# Recombinant Beta-defensin 126 Promotes Bull Sperm Binding to Bovine Oviductal Epithelia

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Recombinant Beta-defensin 126 Promotes Bull Sperm Binding to Bovine Oviductal Epithelia

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ABSTRACT

Primate β-defensin 126 regulates the ability of sperm to bind to oviductal epithelial cells in vitro. Bovine β-defensin 126 (BBD126) exhibits preferential expression in the cauda epididymis of the bull but there have been few studies on its functional role in cattle. The aim of this study was to examine the role of BBD126 in bull sperm binding to bovine oviductal epithelial cell (BOEC) explants. BBD126 has been shown to be highly resistant to the standard methods of dissociation used in other species and, as a result, corpus epididymal sperm, which have not been exposed to the protein, were used to study the functional role of BBD126. Corpus epididymal sperm were incubated with recombinant BBD126 (rBBD126) in the absence or presence of BBD126 antibody. Addition of rBBD126 significantly enhanced the ability of epididymal sperm to bind to BOEC explants (P<0.05). Anti-BBD126 blocked the BBD126-mediated increase in sperm binding capacity. Ejaculated sperm, which is coated with native BBD126 protein but also a large number of seminal plasma proteins in vivo, was incubated with rBBD126 in the absence or presence of BBD126 antibody. Addition of rBBD126 significantly enhanced the ability of ejaculated sperm to bind to BOEC explants (P<0.05), while, rBBD126 reduced corpus sperm agglutination (P<0.05). These results suggest that similar to the role of its analogue in macaque, sperm with more BBD126 in their acrosome may represent sperm with more oviduct binding capacity.
β–Defensins belong to a family of antimicrobial peptides – short, structurally diverse proteins that kill microbes and have specific and specialised functions in innate immunity (Sass et al. 2010; Meade et al. 2014). The microcidal properties of the β–defensin family are well documented in mice and humans (Semple et al. 2015). In humans, β–defensin 3 has been found to disrupt staphylococcal cell wall biosynthesis (Sass et al. 2010) while, in mice, β–defensin 10 has been shown to have antimicrobial effects in vivo (Peyrin-Biroulet et al. 2010). Although most studies on these proteins have focused on their antimicrobial characteristics, there is a growing body of evidence for a role for β–defensins in reproductive function (Tollner et al. 2008a; Tollner et al. 2008b; Fernandez-Fuertes et al. 2016; Legare et al. 2017).

Sperm transit through the epididymis of the male reproductive tract evokes a restructuring of membrane proteins as part of the sperm maturation process (Girouard et al. 2011). Proteins secreted by the epididymal epithelium include membrane surface proteins involved in sperm membrane protection and interaction with the female reproductive tract (Nagdas et al. 2014) and the oocyte (Netzel-Arnett et al. 2009). Amongst the proteins that are secreted by the epididymal epithelium and that bind to the sperm surface, several members of the β-defensin family have been identified (Zhou et al. 2004; Yudin et al. 2008). It is postulated that these proteins may provide immune privilege to sperm as they navigate their way along the female reproductive tract to the site of fertilisation. In primates, there is evidence that one of these protein additions, Defb126, is preferentially expressed in the male reproductive tract and plays an important role in sperm motility, mucus penetration and sperm binding to oviductal explants in vitro (Tollner et al. 2008a; Tollner et al. 2012; Cornwall 2014; Dorin and Barratt 2014). In the macaque, Defb126 is secreted in the corpus and cauda epididymis where it has been reported to cover the entire surface of ejaculated sperm (Yudin et al. 2005). Defb126 is a major component of the sperm surface glycocalyx (Yudin et al. 2005), a dense coating of carbohydrate-rich molecules and glycoproteins (Schroter et al. 1999). This coating protein contains multiple sialylated oligosaccharides and has been shown to enhance migration of macaque sperm through cervical mucus in vitro (Tollner et al. 2008b) by conferring an increased negative charge.
(Tollner et al. 2008a). Upon arriving in the isthmus of the oviduct, sperm bind to the epithelial cells, thereby forming a sperm reservoir (Gwathmey et al. 2003; Hunter 2008; Holt and Fazeli 2010). This reservoir plays a critical role in successful fertilisation as sperm binding has been shown to select a fertile sperm population (Teijeiro et al. 2011; Coy et al. 2012; Holt and Fazeli 2015), extend sperm viability (Suarez and Pacey 2006) and regulate sperm function (Miller 2015). It is postulated that sperm bind to the oviductal epithelium via the plasma membrane overlying the sperm acrosome (Suarez 2002) and this binding usually, but not exclusively occurs on ciliated cells (Hunter et al. 1991; Lefebvre et al. 1995). There is evidence in several species that sperm bind to the cilia via glycans found on oviductal epithelial cells (DeMott et al. 1995; Lefebvre et al. 1997; Suarez 2001; Wagner et al. 2002) and in cattle, sperm binding is thought to be mediated by fucose recognition (Lefebvre et al. 1997). Fucose, found specifically in the Lewis A trisaccharide has been shown to have a regulatory effect on bull sperm binding (Suarez 2016). It has been proposed that oviductal plasma membrane receptor proteins containing Lewis A bind to surface proteins deposited on sperm during epididymal transit as well as from the seminal plasma at ejaculation (Ignottz et al. 2007). In macaque, Defb126 has been reported to play an integral role in sperm binding to oviduct epithelial cell explants in vitro (Yudin et al. 2005; Tollner et al. 2008a) while loss of Defb126 from the sperm head during capacitation is associated with a significant loss of the ability of sperm to bind (Yudin et al. 2005).

Furthermore, Tollner et al. (2004) reported that Defb126 must be released from the sperm surface in order for sperm to bind to the zona pellucida (Tollner et al. 2004). In humans, mutation in the Defb126 gene has been found to correlate with subfertility in men; sperm from individuals with the Defb126 variant exhibited reduced glycosylation and a reduced ability to penetrate mucus in comparison to those without the Defb126 variant (Tollner et al. 2011).

Using a comparative genomics approach, our group has recently discovered and profiled the expression of a cluster of novel β-defensin genes along the male and female reproductive tracts in cattle (Meade et al. 2014), horses (Johnson et al. 2016) and sheep (Hall et al. 2017). We have shown that bovine β-defensin 126 (BBD126) peptide, the ortholog of human Defb126, is expressed solely in the cauda epididymis of the mature bulls (Narcandi et al. 2011). It is not secreted in other regions of
the epididymis or in the testes nor is it present in seminal plasma of vasectomised bulls (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). BBD126 coats bull sperm predominantly on the tail and post acrosomal region of the sperm head (Fernandez-Fuertes et al. 2016), a pattern which differs from that described for the macaque ortholog which coats the entire sperm surface (Fernandez-Fuertes et al. 2016). The dissociation-resistant dimeric structure of BBD126 (Narciandi et al. 2016) has meant that capacitating agents previously reported to remove Defb126 from macaque sperm failed to remove the bovine ortholog (Fernandez-Fuertes et al. 2016). Furthermore, the addition of recombinant BBD126 (rBBD126) to corpus epididymal bull sperm, where the protein is not present, increased their motility and mucus penetration in vitro (Fernandez-Fuertes et al. 2016). Working with a large population (>7,000) of bulls used in artificial insemination our group has recently demonstrated that a haplotype spanning multiple β-defensin genes, including Defb126, was significantly associated with in vivo fertility. Functional analysis confirmed that sperm from bulls possessing this multi-defensin haplotype showed significantly enhanced binding to oviductal epithelium (Whiston et al. 2017).

Given the growing body of evidence linking Defb126 to sperm maturation and mucus penetration in rodents and primates, as well as the documented evidence of a role in mediating sperm binding (Tollner et al. 2004) the aim of this study was to characterise the effect of recombinant BBD126 (rBBD126) on the binding of bull sperm to oviductal epithelia.

MATERIALS AND METHODS

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (Arklow, Co Wicklow, Ireland) unless stated otherwise.

Preparation of Oviductal Explants

Reproductive tracts from non-pregnant crossbred heifers of beef breeds, were collected at a commercial abattoir immediately post-mortem and transported to the laboratory within 1 h in
phosphate buffered saline (PBS) supplemented with Gentamicin sulphate (0.25 mg/ml, Sigma Aldrich, Wicklow, Ireland) at 4 °C. Reproductive tracts from heifers at various stages of the oestrous cycle were used, as stage has been shown to have no effect on sperm binding in vitro in preliminary experiments (data not shown) and the literature (Lefebvre et al. 1997; Suarez 2002; Gwathmey et al. 2003; Gwathmey et al. 2006; Ignotz et al. 2007). At the laboratory, oviducts were trimmed free of connective tissue, washed twice with PBS and the isthmic segment isolated at ambient temperature. The epithelial cells were extruded in sheets by squeezing the oviduct with a sterile glass slide, fragmented by pipetting, centrifuged for 1 min (200 g), transferred to M199 culture media supplemented with fetal calf serum (10%) and gentamicin sulphate (0.25 mg/ml, Sigma Aldrich), and incubated for 1 h at 38 °C in 5% CO₂ in air to form everted vesicles with apical ciliated surfaces oriented outwardly (Ignotz et al. 2007). Explants were used for binding assessments within 5 h of animal slaughter. Three reproductive tracts were flushed on each day and whilst the explants from individual heifers were pooled, explants from different heifers were processed separately.

Preparation of Cauda and Corpus Sperm

To obtain epididymal sperm, both testes were recovered from mature bulls (n=3, per replicate) post slaughter at a commercial abattoir and transported, within 1 h, to the laboratory at 4 °C. To recover sperm from the cauda epididymis, a small incision was made in the cauda and the lumen of the deferent duct was cannulated with a blunted 22 G needle. Sperm were then gently flushed through the cauda with a 5 mL syringe loaded with PBS at 37°C (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). Due to the small diameter of the epididymal tubule, flushing of the corpus epididymis was not possible; therefore the corpus epididymis was minced with a scalpel blade in a dish of PBS at 37°C (Fernandez-Fuertes et al. 2016). Sperm from the cauda and corpus epididymis from each bull were kept separate and sperm motility was assessed subjectively on a minimum of 100 sperm per sample; only samples with >50% total motile sperm were used. Post motility assessment, sperm from the different bulls were pooled as separate corpus and cauda samples. The pool was then treated as the experimental unit.
Sperm Binding Assay

In this study, an epithelial explant model was used as it has been shown previously to be a more physiological assay in terms of maintaining beating cilia and in vivo cell-to-cell interactions in comparison to monolayer cell culture (Walter 1995; Teijeiro et al. 2011). Stock M199 culture media was added (5 mL) to explants of each reproductive tract and centrifuged at 200 g for 5 min. Post-centrifugation, the supernatant was removed and explants (20 μL) from each tract were added to sperm aliquots (140 μL) which were pre-stained for 30 min with 1% Hoechst 33342 at 38°C for enhanced binding visualisation (Al Naib et al. 2011). Final concentration of sperm in binding assay was 5 x 10⁶ sperm per mL. After 30 min incubation at 38°C in 5% CO₂, loosely bound sperm were removed from explants by gently pipetting through two 75 μL droplets of stock M199 media solution on a warmed 24-well culture plate (38°C) (Gwathmey et al. 2003). A droplet of each treatment (10 μL) was placed on a slide, a coverslip added, and viewed using a microscope at 400X fitted with a heated stage at 38°C (BX60; Olympus, Centre Valley, PA, USA) under half-light and half-fluorescence (Figure 1). The number of sperm bound was recorded and relative surface area of each explant was determined using a micrometre. Ten explants of each treatment were assessed at random and sperm binding density was calculated by determining the number of sperm bound per 0.1 mm² of explant surface. The evaluator was blinded to treatment for all sperm binding assessments. This was the method used for all binding assays in this study with the only variable between experiments being sperm treatment prior to binding. The sperm treatments used are detailed in their relevant experimental sections below.

Experiment 1: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

The aim of this experiment was to assess the ability of (i) freshly ejaculated (ii) cauda and (iii) corpus bull sperm to bind epithelial cell explants. Heifer reproductive tracts (n=3) were collected and prepared as described above. Freshly ejaculated semen from mature Holstein Friesian bulls (n=3) was
obtained from National Cattle Breeding Centre (NCBC), Naas, Co Kildare, Ireland, diluted to a concentration of 20 x 10^6 sperm per mL in INRA96, (IMV Technologies, L’Aigle, France), stored at an ambient temperature and delivered to the laboratory the morning after collection. Sperm from each bull was kept separate prior to assessment of sperm motility using the previously described method. Post motility assessment, sperm from the different bulls was pooled. Cauda and corpus sperm were prepared as previously described and sperm binding density was assessed using the explant assay. All treatments were assessed at a final sperm concentration of 5 x10^6 sperm per mL for sperm binding density using the BOEC explant assay. Three replicates were completed on three separate days. A replicate consisted of oviducts from three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In total, nine heifers and nine bulls were used.

Experiment 2: Assessment of the effect of rBBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

BBD126 is highly resistant to the methods of dissociation reported in other species and it was not possible to remove BBD126 from sperm once attached to the sperm surface (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). BBD126 is expressed in the cauda only, therefore, we used corpus sperm which have not yet acquired BBD126 (Narciandi et al. 2016). The aim of this experiment was to assess the effect of rBBD126 on the ability of corpus bull sperm to bind to BOEC explants. BOEC explants and epididymal sperm were collected and prepared using the same methods as described earlier. The following treatments were assessed (i) Cauda sperm (ii) Corpus sperm, (iii) Corpus sperm +10 µg/mL rBBD126, (iv) Corpus sperm +30 µg/mL rBBD126 and (v) Corpus sperm +90 µg/mL rBBD126. The concentration of rBBD126 added to the corpus sperm was based on previous studies of Defb126 in the macaque by Tollner et al. (Tollner et al. 2004; Tollner et al. 2008a; Tollner et al. 2008b). Caudal and corpus sperm treatments were assessed at a final concentration of 5 x10^6 sperm
per mL for sperm binding density using the BOEC explant assay. The control in this experiment was the untreated corpus sperm. Five replicates were carried out. A replicate consisted of oviducts from three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In total, twelve heifers and twelve bulls were used.

Recombinant BBD126 Expression

As BBD126 is an antimicrobial peptide, protein expression in an *E. coli* host was only possible with the aid of a carrier protein, as described by (Narciandi et al. 2016). It has been established previously that BBD126 is not glycosylated and therefore is suitable for prokaryotic expression (Narciandi et al. 2011; Fernandez-Fuertes et al. 2016). Briefly, the BBD126 coding sequence was amplified by PCR (Forward primer: 5’-GGTAATTGGTATGTGAGA 3’; Reverse primer: 5’-AGCAATGCTGTTGTAGATC 3’) using a Platinum Taq DNA Polymerase (Life Technologies, NY, USA). The resulting PCR product was cloned with the pBAD/TOPO Thiofusion kit (Invitrogen Ltd., Paisley, UK) following the manufacturer’s instructions (Narciandi et al. 2011; Narciandi et al. 2016). The resulting rBBD126 protein has a thioredoxin fusion at the N-terminus and 6x histidine fusion at the C-terminus. Sequence was confirmed by Sanger sequencing of the resulting plasmid (GATC Biotech, Konstanz, Germany). Luria-Bertani (LB) broth supplemented with 100 µg/mL ampicillin was used as culturing media. Transformed bacteria were cultured in 1 litre of media at 37 °C and 250 rpm to a density of 0.5 at 600 nm. Protein expression was induced by adding 1 mL 20% L-arabinose, the culture was grown at 28 °C for 4 h. After production, the bacteria were sonicated using a sonicator equipped with a microtip. The sample was then centrifuged to recover the rBBD126-rich supernatant.

The extracted solution was mixed with 1.5 ml nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Manchester, UK); resin was washed several times with PBS and protein was eluted with a 300 mM imidazole solution. The eluted fraction was injected into an AKTA gel filtration system set up with a Hiprep 16/60 Sephacryl S100HR column (GE Healthcare, Little Chalfont, UK). Fractions were tested by Western blot using α-BBD126 mAb (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). The purified fusion protein was treated with enterokinase protease 7 (New England Biolabs, Ipswich, MA,
USA) to cleave the N-terminus Thioredoxin tag following the manufacturer’s protocol. The resulting rBBBD126 without thioredoxin was purified by using Ni-NTA resin purification as carried out previously. The eluted fraction was then further purified by using a Hiprep 16/60 Sephacryl S100HR column (GE Healthcare). Coomassie gel staining and Western blot using anti-BBD126 were analysed to determine the purity of the resulting rBBBD126.

(b) Effect of rBBBD126 and Anti-BBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

Based on observations made in Experiment 2a it was hypothesised that the incubation of rBBBD126 with Anti-BBD126 would inhibit the effect of rBBBD126. The aim of this experiment was to assess the effect of exposure of corpus sperm to rBBBD126 and BBD126 antibody (Anti-BBD126) on the ability to bind to BOEC explants. Explants and epididymal sperm were collected and prepared as described above. The following treatments were assessed (i) Cauda sperm, (ii) Corpus sperm + 0 µg/mL rBBBD126, (iii) Corpus sperm + 10 µg/mL rBBBD126, (iv) Corpus sperm + 30 µg/mL Anti-BBD126 and +10 µg/mL rBBBD126, (v) Corpus sperm + 30 µg/mL Anti-BBD126, (vi) Corpus sperm +7 µg/mL IgG Ab and +10 µg/mL rBBBD126 and (vii) Corpus sperm +7 µg/mL IgG. The concentration of Anti-BBD126 added to the corpus sperm was based on previous studies of Defb126 in the macaque by Tollner et al. (Tollner et al. 2004; Tollner et al. 2008a; Tollner et al. 2008b). Sperm were incubated with Anti-BBD126 for 1 h prior to incubation with rBBBD126 for 1 h. The IgG Ab control was included to demonstrate specificity of the antibody. IgG is a glycoprotein antibody and was used as a control as it does not react with any proteins other than anti-mouse whole serum. All treatments were assessed for sperm binding density using the oviductal explant assay. Five replicates were carried out. A replicate consisted of oviducts from three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In total, fifteen heifers and fifteen bulls were used.
A custom monoclonal antibody specific for BBD126 was ordered from GeneScript (Piscataway, NJ, USA), and generated as described previously (Narciandi et al. 2011). Briefly, 5 BALB/c mice were inoculated with a 14-amino acid chemically produced peptide (RNGERVINPPTGMC). Immune response was confirmed by binding of serum to the antigen in an ELISA type assay and the cells were isolated for cell fusion and hybridoma production. Unpurified antibodies produced by each of the four hybridoma clones, selected and tested in an ELISA against the peptide, were tested against BBD126 on Western blot (Narciandi et al. 2011). Clone 6A11E2 was selected for large-scale production and purification. The specificity of the antibody was validated using a peptide competition assay where a sperm lysate sample was blotted with Anti-BBD126 in the presence of recombinant rBBD126. The specificity of the antibody was tested further by transfecting human embryonic kidney-derived cells (HEK293) with a transient expression vector containing the coding sequence for BBD126. When analyzed by Western blot, only cells transfected with BBD126 showed a band of the predicted protein size (Narciandi et al. 2011).

Experiment 3: Assessment of the effect of rBBD126 and Anti-BBD126 on the Ability of Ejaculated Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

The aim of this experiment was to assess the effect of exposure of ejaculated sperm which is coated in native BBD126 protein as well as seminal plasma proteins in vivo, to rBBD126 and Anti-BBD126 on the ability to bind to BOEC explants. It was hypothesised that rBBD126 would promote sperm binding and that Anti-BBD126 would bind to the epitope and block the effects of the native BBD126. Explants and ejaculated sperm were collected and prepared as described previously. The following treatments were assessed (i) Ejaculated sperm, (ii) Ejaculated sperm + 10 µg/mL rBBD126, (iii) Ejaculated sperm + 10 µg/mL rBBD126 and + 30 µg/mL Anti-BBD126 (iv) Ejaculated sperm + 30 µg/mL Anti-BBD126. Three replicates were completed.

Experiment 4: Effect of rBBD126 on Corpus Sperm Agglutination
It was observed in Experiment 2a that rBBD126 appeared to decrease agglutination of corpus sperm, as was observed recently in a previous study (Fernandez-Fuertes et al. 2016). Therefore, the aim of this experiment was to quantify the effect of rBBD126 concentration on corpus sperm agglutination. Caudal and corpus sperm were recovered, assessed for concentration and diluted to 20 x 10^6 sperm per mL as described above. The following treatments were assessed: (i) Cauda sperm (ii) Corpus sperm (iii) Corpus sperm + 10 µg/mL rBBD126, (iv) Corpus sperm +30 µg/mL rBBD126 and (v) Corpus sperm + 90 µg/mL rBBD126. Sperm were incubated with Anti-BBD126 for 1 h prior to incubation with rBBD126 for 1 h. The incidence of sperm agglutination were assessed using nigrosin–eosin staining for enhanced visualisation as described previously (Druart et al. 2009; Kiernan et al. 2013; Holden et al. 2017). Briefly, nigrosin-eosin stain (30 µL) was added to eppendorfs of each sperm treatment (30 µL) and a droplet of each treatment (10 µL) was smeared on to a glass slide, allowed to dry at room temperature and then viewed under a phase contrast microscope (x400) by one evaluator blinded to treatment. Fifty events were assessed in each count and the average of two counts was recorded. An event was described as the occurrence of a single sperm cell or an agglutinated mass of sperm. The number of sperm per agglutinated mass was also recorded (Holden et al. 2017). Agglutination percentage was calculated as the number of sperm cells present in fifty agglutinated masses as a percentage of the total sperm population assessed in the fifty events as per the following equation: number of sperm in agglutinated masses/total number of spermatozoa counted 100 (Holden et al. 2017). Five replicates were completed. In total, fifteen bulls were used.

**Statistical Analysis**

Data were examined for normality of distribution, tested for homogeneity of variance, transformed where appropriate and analysed in the Statistical Package for the Social Sciences (SPSS software, version 22, IBM, Chicago, IL). Univariate (ANOVA) was used to analyse all data. Sperm agglutination (%) data were transformed using a square root transformation. The transformed data were used to calculate the P values; however, the corresponding means and standard error of the non-transformed data are presented in the results. Post hoc tests were carried out using the Tukey test; a P
value < 0.05 was considered statistically significant and results were reported as the mean ± the standard error of the mean (s.e.m.).

RESULTS

Experiment 1: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

A representative image of corpus epididymal sperm bound to a bovine oviduct epithelial cell explant was captured in this experiment (Figure 1). A significant effect of stage of sperm maturity on their ability to bind to BOEC explants was detected (P<0.05; Figure 2). Ejaculated sperm had greater sperm binding density to BOEC explants in comparison to cauda and corpus sperm (P<0.05). There was no difference in sperm binding density between cauda and corpus sperm (P>0.05). Sperm binding density was 23.2 ± 2.97, 15.2 ± 2.88 and 13.2 ± 1.56 sperm/0.1mm² for ejaculated sperm, cauda sperm and corpus sperm, respectively.

Experiment 2: Assessment of the Effect of rBBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

There was a significant effect of treatment on binding ability. Incubation of corpus sperm with 10 µg/mL rBBD126 resulted in a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 3). There was no difference in sperm binding density between the other treatments (P>0.05).
(b) Effect of rBBD126 and Anti-BBD126 on the Ability of Corpus Sperm to Bind Bovine Oviductal Epithelial Cell Explants in vitro

Corpus sperm incubated with 10 µg/mL rBBD126 had a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 4). Furthermore, the presence of BBD126 antibody (+30 µg/mL) inhibited the increase in sperm binding density associated with rBBD126 (+10 µg/mL; P<0.05). The presence of the control IgG Ab (+7 µg/mL) did not inhibit the stimulatory effect of rBBD126 (+10 µg/mL; P>0.05). There was no difference in sperm binding density between the other treatments (P>0.05).

Experiment 3: Assessment of the effect of rBBD126 and Anti-BBD126 on the Ability of Ejaculated Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

Ejaculated sperm incubated with 10 µg/mL rBBD126 had a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 5). However, whilst BBD126 antibody (+30 µg/mL) did have an inhibitory effect on the increase in sperm binding density associated with rBBD126 (+10 µg/mL), this effect was not statistically significant (P>0.05). There was no difference in sperm binding density between the other treatments (P>0.05).

Experiment 4: Effect of rBBD126 on Corpus Sperm Agglutination

Corpus sperm exhibited higher sperm agglutination than cauda sperm (Figure 6; P<0.05). While the addition of rBBD126 to corpus sperm reduced sperm agglutination, the difference was only significant at a concentration of 90 µg/mL rBBD126 (Figure 6; P<0.05).

DISCUSSION

During maturation, immature sperm migrate through the epididymis where they are bathed in region-specific epididymal fluid which leads to a sequential addition, deletion, and modification of sperm surface proteins. These modifications lead to the acquisition of properties vital for survival and
interaction with the female reproductive tract (Tollner et al. 2012). BBD126 has been recently characterised on bull sperm and is preferentially expressed in the caudal epithelium of the epididymis. However, outside of our group (Fernandez-Fuertes et al. 2016), the role of BBD126 in mediating sperm function has not previously been investigated in cattle. To the best of our knowledge, this is the first study to assess the potential role of BBD126 in bull sperm binding to oviductal epithelia, thus shedding additional light on the functional roles of this protein family in bovine reproduction. This novel study has shown for the first time that i) corpus epididymal sperm can bind to epithelial cell explants but at a reduced rate to that of ejaculated bull sperm; ii) rBBD126 increases the ability of corpus bull sperm to bind to epithelial cell explants, an effect that is inhibited by Anti-BBD126; (iii) rBBD126 increases the ability of ejaculated bull sperm to bind epithelial cell explants and iv) rBBD126 reduces corpus sperm agglutination.

While previous studies have shown that cauda epididymal sperm have the ability to bind BOEC in vitro (Gwathmey et al. 2003; Gualtieri et al. 2010), there is a paucity of data in the literature relating to the binding ability of corpus sperm. Identification of the specific molecules regulating these cellular interactions has also been limited due to the difficulty in removing BBD126 from the surface of bull sperm (Fernandez-Fuertes et al. 2016), corpus epididymal sperm, which have not yet acquired the protein (Narciandi et al. 2016), were used as a model to study the role of BBD126 protein in sperm binding. In this study, rBBD126 increased the binding ability of corpus sperm to BOEC explants. This stimulatory effect was inhibited by the presence of BBD126 antibody but not by the presence of the control IgG antibody. Whilst rBBD126 increased corpus sperm binding, it did not increase binding to the levels exhibited by ejaculated sperm. When incubated with ejaculated sperm, rBBD126 increased their binding ability to BOEC explants, however, Anti-BBD126 failed to fully abrogate this increase. The reason for this reduced inhibitory effect, compared to what was evident with corpus sperm, is unclear but may be due to the interaction of seminal plasma proteins with the antibody on ejaculated sperm. Alternatively, as ejaculated sperm are coated in native BBD126, of caudal origin, it may be due to insufficient levels of Anti-BBD126 being used (as the concentration was selected based on experiments with corpus sperm). Thus, similar to the role of its analogue in

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macaque, ejaculated sperm with more BBD126 in their acrosome may represent sperm with more oviduct binding capacity; however, it is most likely as an additional mechanism to a number of other molecules that have been linked to oviductal binding in bovines (Suarez 2002; Boilard et al. 2004; Gwathmey et al. 2006; Ignoz et al. 2007; Suarez 2016; Osycka-Salut et al. 2017).

One such group of molecules also believed to mediate the complex sperm binding mechanisms are the binder of sperm proteins (BSP) described in previous studies (Lefebvre et al. 1997; Suarez 2002; Ignoz et al. 2007; Suarez 2016). In cattle, a number of lectin-like sperm binding proteins including binder of sperm 1, 3 and 5 (BSP1, BSP3, BSP5) - which are produced by the seminal vesicles and adhere to the surface of sperm are believed to bind to several oviductal receptor proteins (Ignoz et al. 2007). However, the concentrations of BSP3 and BSP5 (2–6 mg/ml) are only about one-tenth that of BSP1 (Nauc and Manjunath 2000). Each BSP has been shown to enhance sperm binding to oviductal epithelium independently (Suarez 2016). As a direct consequence, since each BSP can act independently, each may play a different, if overlapping, role in mediating complex sperm interactions with oviductal epithelium (Suarez 2016).

In addition, α5β1 – an integrin protein found on the sperm surface is believed to play a role in sperm binding. Using monolayer co-culture experiments, it was shown that α5β1 on sperm binds to fibronectin – a glycoprotein which is expressed on the apical surface of oviductal cells (Osycka-Salut et al. 2017). However, several other integrins such as α4β1, αIIb3, αvβ3, and αvβ6 have also been found to interact with fibronectin, thus the participation of other oviductal integrins cannot be discounted (Lodish et al. 2008; Osycka-Salut et al. 2017). Furthermore, a study by another group has indicated that two bovine oviductal proteins - the chaperones GRP78 and HSP60 - may also have a role in the complex binding mechanism. However, whilst both of these molecules have been identified on the surface of epithelial cells and sperm respectively, currently there is limited data on the functionality of these proteins in bull sperm binding (Boilard et al. 2004).
In this study, cauda and corpus epididymal sperm, which lack BSP, α5β1 and chaperone proteins, retain the ability to bind BOEC explants \textit{in vitro}; this indicates that additional proteins such as BBD126 may be involved in this adhesion event (Boilard \textit{et al.} 2004; Gwathmey \textit{et al.} 2006; Ignotz \textit{et al.} 2007; Thys \textit{et al.} 2009; Gualtieri \textit{et al.} 2010). Currently, the identity of binding molecule(s) for BBD126 on epithelial cells is unknown (Tollner \textit{et al.} 2008a); however, the absorption pattern of BBD126 on the bull sperm head (Fernandez-Fuertes \textit{et al.} 2016) appears to be consistent with the location and pattern of sperm attachment to BOEC. Whilst several other binding proteins on sperm have been identified, understanding of the complex interaction of these molecules remains limited (Suarez 2016).

In this study there was no significant difference between untreated cauda and corpus sperm treatments. We believe that due to the large number of proteins/molecules linked to oviductal binding previously (Boilard \textit{et al.} 2004; Gwathmey \textit{et al.} 2006; Ignotz \textit{et al.} 2007; Miller 2015; Suarez 2016; Osycka-Salut \textit{et al.} 2017), there may be proteins expressed in the epididymis which also mediate this complex binding mechanism in conjunction with BBD126 and the other known proteins.

In this study, concentration of rBBD126 used was found to influence the function of the BBD126 protein. At lower concentrations, rBBD126 significantly enhanced sperm binding ability, whereas at higher concentrations this effect was not statistically significant. In contrast, higher concentrations of rBBD126 reduced the incidence of sperm agglutination, whereas, at lower concentrations this effect was not statistically significant. It is likely that protein concentration mediates the multi-functional role of BBD126 \textit{in vivo}. Whilst the concentration of BBD126 in the cauda is currently unknown, it would be at higher concentrations in the cauda in comparison to the BBD126 concentration present in the female reproductive tract. This dilution effect occurs due to the exposure of sperm to seminal plasma, which does not contain BBD126 (Narciandi \textit{et al.}, 2016), as well as a variety of secretions from the female reproductive tract post ejaculation (Hunter 2012; Holt and Fazeli 2015). Thus, higher concentrations in the cauda of the epididymis, BBD126 may have a functional role in preventing sperm agglutination, whilst at lower concentrations in the female reproductive tract \textit{in vivo}, BBD126 may mediate sperm binding. Corpus sperm agglutination is documented in several species including

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rams, boars and bovines (Dacheux et al. 1983; Sinowatz et al. 1984; Fernandez-Fuertes et al. 2016). In this study, rBBD126 was found to reduce corpus sperm agglutination to the very low levels comparable with those observed in cauda sperm, where BBD126 is expressed in vivo. It is possible that the increase in sperm binding observed after incubation with rBBD126 is due to the sperm being disengaged from one another and being allowed to swim freely rather than a direct effect on the sperm binding mechanism itself. This would be consistent with the observation of reduced corpus sperm agglutination and a concurrent increase in overall motility after incubation with rBBD126 which was found in a related study by our group (Fernandez-Fuertes et al. 2016). However, this hypothesis does not account for the ability of corpus sperm to bind in the absence of other binding proteins. In the current study, in contrast to sperm binding, agglutination was found to be inversely proportional to increasing BBD126 concentration. Previous studies, including one in rhesus monkeys, have reported that glycoproteins that are present in the cauda epididymis also reduce agglutination (Srivastav et al. 2004).

In conclusion, this is the first published study to demonstrated that rBBD126 enhances the ability of bull sperm to bind to oviduct epithelial cell explants and to reduce sperm agglutination. Further research on BBD126 and related β-defensins will help expand our understanding of the complex interactions between sperm binding proteins and the female reproductive tract as well as evaluating their relevance to bull fertility.

ACKNOWLEDGEMENTS

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest
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Figure 1: Representative image of corpus epididymal sperm bound to a bovine oviduct epithelial cell explant (BOEC). The green line indicates the overall BOEC explant, blue lines indicate sperm bound to epithelial cells (both ciliated and non-ciliated), the orange line indicates a group of cilia on a ciliated epithelial cell, the yellow line indicates a non-ciliated epithelial cell and the purple lines indicate unbound sperm. Image captured at 400X using a fluorescent microscope at half light.
Figure 2: Binding density of ejaculated, cauda and corpus sperm to bovine oviduct epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1 mm$^2$ of explant. Vertical error bars represent s.e.m. $^{ab}$Different superscripts between treatment groups differ significantly (P<0.05). n = 3 replicates. Experiment 1.
Figure 3: Binding density of cauda and corpus sperm incubated with various concentrations of recombinant BBD126 (rBBD126) to bovine oviduct epithelial cell explants. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. Vertical error bars represent s.e.m. Different superscripts between treatment groups differ significantly (P<0.05). n = 5 replicates. Experiment 2a.
Figure 4: Binding density of cauda and corpus sperm to bovine oviduct epithelial cell explants following incubation with and without recombinant BBD126, BBD126 antibody (Ab) and a control IgG mouse antibody. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm$^2$ of explant. Vertical error bars represent s.e.m. \textsuperscript{abc}Treatment groups with different superscripts differ significantly ($P<0.05$). $n = 5$ replicates. Experiment 2b.
Figure 5: Binding density of ejaculated sperm to bovine oviduct epithelial cell explants following incubation with and without recombinant BBD126 and BBD126 antibody (Ab). Ejaculated sperm without rBBD126 or BBD126 antibody was used as a control. Binding density is expressed as the number of bound sperm per 0.1 mm$^2$ of explant. Vertical error bars represent s.e.m. Treatment groups with different superscripts differ significantly (P<0.05). n = 3 replicates. Experiment 3.
Figure 6: Sperm agglutination of cauda and corpus sperm following incubation with various concentrations of recombinant BBD126. Vertical error bars represent s.e.m. a,b Different superscripts between treatment groups differ significantly (P<0.05). n = 5 replicates. Experiment 4.
Recombinant Beta-defensin 126 Promotes Bull Sperm Binding to Bovine Oviductal Epithelia

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Running Title:

β-defensin 126 on bovine sperm

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Primate β-defensin 126 regulates the ability of sperm to bind to oviductal epithelial cells \textit{in vitro}.

Bovine β-defensin 126 (BBD126) exhibits preferential expression in the cauda epididymis of the bull but there have been few studies on its functional role in cattle. The aim of this study was to examine the role of BBD126 in bull sperm binding to bovine oviductal epithelial cell (BOEC) explants. BBD126 has been shown to be highly resistant to the standard methods of dissociation used in other species and, as a result, corpus epididymal sperm, which have not been exposed to the protein, were used to study the functional role of BBD126. Corpus epididymal sperm were incubated with recombinant BBD126 (rBBD126) in the absence or presence of BBD126 antibody. Addition of rBBD126 significantly enhanced the ability of epididymal sperm to bind to BOEC explants (P<0.05). Anti-BBD126 blocked the BBD126-mediated increase in sperm binding capacity. Ejaculated sperm, which is coated with native BBD126 protein but also a large number of seminal plasma proteins \textit{in vivo}, was incubated with rBBD126 in the absence or presence of BBD126 antibody. Addition of rBBD126 significantly enhanced the ability of ejaculated sperm to bind to BOEC explants (P<0.05), while, rBBD126 reduced corpus sperm agglutination (P<0.05). These results suggest that similar to the role of its analogue in macaque, sperm with more BBD126 in their acrosome may represent sperm with more oviduct binding capacity.
INTRODUCTION

β–Defensins belong to a family of antimicrobial peptides – short, structurally diverse proteins that kill microbes and have specific and specialised functions in innate immunity (Sass et al. 2010; Meade et al. 2014). The microcidal properties of the β–defensin family are well documented in mice and humans (Semple et al. 2015). In humans, β–defensin 3 has been found to disrupt staphylococcal cell wall biosynthesis (Sass et al. 2010) while, in mice, β–defensin 10 has been shown to have antimicrobial effects in vivo (Peyrin-Biroulet et al. 2010). Although most studies on these proteins have focused on their antimicrobial characteristics, there is a growing body of evidence for a role for β–defensins in reproductive function (Tollner et al. 2008a; Tollner et al. 2008b; Fernandez-Fuertes et al. 2016; Legare et al. 2017).

Sperm transit through the epididymis of the male reproductive tract evokes a restructuring of membrane proteins as part of the sperm maturation process (Girouard et al. 2011). Proteins secreted by the epididymal epithelium include membrane surface proteins involved in sperm membrane protection and interaction with the female reproductive tract (Nagdas et al. 2014) and the oocyte (Netzel-Arnett et al. 2009). Amongst the proteins that are secreted by the epididymal epithelium and that bind to the sperm surface, several members of the β–defensin family have been identified (Zhou et al. 2004; Yudin et al. 2008). It is postulated that these proteins may provide immune privilege to sperm as they navigate their way along the female reproductive tract to the site of fertilisation. In primates, there is evidence that one of these protein additions, Defb126, is preferentially expressed in the male reproductive tract and plays an important role in sperm motility, mucus penetration and sperm binding to oviductal explants in vitro (Tollner et al. 2008a; Tollner et al. 2012; Cornwall 2014; Dorin and Barratt 2014). In the macaque, Defb126 is secreted in the corpus and cauda epididymis where it has been reported to cover the entire surface of ejaculated sperm (Yudin et al. 2005). Defb126 is a major component of the sperm surface glycocalyx (Yudin et al. 2005), a dense coating of carbohydrate-rich molecules and glycoproteins (Schroter et al. 1999). This coating protein contains multiple sialylated oligosaccharides and has been shown to enhance migration of macaque sperm through cervical mucus in vitro (Tollner et al. 2008b) by conferring an increased negative charge.
Upon arriving in the isthmus of the oviduct, sperm bind to the epithelial cells, thereby forming a sperm reservoir (Gwathmey et al. 2003; Hunter 2008; Holt and Fazeli 2010). This reservoir plays a critical role in successful fertilisation as sperm binding has been shown to select a fertile sperm population (Teijeiro et al. 2011; Coy et al. 2012; Holt and Fazeli 2015), extend sperm viability (Suarez and Pacey 2006) and regulate sperm function (Miller 2015). It is postulated that sperm bind to the oviductal epithelium via the plasma membrane overlying the sperm acrosome (Suarez 2002) and this binding usually, but not exclusively occurs on ciliated cells (Hunter et al. 1991; Lefebvre et al. 1995). There is evidence in several species that sperm bind to the cilia via glycans found on oviductal epithelial cells (DeMott et al. 1995; Lefebvre et al. 1997; Suarez 2001; Wagner et al. 2002) and in cattle, sperm binding is thought to be mediated by fucose recognition (Lefebvre et al. 1997). Fucose, found specifically in the Lewis A trisaccharide has been shown to have a regulatory effect on bull sperm binding (Suarez 2016). It has been proposed that oviductal plasma membrane receptor proteins containing Lewis A bind to surface proteins deposited on sperm during epididymal transit as well as from the seminal plasma at ejaculation (Ignotz et al. 2007). In macaque, Defb126 has been reported to play an integral role in sperm binding to oviduct epithelial cell explants in vitro (Yudin et al. 2005; Tollner et al. 2008a) while loss of Defb126 from the sperm head during capacitation is associated with a significant loss of the ability of sperm to bind (Yudin et al. 2005). Furthermore, Tollner et al. (2004) reported that Defb126 must be released from the sperm surface in order for sperm to bind to the zona pellucida (Tollner et al. 2004). In humans, mutation in the Defb126 gene has been found to correlate with subfertility in men; sperm from individuals with the Defb126 variant exhibited reduced glycosylation and a reduced ability to penetrate mucus in comparison to those without the Defb126 variant (Tollner et al. 2011).

Using a comparative genomics approach, our group has recently discovered and profiled the expression of a cluster of novel β-defensin genes along the male and female reproductive tracts in cattle (Meade et al. 2014), horses (Johnson et al. 2016) and sheep (Hall et al. 2017). We have shown that bovine β-defensin 126 (BBD126) peptide, the ortholog of human Defb126, is expressed solely in the cauda epididymis of the mature bulls (Narciandi et al. 2011). It is not secreted in other regions of
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the epididymis or in the testes nor is it present in seminal plasma of vasectomised bulls (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). BBD126 coats bull sperm predominantly on the tail and post acrosomal region of the sperm head (Fernandez-Fuertes et al. 2016), a pattern which differs from that described for the macaque ortholog which coats the entire sperm surface (Fernandez-Fuertes et al. 2016). The dissociation-resistant dimeric structure of BBD126 (Narciandi et al. 2016) has meant that capacitating agents previously reported to remove Defb126 from macaque sperm failed to remove the bovine ortholog (Fernandez-Fuertes et al. 2016). Furthermore, the addition of recombinant BBD126 (rBBD126) to corpus epididymal bull sperm, where the protein is not present, increased their motility and mucus penetration in vitro (Fernandez-Fuertes et al. 2016). Working with a large population (>7,000) of bulls used in artificial insemination our group has recently demonstrated that a haplotype spanning multiple β-defensin genes, including Defb126, was significantly associated with in vivo fertility. Functional analysis confirmed that sperm from bulls possessing this multi-defensin haplotype showed significantly enhanced binding to oviductal epithelium (Whiston et al. 2017).

Given the growing body of evidence linking Defb126 to sperm maturation and mucus penetration in rodents and primates, as well as the documented evidence of a role in mediating sperm binding (Tollner et al. 2004) the aim of this study was to characterise the effect of recombinant BBD126 (rBBD126) on the binding of bull sperm to oviductal epithelia.

MATERIALS AND METHODS

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (Arklow, Co Wicklow, Ireland) unless stated otherwise.

Preparation of Oviductal Explants

Reproductive tracts from non-pregnant crossbred heifers of beef breeds, were collected at a commercial abattoir immediately post-mortem and transported to the laboratory within 1 h in

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phosphate buffered saline (PBS) supplemented with Gentamicin sulphate (0.25 mg/ml, Sigma Aldrich, Wicklow, Ireland) at 4 °C. Reproductive tracts from heifers at various stages of the oestrous cycle were used, as stage has been shown to have no effect on sperm binding in vitro in preliminary experiments (data not shown) and the literature (Lefebvre et al. 1997; Suarez 2002; Gwathmey et al. 2003; Gwathmey et al. 2006; Ignotz et al. 2007). At the laboratory, oviducts were trimmed free of connective tissue, washed twice with PBS and the isthmic segment isolated at ambient temperature. The epithelial cells were extruded in sheets by squeezing the oviduct with a sterile glass slide, fragmented by pipetting, centrifuged for 1 min (200 g), transferred to M199 culture media supplemented with fetal calf serum (10%) and gentamicin sulphate (0.25 mg/ml, Sigma Aldrich), and incubated for 1 h at 38 °C in 5% CO₂ in air to form everted vesicles with apical ciliated surfaces oriented outwardly (Ignotz et al. 2007). Explants were used for binding assessments within 5 h of animal slaughter. Three reproductive tracts were flushed on each day and whilst the explants from individual heifers were pooled, explants from different heifers were processed separately.

**Preparation of Cauda and Corpus Sperm**

To obtain epididymal sperm, both testes were recovered from mature bulls (n=3, per replicate) post slaughter at a commercial abattoir and transported, within 1 h, to the laboratory at 4 °C. To recover sperm from the cauda epididymis, a small incision was made in the cauda and the lumen of the deferent duct was cannulated with a blunted 22 G needle. Sperm were then gently flushed through the cauda with a 5 mL syringe loaded with PBS at 37ºC (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). Due to the small diameter of the epididymal tubule, flushing of the corpus epididymis was not possible; therefore the corpus epididymis was minced with a scalpel blade in a dish of PBS at 37ºC (Fernandez-Fuertes et al. 2016). Sperm from the cauda and corpus epididymis from each bull were kept separate and sperm motility was assessed subjectively on a minimum of 100 sperm per sample; only samples with >50% total motile sperm were used. Post motility assessment, sperm from the different bulls were pooled as separate corpus and cauda samples. The pool was then treated as the experimental unit.
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*Sperm Binding Assay*

In this study, an epithelial explant model was used as it has been shown previously to be a more physiological assay in terms of maintaining beating cilia and *in vivo* cell-to-cell interactions in comparison to monolayer cell culture (Walter 1995; Teijeiro *et al.* 2011). Stock M199 culture media was added (5 mL) to explants of each reproductive tract and centrifuged at 200 g for 5 min. Post-centrifugation, the supernatant was removed and explants (20 μL) from each tract were added to sperm aliquots (140 μL) which were pre-stained for 30 min with 1% Hoechst 33342 at 38°C for enhanced binding visualisation (Al Naib *et al.* 2011). Final concentration of sperm in binding assay was 5 x 10^6 sperm per mL. After 30 min incubation at 38°C in 5% CO₂, loosely bound sperm were removed from explants by gently pipetting through two 75 μL droplets of stock M199 media solution on a warmed 24-well culture plate (38°C) (Gwathmey *et al.* 2003). A droplet of each treatment (10 μL) was placed on a slide, a coverslip added, and viewed using a microscope at 400X fitted with a heated stage at 38°C (BX60; Olympus, Centre Valley, PA, USA) under half-light and half-fluorescence (Figure 1). The number of sperm bound was recorded and relative surface area of each explant was determined using a micrometre. Ten explants of each treatment were assessed at random and sperm binding density was calculated by determining the number of sperm bound per 0.1 mm² of explant surface. The evaluator was blinded to treatment for all sperm binding assessments. This was the method used for all binding assays in this study with the only variable between experiments being sperm treatment prior to binding. The sperm treatments used are detailed in their relevant experimental sections below.

*Experiment 1: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.*

The aim of this experiment was to assess the ability of (i) freshly ejaculated (ii) cauda and (iii) corpus bull sperm to bind epithelial cell explants. Heifer reproductive tracts (n=3) were collected and prepared as described above. Freshly ejaculated semen from mature Holstein Friesian bulls (n=3) was
obtained from National Cattle Breeding Centre (NCBC), Naas, Co Kildare, Ireland, diluted to a concentration of 20 x 10^6 sperm per mL in INRA96, (IMV Technologies, L’Aigle, France), stored at an ambient temperature and delivered to the laboratory the morning after collection. Sperm from each bull was kept separate prior to assessment of sperm motility using the previously described method. Post motility assessment, sperm from the different bulls was pooled. Cauda and corpus sperm were prepared as previously described and sperm binding density was assessed using the explant assay. All treatments were assessed at a final sperm concentration of 5 x 10^6 sperm per mL for sperm binding density using the BOEC explant assay. Three replicates were completed on three separate days. A replicate consisted of oviducts from three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In total, nine heifers and nine bulls were used.

Experiment 2: Assessment of the effect of rBBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

BBD126 is highly resistant to the methods of dissociation reported in other species and it was not possible to remove BBD126 from sperm once attached to the sperm surface (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). BBD126 is expressed in the cauda only, therefore, we used corpus sperm which have not yet acquired BBD126 (Narciandi et al. 2016). The aim of this experiment was to assess the effect of rBBD126 on the ability of corpus bull sperm to bind to BOEC explants. BOEC explants and epididymal sperm were collected and prepared using the same methods as described earlier. The following treatments were assessed (i) Cauda sperm (ii) Corpus sperm, (iii) Corpus sperm +10 µg/mL rBBD126, (iv) Corpus sperm +30 µg/mL rBBD126 and (v) Corpus sperm +90 µg/mL rBBD126. The concentration of rBBD126 added to the corpus sperm was based on previous studies of Defb126 in the macaque by Tollner et al. (Tollner et al. 2004; Tollner et al. 2008a; Tollner et al. 2008b). Caudal and corpus sperm treatments were assessed at a final concentration of 5 x 10^6 sperm.
per mL for sperm binding density using the BOEC explant assay. The control in this experiment was
the untreated corpus sperm. Five replicates were carried out. A replicate consisted of oviducts from
three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In
total, twelve heifers and twelve bulls were used.

Recombinant BBD126 Expression

As BBD126 is an antimicrobial peptide, protein expression in an *E. coli* host was only possible with
the aid of a carrier protein, as described by (Narciandi et al. 2016). It has been established previously
that BBD126 is not glycosylated and therefore is suitable for prokaryotic expression (Narciandi et al.
2011; Fernandez-Fuertes et al. 2016). Briefly, the BBD126 coding sequence was amplified by PCR
(Forward primer: 5’-GGTAGGGATCTGTCGTTTAAG-3’; Reverse primer: 5’-
AGCAATGCCTGTGTAGATCT-3’) using a Platinum Taq DNA Polymerase (Life Technologies, NY,
USA). The resulting PCR product was cloned with the pBAD/TOPO Thiofusion kit (Invitrogen Ltd.,
Paisley, UK) following the manufacturer’s instructions (Narciandi et al. 2011; Narciandi et al. 2016).
The resulting rBBD126 protein has a thioredoxin fusion at the N-terminus and 6x histidine fusion at
the C-terminus. Sequence was confirmed by Sanger sequencing of the resulting plasmid (GATC
Biotech, Konstanz, Germany). Luria-Bertani (LB) broth supplemented with 100 µg/mL ampicillin
was used as culturing media. Transformed bacteria were cultured in 1 litre of media at 37 ºC and 250
rpm to a density of 0.5 at 600 nm. Protein expression was induced by adding 1 mL 20% L-arabinose,
the culture was grown at 28 ºC for 4 h. After production, the bacteria were sonicated using a sonicator
equipped with a microtip. The sample was then centrifuged to recover the rBBD126-rich supernatant.
The extracted solution was mixed with 1.5 ml nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen,
Manchester, UK); resin was washed several times with PBS and protein was eluted with a 300 mM
imidazole solution. The eluted fraction was injected into an AKTA gel filtration system set up with a
Hiprep 16/60 Sephacryl S100HR column (GE Healthcare, Little Chalfont, UK). Fractions were tested
by Western blot using α-BBD126 mAb (Fernandez-Fuertes et al. 2016; Narciandi et al. 2016). The
purified fusion protein was treated with enterokinase protease 7 (New England Biolabs, Ipswich, MA,
USA) to cleave the N-terminus Thioredoxin tag following the manufacturer’s protocol. The resulting rBBD126 without thioredoxin was purified by using Ni-NTA resin purification as carried out previously. The eluted fraction was then further purified by using a Hiprep 16/60 Sephacryl S100HR column (GE Healthcare). Coomassie gel staining and Western blot using anti-BBD126 were analysed to determine the purity of the resulting rBBD126.

(b) Effect of rBBD126 and Anti-BBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

Based on observations made in Experiment 2a it was hypothesised that the incubation of rBBD126 with Anti-BBD126 would inhibit the effect of rBBD126. The aim of this experiment was to assess the effect of exposure of corpus sperm to rBBD126 and BBD126 antibody (Anti-BBD126) on the ability to bind to BOEC explants. Explants and epididymal sperm were collected and prepared as described above. The following treatments were assessed (i) Cauda sperm, (ii) Corpus sperm + 0 µg/mL rBBD126, (iii) Corpus sperm + 10 µg/mL rBBD126, (iv) Corpus sperm + 30 µg/mL Anti-BBD126 and +10 µg/mL rBBD126, (v) Corpus sperm + 30 µg/mL Anti-BBD126, (vi) Corpus sperm +7 µg/mL IgG Ab and +10 µg/mL rBBD126 and (vii) Corpus sperm +7 µg/mL IgG. The concentration of Anti-BBD126 added to the corpus sperm was based on previous studies of Defb126 in the macaque by Tollner et al. (Tollner et al. 2004; Tollner et al. 2008a; Tollner et al. 2008b). Sperm were incubated with Anti-BBD126 for 1 h prior to incubation with rBBD126 for 1 h. The IgG Ab control was included to demonstrate specificity of the antibody. IgG is a glycoprotein antibody and was used as a control as it does not react with any proteins other than anti-mouse whole serum. All treatments were assessed for sperm binding density using the oviductal explant assay. Five replicates were carried out. A replicate consisted of oviducts from three heifers with all sperm treatments performed in duplicate on each individual tract on each day. In total, fifteen heifers and fifteen bulls were used.
A custom monoclonal antibody specific for BBD126 was ordered from GeneScript (Piscataway, NJ, USA) and generated as described previously (Narciandi et al. 2011). Briefly, 5 BALB/c mice were inoculated with a 14-amino-acid chemically produced peptide (RNGERVINPPTGMC). Immune response was confirmed by binding of serum to the antigen in an ELISA type assay and the cells were isolated for cell fusion and hybridoma production. Unpurified antibodies produced by each of the four hybridoma clones, selected and tested in an ELISA against BBD126 on Western blot (Narciandi et al. 2011). Clone 6A11E2 was selected for large-scale production and purification. The specificity of the antibody was validated using a peptide competition assay where a sperm lysate sample was blotted with Anti-BBD126 in the presence of recombinant rBBD126. The specificity of the antibody was tested further by transfecting human embryonic kidney-derived cells (HEK293) with a transient expression vector containing the coding sequence for BBD126. When analyzed by Western blot, only cells transfected with BBD126 showed a band of the predicted protein size (Narciandi et al. 2011).

Experiment 3: Assessment of the effect of rBBD126 and Anti-BBD126 on the Ability of Ejaculated Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

The aim of this experiment was to assess the effect of exposure of ejaculated sperm which is coated in native BBD126 protein as well as seminal plasma proteins in vivo, to rBBD126 and Anti-BBD126 on the ability to bind to BOEC explants. It was hypothesised that rBBD126 would promote sperm binding and that Anti-BBD126 would block the effects of the native BBD126. Experiments and ejaculated sperm were collected and prepared as described previously. The following treatments were assessed: (i) Ejaculated sperm, (ii) Ejaculated sperm + 10 µg/mL rBBD126, (iii) Ejaculated sperm + 10 µg/mL rBBD126 + 30 µg/mL Anti-BBD126, (iv) Ejaculated sperm + 30 µg/mL Anti-BBD126. Three replicates were completed.

Experiment 4: Effect of rBBD126 on Corpus Sperm Agglutination
It was observed in Experiment 2a that rBBD126 appeared to decrease agglutination of corpus sperm, as was observed recently in a previous study (Fernandez-Fuertes et al. 2016). Therefore, the aim of this experiment was to quantify the effect of rBBD126 concentration on corpus sperm agglutination. Caudal and corpus sperm were recovered, assessed for concentration and diluted to 20 x 10^6 sperm per mL as described above. The following treatments were assessed: (i) Cauda sperm (ii) Corpus sperm (iii) Corpus sperm + 10 µg/mL rBBD126, (iv) Corpus sperm +30 µg/mL rBBD126 and (v) Corpus sperm + 90 µg/mL rBBD126. Sperm were incubated with Anti-BBD126 for 1 h prior to incubation with rBBD126 for 1 h. The incidence of sperm agglutination were assessed using nigrosin–eosin staining for enhanced visualisation as described previously (Druart et al. 2009; Kiernan et al. 2013; Holden et al. 2017). Briefly, nigrosin-eosin stain (30 µL) was added to eppendorfs of each sperm treatment (30 µL) and a droplet of each treatment (10 µL) was smeared on to a glass slide, allowed to dry at room temperature and then viewed under a phase contrast microscope (x400) by one evaluator blinded to treatment. Fifty events were assessed in each count and the average of two counts was recorded. An event was described as the occurrence of a single sperm cell or an agglutinated mass of sperm. The number of sperm per agglutinated mass was also recorded (Holden et al. 2017). Agglutination percentage was calculated as the number of sperm cells present in fifty agglutinated masses as a percentage of the total sperm population assessed in the fifty events as per the following equation: number of sperm in agglutinated masses/total number of spermatozoa counted 100 (Holden et al. 2017). Five replicates were completed. In total, fifteen bulls were used.

**Statistical Analysis**

Data were examined for normality of distribution, tested for homogeneity of variance, transformed where appropriate and analysed in the Statistical Package for the Social Sciences (SPSS software, version 22, IBM, Chicago, IL). Univariate (ANOVA) was used to analyse all data. Sperm agglutination (%) data were transformed using a square root transformation. The transformed data were used to calculate the P values; however, the corresponding means and standard error of the non-transformed data are presented in the results. Post hoc tests were carried out using the Tukey test; a P
value < 0.05 was considered statistically significant and results were reported as the mean ± the standard error of the mean (s.e.m.).

RESULTS

Experiment 1: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

A representative image of corpus epididymal sperm bound to a bovine oviduct epithelial cell explant was captured in this experiment (Figure 1). A significant effect of stage of sperm maturity on their ability to bind to BOEC explants was detected (P<0.05; Figure 2). Ejaculated sperm had greater sperm binding density to BOEC explants in comparison to cauda and corpus sperm (P<0.05). There was no difference in sperm binding density between cauda and corpus sperm (P>0.05). Sperm binding density was 23.2 ± 2.97, 15.2 ± 2.88 and 13.2 ± 1.56 sperm/0.1mm² for ejaculated sperm, cauda sperm and corpus sperm, respectively.

Experiment 2: Assessment of the Effect of rBBD126 on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind to Bovine Oviductal Epithelial Cell Explants in vitro

There was a significant effect of treatment on binding ability. Incubation of corpus sperm with 10 µg/mL rBBD126 resulted in a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 3). There was no difference in sperm binding density between the other treatments (P>0.05).
(b) Effect of rBBD126 and Anti-BBD126 on the Ability of Corpus Sperm to Bind Bovine Oviductal Epithelial Cell Explants in vitro

Corpus sperm incubated with 10 µg/mL rBBD126 had a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 4). Furthermore, the presence of BBD126 antibody (+30 µg/mL) inhibited the increase in sperm binding density associated with rBBD126 (+10 µg/mL; P<0.05). The presence of the control IgG Ab (+7 µg/mL) did not inhibit the stimulatory effect of rBBD126 (+10 µg/mL; P>0.05). There was no difference in sperm binding density between the other treatments (P>0.05).

Experiment 3: Assessment of the effect of rBBD126 and Anti-BBD126 on the Ability of Ejaculated Sperm to Bind to Bovine Oviductal Epithelial Cell Explants.

Ejaculated sperm incubated with 10 µg/mL rBBD126 had a greater sperm binding density to BOEC explants than the control (P<0.05; Figure 5). However, whilst BBD126 antibody (+30 µg/mL) did have an inhibitory effect on the increase in sperm binding density associated with rBBD126 (+10 µg/mL), this effect was not statistically significant (P>0.05). There was no difference in sperm binding density between the other treatments (P>0.05).

Experiment 4: Effect of rBBD126 on Corpus Sperm Agglutination

Corpus sperm exhibited higher sperm agglutination than cauda sperm (Figure 6; P<0.05). While the addition of rBBD126 to corpus sperm reduced sperm agglutination, the difference was only significant at a concentration of 90 µg/mL rBBD126 (Figure 6; P<0.05).

DISCUSSION

During maturation, immature sperm migrate through the epididymis where they are bathed in region-specific epididymal fluid which leads to a sequential addition, deletion, and modification of sperm surface proteins. These modifications lead to the acquisition of properties vital for survival and
interaction with the female reproductive tract (ToIlner et al. 2012). BBD126 has been recently characterised on bull sperm and is preferentially expressed in the caudal epithelium of the epididymis. However, outside of our group (Fernandez-Fuertes et al. 2016), the role of BBD126 in mediating sperm function has not previously been investigated in cattle. To the best of our knowledge, this is the first study to assess the potential role of BBD126 in bull sperm binding to oviductal epithelia, thus shedding additional light on the functional roles of this protein family in bovine reproduction. This novel study has shown for the first time that i) corpus epididymal sperm can bind to epithelial cell explants but at a reduced rate to that of ejaculated bull sperm; ii) rBBD126 increases the ability of corpus bull sperm to bind to epithelial cell explants, an effect that is inhibited by Anti-BBD126; (iii) rBBD126 increases the ability of ejaculated bull sperm to bind epithelial cell explants and iv) rBBD126 reduces corpus sperm agglutination.

While previous studies have shown that cauda epididymal sperm have the ability to bind BOEC in vitro (Gwathmey et al. 2003; Gualtieri et al. 2010), there is a paucity of data in the literature relating to the binding ability of corpus sperm. Identification of the specific molecules regulating these cellular interactions has also been limited due to the difficulty in removing BBD126 from the surface of bull sperm (Fernandez-Fuertes et al. 2016), corpus epididymal sperm, which have not yet acquired the protein (Narciandi et al. 2016), were used as a model to study the role of BBD126 protein in sperm binding. In this study, rBBD126 increased the binding ability of corpus sperm to BOEC explants. This stimulatory effect was inhibited by the presence of BBD126 antibody but not by the presence of the control IgG antibody. Whilst rBBD126 increased corpus sperm binding, it did not increase binding to the levels exhibited by ejaculated sperm. When incubated with ejaculated sperm, rBBD126 increased their binding ability to BOEC explants, however, Anti-BBD126 failed to fully abrogate this increase. The reason for this reduced inhibitory effect, compared to what was evident with corpus sperm, is unclear but may be due to the interaction of seminal plasma proteins with the antibody on ejaculated sperm. Alternatively, as ejaculated sperm are coated in native BBD126, of caudal origin, it may be due to insufficient levels of Anti-BBD126 being used (as the concentration was selected based on experiments with corpus sperm). Thus, similar to the role of its analogue in
macaque, ejaculated sperm with more BBD126 in their acrosome may represent sperm with more oviduct binding capacity; however, it is most likely as an additional mechanism to a number of other molecules that have been linked to oviductal binding in bovines (Suarez 2002; Boilard et al. 2004; Gwathmey et al. 2006; Ignotz et al. 2007; Suarez 2016; Osyscka-Salut et al. 2017).

One such group of molecules also believed to mediate the complex sperm binding mechanisms are the binder of sperm proteins (BSP) described in previous studies (Lefebvre et al. 1997; Suarez 2002; Ignotz et al. 2007; Suarez 2016). In cattle, a number of lectin-like sperm binding proteins including binder of sperm 1, 3 and 5 (BSP1, BSP3, BSP5) - which are produced by the seminal vesicles and adhere to the surface of sperm are believed to bind to several oviductal receptor proteins (Ignotz et al. 2007). However, the concentrations of BSP3 and BSP5 (2–6 mg/ml) are only about one-tenth that of BSP1 (Nauc and Manjunath 2000). Each BSP has been shown to enhance sperm binding to oviductal epithelium independently (Suarez 2016). As a direct consequence, since each BSP can act independently, each may play a different, if overlapping, role in mediating complex sperm interactions with oviductal epithelium (Suarez 2016).

In addition, α5β1 – an integrin protein found on the sperm surface is believed to play a role in sperm binding. Using monolayer co-culture experiments, it was shown that α5β1 on sperm binds to fibronectin – a glycoprotein which is expressed on the apical surface of oviductal cells (Osyscka-Salut et al. 2017). However, several other integrins such as α4β1, αIIb3, αvβ3, and αvβ6 have also been found to interact with fibronectin, thus the participation of other oviductal integrins cannot be discounted (Lodish et al. 2008; Osyscka-Salut et al. 2017). Furthermore, a study by another group has indicated that two bovine oviductal proteins - the chaperones GRP78 and HSP60 - may also have a role in the complex binding mechanism. However, whilst both of these molecules have been identified on the surface of epithelial cells and sperm respectively, currently there is limited data on the functionality of these proteins in bull sperm binding (Boilard et al. 2004).
In this study, cauda and corpus epididymal sperm, which lack BSP, α5β1 and chaperone proteins, retain the ability to bind BOEC explants \textit{in vitro}; this indicates that additional proteins such as BBD126 may be involved in this adhesion event (Boilard \textit{et al.} 2004; Gwathmey \textit{et al.} 2006; Ignotz \textit{et al.} 2007; Thys \textit{et al.} 2009; Gualtieri \textit{et al.} 2010). Currently, the identity of binding molecule(s) for BBD126 on epithelial cells is unknown (Tollner \textit{et al.} 2008a); however, the absorption pattern of BBD126 on the bull sperm head (Fernandez-Fuertes \textit{et al.} 2016) appears to be consistent with the location and pattern of sperm attachment to BOEC. Whilst several other binding proteins on sperm have been identified, understanding of the complex interaction of these molecules remains limited (Suarez 2016).

In this study there was no significant difference between untreated cauda and corpus sperm treatments. We believe that due to the large number of proteins/molecules linked to oviductal binding previously (Boilard \textit{et al.} 2004; Gwathmey \textit{et al.} 2006; Ignotz \textit{et al.} 2007; Miller 2015; Suarez 2016; Osycka-Salut \textit{et al.} 2017), there may be proteins expressed in the epididymis which also mediate this complex binding mechanism in conjunction with BBD126 and the other known proteins.

In this study, concentration of rBBD126 used was found to influence the function of the BBD126 protein. At lower concentrations, rBBD126 significantly enhanced sperm binding ability, whereas at higher concentrations this effect was not statistically significant. In contrast, higher concentrations of rBBD126 reduced the incidence of sperm agglutination, whereas, at lower concentrations this effect was not statistically significant. It is likely that protein concentration mediates the multi-functional role of BBD126 \textit{in vivo}. Whilst the concentration of BBD126 in the cauda is currently unknown, it would be at higher concentrations in the cauda in comparison to the BBD126 concentration present in the female reproductive tract. This dilution effect occurs due to the exposure of sperm to seminal plasma, which does not contain BBD126 (Narciandi \textit{et al.}, 2016), as well as a variety of secretions from the female reproductive tract post ejaculation (Hunter 2012; Holt and Fazeli 2015). Thus, higher concentrations in the cauda of the epididymis, BBD126 may have a functional role in preventing sperm agglutination, whilst at lower concentrations in the female reproductive tract \textit{in vivo}, BBD126 may mediates sperm binding. Corpus sperm agglutination is documented in several species including...
rams, boars and bovines (Dacheux et al. 1983; Sinowitz et al. 1984; Fernandez-Fuertes et al. 2016). In this study, rBBD126 was found to reduce corpus sperm agglutination to the very low levels comparable with those observed in cauda sperm, where BBD126 is expressed in vivo. It is possible that the increase in sperm binding observed after incubation with rBBD126 is due to the sperm being disengaged from one another and being allowed to swim freely rather than a direct effect on the sperm binding mechanism itself. This would be consistent with the observation of reduced corpus sperm agglutination and a concurrent increase in overall motility after incubation with rBBD126 which was found in a related study by our group (Fernandez-Fuertes et al. 2016). However, this hypothesis does not account for the ability of corpus sperm to bind in the absence of other binding proteins. In the current study, in contrast to sperm binding, agglutination was found to be inversely proportional to increasing BBD126 concentration. Previous studies, including one in rhesus monkeys, have reported that glycoproteins that are present in the cauda epididymis also reduce agglutination (Srivastav et al. 2004).

In conclusion, this is the first published study to demonstrated that rBBD126 enhances the ability of bull sperm to bind to oviduct epithelial cell explants and to reduce sperm agglutination. Further research on BBD126 and related β-defensins will help expand our understanding of the complex interactions between sperm binding proteins and the female reproductive tract as well as evaluating their relevance to bull fertility.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest
REFERENCES


Figure 1: Representative image of corpus epididymal sperm bound to a bovine oviduct epithelial cell explant (BOEC). The green line indicates the overall BOEC explant, blue lines indicate sperm bound to epithelial cells (both ciliated and non-ciliated), the orange line indicates a group of cilia on a ciliated epithelial cell, the yellow line indicates a non-ciliated epithelial cell and the purple lines indicate unbound sperm. Image captured at 400X using a fluorescent microscope at half light.
Figure 2: Binding density of ejaculated, cauda and corpus sperm to bovine oviduct epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. Vertical error bars represent s.e.m. abDifferent superscripts between treatment groups differ significantly (P<0.05). n = 3 replicates. Experiment 1.
Figure 3: Binding density of cauda and corpus sperm incubated with various concentrations of recombinant BBD126 (rBBD126) to bovine oviduct epithelial cell explants. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm$^2$ of explant. Vertical error bars represent s.e.m. Different superscripts between treatment groups differ significantly (P<0.05). n = 5 replicates. Experiment 2a.
Figure 4: Binding density of cauda and corpus sperm to bovine oviduct epithelial cell explants following incubation with and without recombinant BBD126, BBD126 antibody (Ab) and a control IgG mouse antibody. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. Vertical error bars represent s.e.m. abc Treatment groups with different superscripts differ significantly (P<0.05). n = 5 replicates. Experiment 2b.
Figure 5: Binding density of ejaculated sperm to bovine oviduct epithelial cell explants following incubation with and without recombinant BBD126 and BBD126 antibody (Ab). Ejaculated sperm without rBBD126 or BBD126 antibody was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. Vertical error bars represent s.e.m. abcTreatment groups with different superscripts differ significantly (P<0.05). n = 3 replicates. Experiment 3.
Figure 6: Sperm agglutination of cauda and corpus sperm following incubation with various concentrations of recombinant BBD126. Vertical error bars represent s.e.m. \(^{a,b}\)Different superscripts between treatment groups differ significantly (\(P<0.05\)). \(n = 5\) replicates. Experiment 4.