band in the sperm membrane specifically binds to HSP70. (B) The deduced amino acid sequence of voltage-dependent anion selective channel protein 2 (GenBank accession number; P82013). Peptide fragments detected by MS/MS analysis appear in bold. (C-D) Linear velocity of the sperm (C) and maximum amplitude of flagellar bending (D) at 30 min of incubation in the presence of recombinant HSP70 (30 μg/ml), erastin (10 μM) or both were measured. The control sperm were incubated in the medium supplemented with vehicle alone. Data were expressed as mean ± SD of three independent experiments with 10 sperm. Values with different letters are significantly different ($P < 0.05$).

Table caption

Table 1

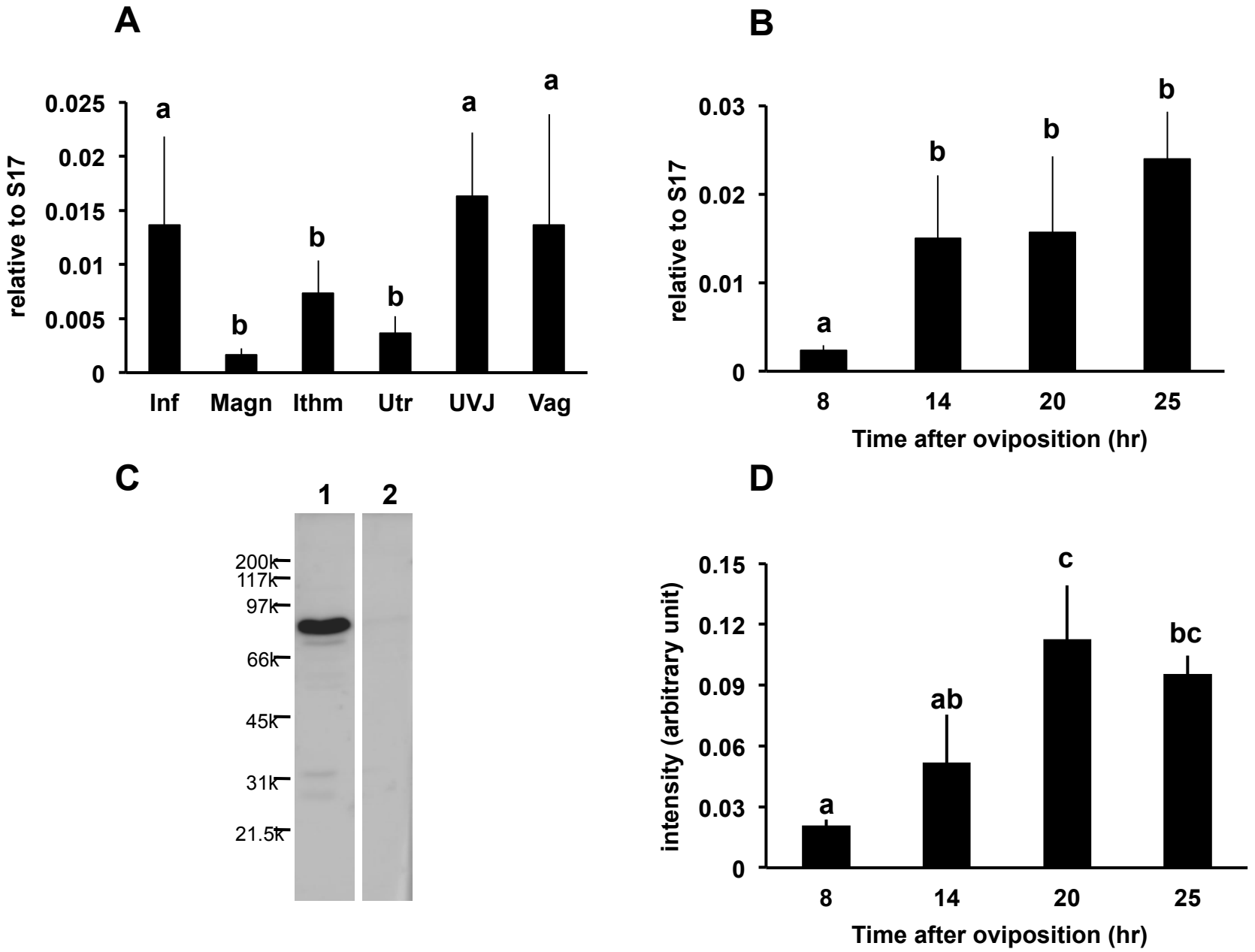
Summary of MS/MS analysis of HSP70-binding protein on sperm surface in Japanese quail.

Table 1

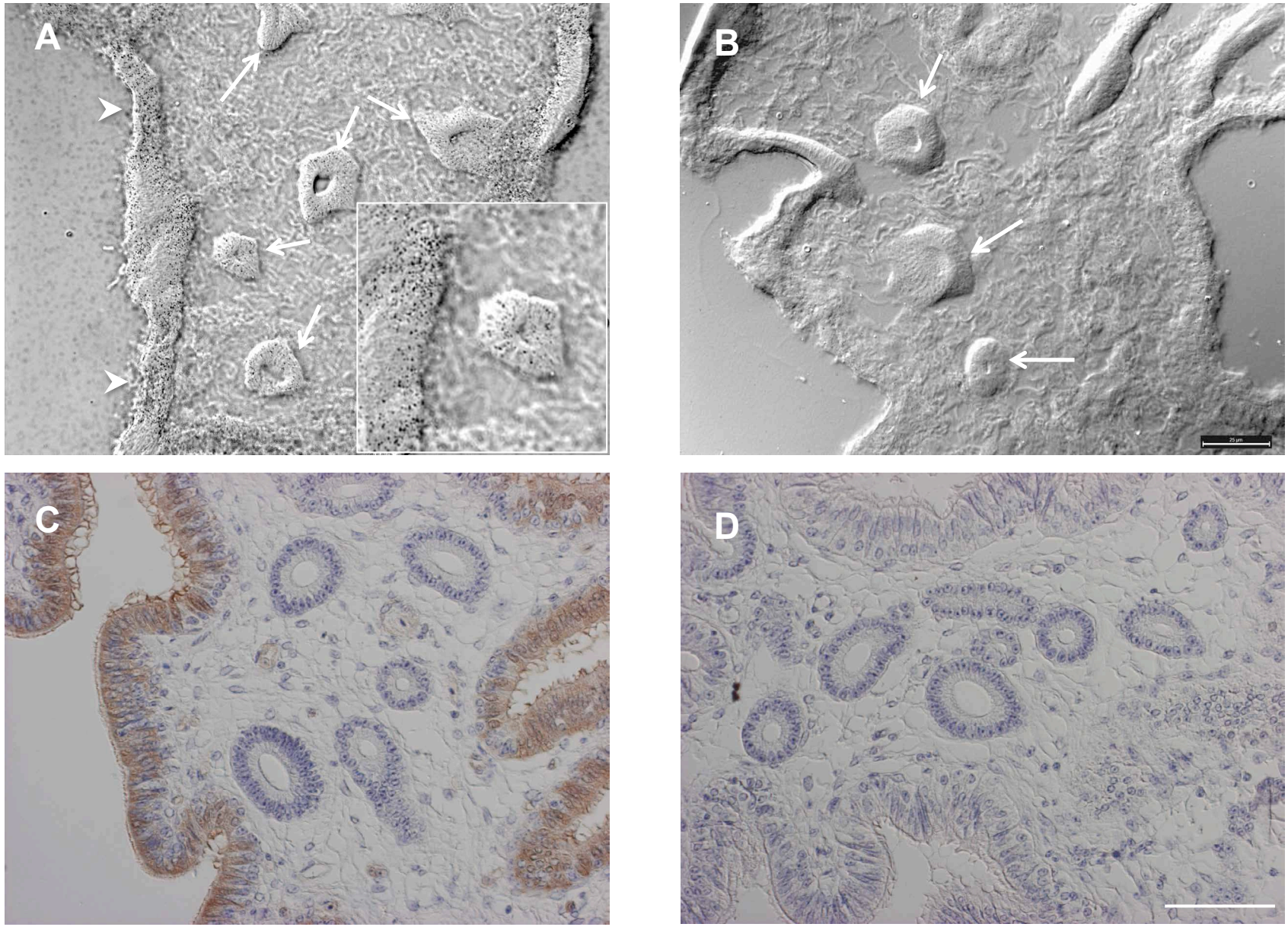
Summary of MS/MS analysis of HSP70-binding protein on sperm surface*

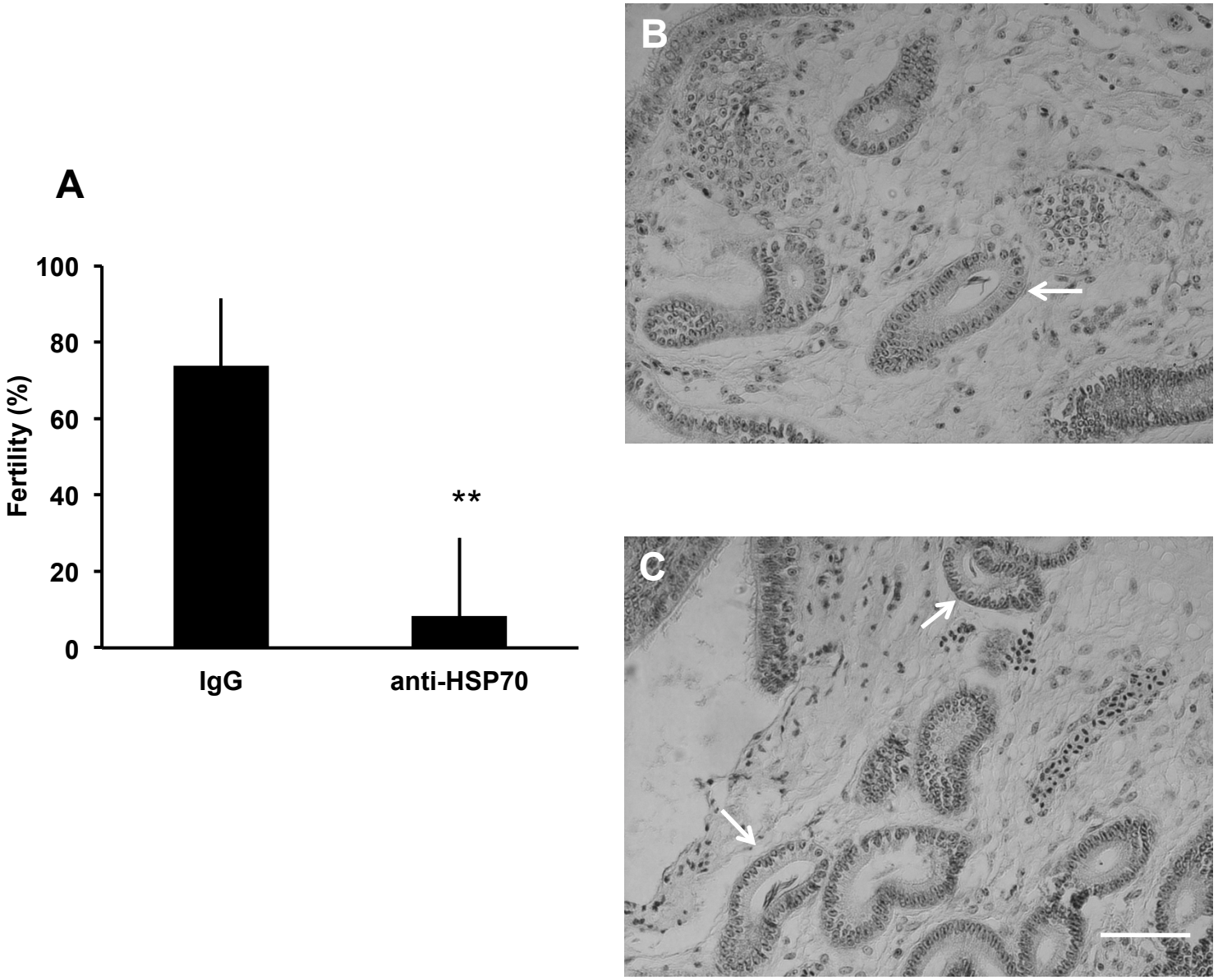
Amino Acid No.	Sequence	m/z observed	Charge	Mr(Calc)	PEAKS (Score %)	SPIDER (Score #)	MASCOT (Score)
20-27	GYGFGLVK	420.7515	2+	839.4541	98.3	31.1	56.2
34-52	SASGVEFTTSGSSNTDTGK	916.9418	2+	1831.8074	99.2	71.6	141.3
63-73	WAEYGLTFTEK	672.8547	2+	1343.6396	99.2	44.1	74.6
74-92	WNTDNTLGTEIAIEDQIAK	1066.572	2+	2131.0432	99.2	67.9	147.7
74-92	WNTDNTLGTEIAIEDQIAK	711.3925	3+	2131.0432	99.2	67.9	75.7
96-108	LTFDTTFSPNTGK	714.889	2+	1427.6934	99.2	46.9	66.1
96-109	LTFDTTFSPNTGKK	519.6307	3+	1555.7883	99.2	51.3	69.3
166-173	NNFSVGYK	464.7489	2+	927.445	93.3	30.4	31.3
174-196	TGDFQLHTNVNDGSEFGGSIYQK	838.7631	3+	2513.1458	99.2	46.0	103.7
256-265	LTLSALIDGK	515.832	2+	1029.6069	90.3	29.2	31.2
274-282	LGLGLELEA	914.554	1+	913.512	45.0	-	16.7

* All peaks are monoisotopic.

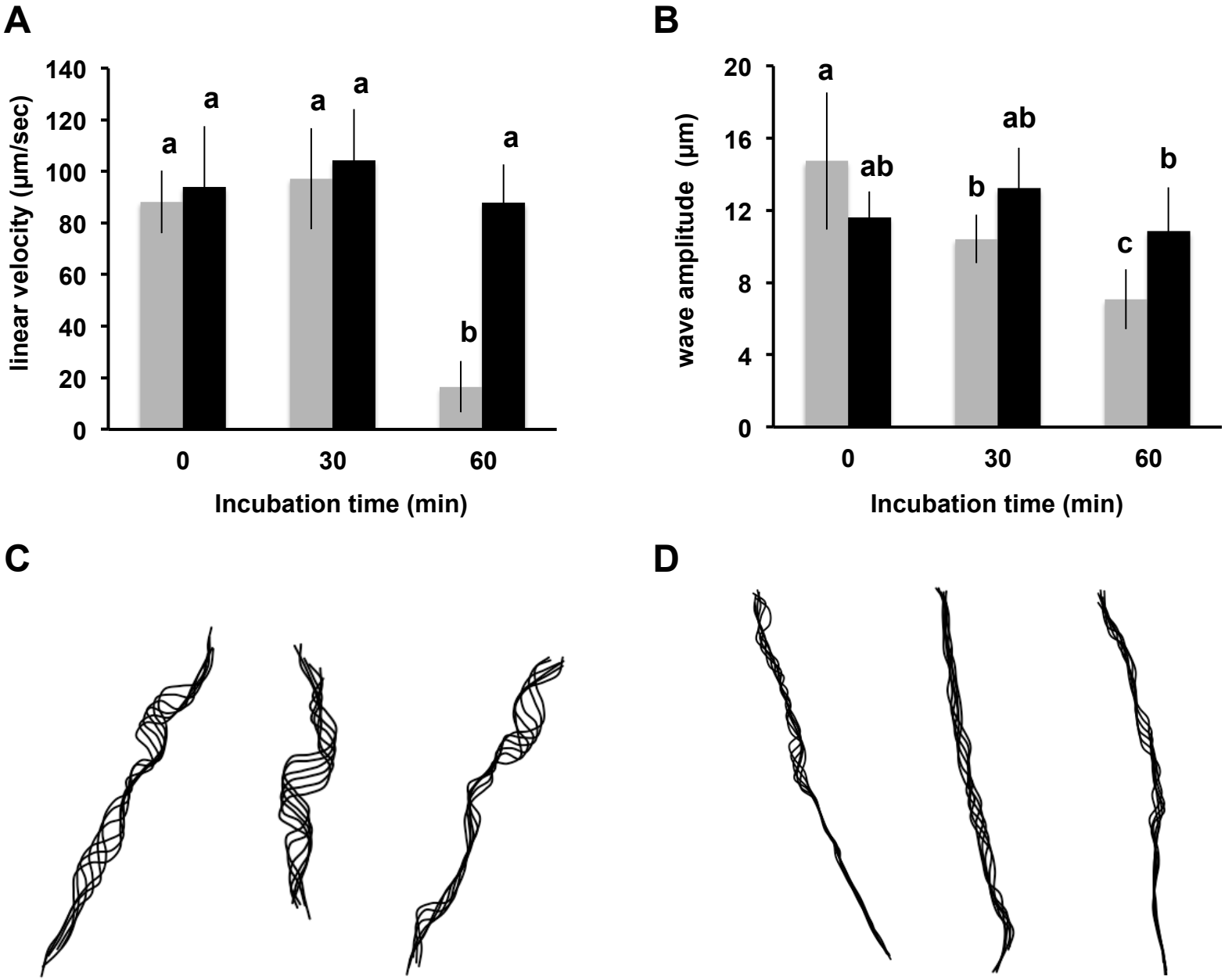

A
relative to S17
0.025
0.02
0.015
0.01
0.005
0
a
b
b
b
a
a
Inf
Magn
Ithm
Utr
UVJ
Vag
B
relative to S17
0.03
0.02
0.01
0
a
b
b
b
8
14
20
25
Time after oviposition (hr)
C
1
2
200k
117k
97k
66k
45k
31k
21.5k
D
intensity (arbitrary unit)
0.15
0.12
0.09
0.06
0.03
0
a
ab
c
bc
8
14
20
25
Time after oviposition (hr)

Hiyama et al., Fig.2

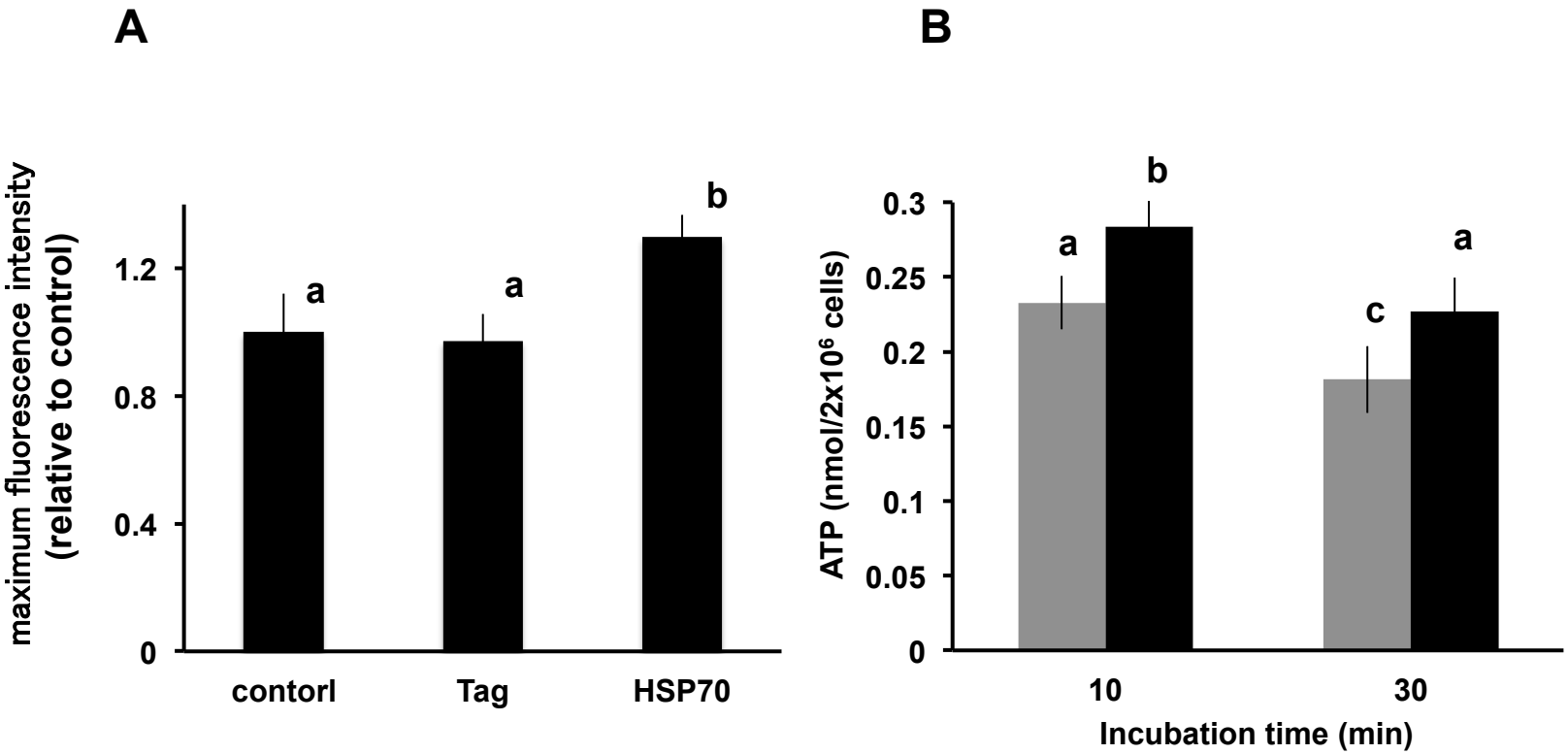



A
100
80
60
40
20
0
Fertility (%)
**
IgG
anti-HSP70
B
C

Hiyama et al., Fig.4



Hiyama et al., Fig.5



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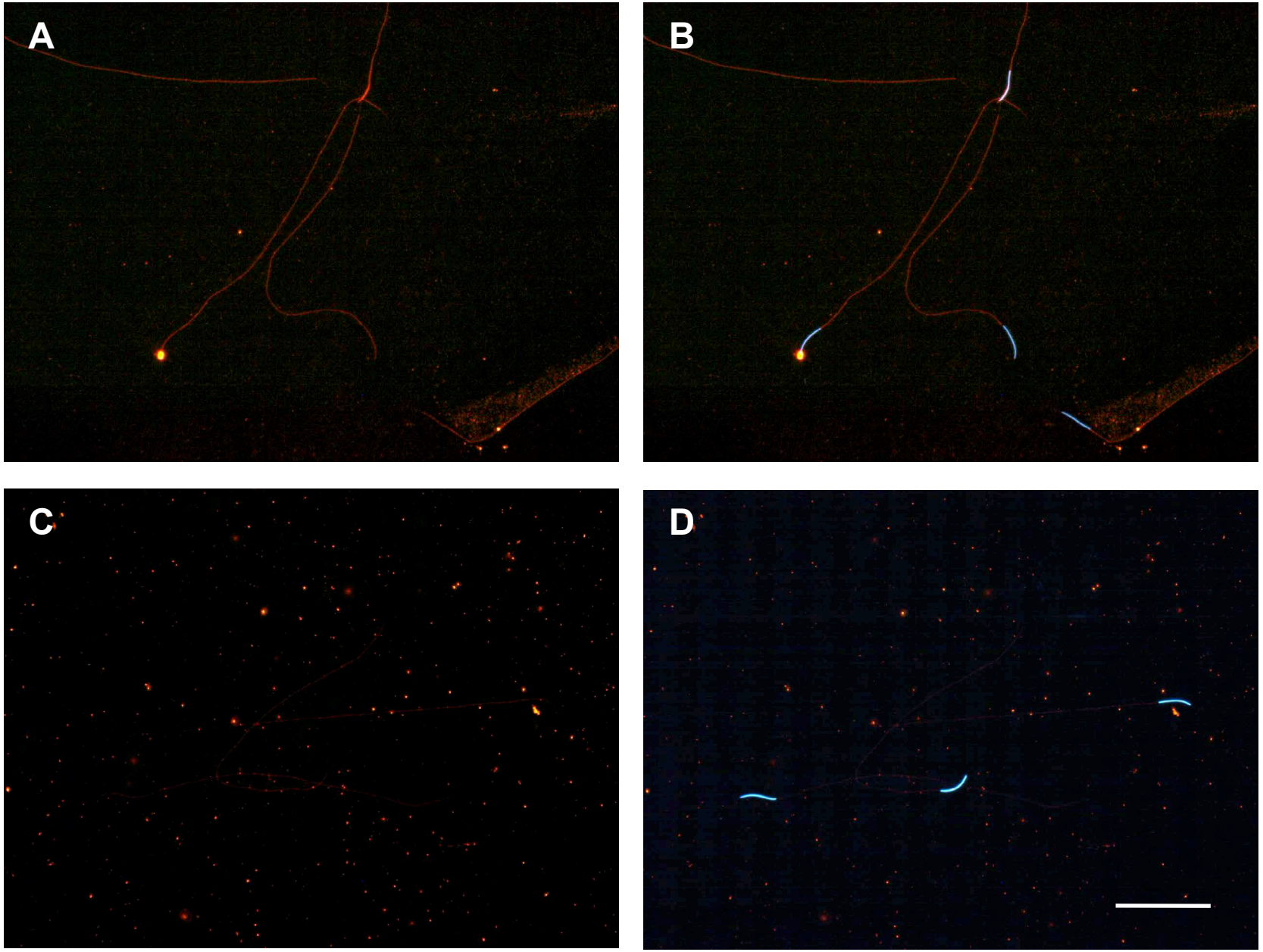

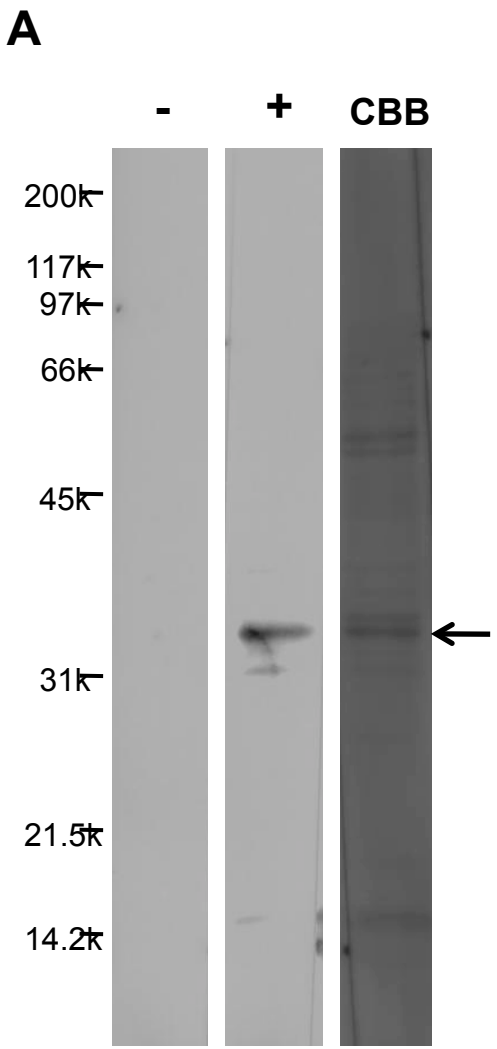

B

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  1 AIPPSYADLG KSARDIFNKG YGFGLVKLDV KTKSASGVEF TTSGSSNTDT
 51 GKVNGSLETK YKWAEYGLTF TEKWNTDNTL GTEIAIEDQI AKGLKLTFDT
101 TFSPNTGKKS GKIKSAYKRE CLNLGCDVDF DFAGPAIHGS AVFGYEGWLA
151 GYQMTFDSAK SKLTRNNFSV GYKTGDFQLH TNVNDGSEFG GSIYQKVSDN
201 LETAVNLAWT AGSNSTRFGI AAKYKLDSTA SISAKVNNSS LVGVGYTQTL
251 RPGVKLTLSA LIDGKSINAG GHKLGLGLEL EA
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