**Size of supernumerary teats in sheep correlates with complexity of the anatomy and
microenvironment**

Short running title:

Microenvironment of ovine supernumerary teats

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Abstract

Supernumerary nipples or teats (polythelia) are congenital accessory structures that may develop at any location along the milk line and have been implicated in the pathogenesis of mastitis. We describe the anatomy and histology of 27 spontaneously occurring supernumerary teats from 16 sheep, delineating two groups of teats – simple and anatomically complex – according to the complexity of the anatomy and microenvironment. Anatomically complex supernumerary teats exhibited significantly increased length and barrel diameter compared to simple supernumerary teats. A teat canal and/or teat cistern was present in anatomically complex teats, with smooth muscle fibres forming a variably well-organised encircling teat sphincter. Complex supernumerary teats also exhibited immune cell infiltrates similar to those of normal teats, including lymphoid follicle-like structures at the folds of the teat cistern – teat canal junction, and macrophages that infiltrated the peri-cisternal glandular tissue. One complex supernumerary teat exhibited teat end hyperkeratosis. These anatomical and histological features allow inference that supernumerary teats may be susceptible to bacterial ingress through the teat canal and we hypothesise that this may be more likely in those teats with less well-organised encircling smooth muscle. The teat cistern of anatomically complex teats may also constitute a focus of milk accumulation and thus a potential nidus for bacterial infection, potentially predisposing to mastitis. We suggest that size of the supernumerary teat, and relationship to the main teats, particularly in the case of ‘cluster teats’, should be considerations if surgical removal is contemplated.

Keywords

Histology; lymphoid follicle; mammary gland; mastitis; microenvironment; supernumerary teat; sheep; teat.

Introduction

Sheep and goats typically have one pair of inguinal mammae, each with one ductal system and one teat (Hughes and Watson, 2018a). Supernumerary nipples or teats (polythelia) are congenital accessory structures which may develop at any location along the milk line (Stone and Wheeler, 2015). In goats, the presence of supernumerary teats is considered to be a heritable characteristic that is likely polygenic (Martin et al., 2016). Similarly, in sheep, teat number is a highly heritable characteristic (Rowson et al., 2012), and development of supernumerary teats is a complex polygenic trait (Peng et al., 2017). Over the centuries, selective sheep breeding programmes have, from time to time, been instigated to attempt to capitalise on the heritability of this trait to increase functional teat number (Bell, 1904; Phillips et al., 1946).

Paradoxically, in dairy cows, supernumerary teats are thought to have the potential to interfere with milking and to represent a mastitis risk, and therefore removal may be considered clinically appropriate in some cases (Roberts and Fishwick, 2010). Indeed, supernumerary teat removal correlates with a reduced incidence of subclinical mastitis in Dutch dairy heifers, although this observation could point to wider management differences between farms implementing different removal policies, rather than necessarily reflecting a direct effect (Santman-Berends et al., 2012). In small ruminants, as in cattle, bacterial mastitis is a major health issue with welfare implications (Hughes and Watson, 2018a), both in dairy sheep (Bramis et al., 2016) and those used for meat production (Phythian et al., 2011; Crump et al., 2019; Phythian et al., 2019). It has been suggested that poor udder conformation, including the presence of supernumerary teats, may also constitute a minor, but notable, risk factor for acquisition of new intramammary infections (Vasileiou et al., 2019), potentially as an indirect effect due to impairment of lamb sucking and consequent inefficient removal of milk (Menzies and Ramanoon, 2001). Whilst removal of supernumerary teats is less frequently performed than in dairy cows, the procedure may be undertaken from time to time (Sargison et al., 2018). On one Spanish farm, comparison of milk yield between dairy sheep in which supernumerary teats were removed, and those in which they were not, suggested that the procedure could be avoided (Palacios and Abecia, 2014).

There is a relative paucity of knowledge regarding the anatomy and histology of ovine supernumerary teats in spite of the dual interest in additional teats both as a target for selective breeding programmes, and conversely as a potential mastitis risk factor requiring removal, with the attendant welfare implications of such a procedure. The purpose of this study was therefore to investigate the anatomy and microenvironment of ovine supernumerary teats, with a particular focus on describing anatomical variations in these structures. We wished to assess whether there was any anatomical evidence to support the notion that these structures may form a risk factor for mastitis, for example, by evaluation of teats for the presence of teat sinuses in which milk could accumulate.

Materials and methods

Animals

Ewes from three separate sources were examined to assemble a panel of supernumerary teats for this study. Udders from adult ewes that were examined by the diagnostic veterinary anatomic pathology service of the Department of Veterinary Medicine, University of Cambridge or the Section for Small Ruminant Research, Norwegian University of Life Sciences, were assessed for the presence of supernumerary teats and those with such structures were included in the study. Additionally, udders were also collected *post mortem* from adult and immature ewes studied experimentally for other purposes (Vaughan et al., 2018) and euthanased under the Animals (Scientific Procedures) Act 1986. The Ethics and Welfare Committee of the Department of Veterinary Medicine, University of Cambridge, reviewed the study plan relating to the use of ovine post mortem material for the study of mammary gland biology (reference: CR223). An application for non-regulated use of animals in scientific procedures was granted for the *post mortem* collection of mammary tissue from the experimental ewes. Ewes with supernumerary teats were selected from the larger population of animals being examined in each of the contexts described above for the three tissue sources.

Animals with a history of mastitis, or with macroscopic evidence of mastitis, were excluded. With the exception of one case, macroscopic *post mortem* examination of each udder was conducted by a

single board-certified veterinary pathologist. In the exceptional case, macroscopic post mortem examination of the udder was carried out by a specialist from the European College of Small Ruminant Health Management, with images of the udder evaluated by the same veterinary pathologist as above. A total of 27 supernumerary teats from 16 ewes were examined. Statistics relating to the sample population were recorded to the nearest one decimal place.

Anatomic assessment

Udders were fixed in 10% neutral-buffered formalin. Following approximately 72 hours of fixation, the supernumerary teat was then incised in a sagittal or transverse plane, and the length of the teat, and the diameter of the teat at the barrel (middle part of the teat) were measured, a technique adapted from that described previously for bovine teats (Guarin and Ruegg, 2016). Supernumerary teats were inspected for internal anatomy and those exhibiting either a teat canal (ductus papillaris), teat cistern (pars papillaris sinus lactifer), or both, were classified as anatomically complex. Those exhibiting no such structure were considered simple. Teat length and barrel diameter were compared between the simple and anatomically complex groups using the two-tailed unpaired student's T-test.

Histology

Supernumerary teats from one immature ewe and 14 mature ewes, together with control non-supernumerary teats and udder skin from three mature ewes, were collected in 10% neutral-buffered formalin. Tissues were processed and subsequently cut at five microns to produce histological sections that were stained with haematoxylin and eosin (H&E).

Immunohistochemistry

Immunohistochemical (IHC) staining for CD3 (dilution 1:150; mouse monoclonal, clone F7.2.38, catalogue number M7254), (both Agilent/Dako Technologies LDA UK, Cheadle, Cheshire, UK) and ionized calcium-binding adapter molecule 1 (Iba-1; dilution 1:800; mouse monoclonal, clone 20A12.1, catalogue number MABN92, Millipore UK, Croxley Green Business Park, Watford, Hertfordshire, UK), followed routine protocols using an automated IHC system (Dako

Pathology/Agilent Technologies). Dual staining for smooth muscle actin (SMA; dilution 1:400; mouse monoclonal, clone 1A4, catalogue number M0851, Agilent/Dako Technologies LDA UK as above) and E-cadherin (dilution 1:500; rabbit monoclonal, clone 24E10, catalogue number 3195, Cell Signaling Technology Europe, Leiden, The Netherlands) was conducted using an ImmPRESS™ Duet Double Staining Polymer Kit (Vector laboratories, Peterborough, UK). Negative control slides were prepared using species-matched immunoglobulins.

Results

The size of supernumerary teats correlates with anatomical complexity

Twenty seven supernumerary teats from 16 ewes were included in the study. Eleven (68.8%) of the ewes exhibited bilateral supernumerary teats (Fig. S1). Animals at varying stages of the mammary postnatal developmental cycle were examined: five animals (31.3%) were pregnant, three (18.8%) were dry but previously parous, four ewes (25%) were sampled during post-lactational regression (mammary gland involution) and one ewe each (6.3%) was immature, mature but nulliparous, lactating, or had no stage of mammary postnatal development recorded. Several different breeds of suckler ewes were represented in the study population, with an overrepresentation of Welsh mountain ewes (11 ewes; 68.8%), and one (6.3%) each of Lleyn, Hebridean, Texel, and Norsk Kvit Sau. For one sheep, no breed information was available. Of the 27 supernumerary teats examined, one was from an immature ewe and was therefore analysed grossly and microscopically but excluded from teat measurements. One supernumerary teat from an adult ewe with bilateral supernumerary teats was examined macroscopically on superficial surface but was lost at the point of sampling and was thus not analysed further.

Of the 25 supernumerary teats examined from adult ewes, 13 (52%) were anatomically simple and had no macro- or microscopically apparent connection to either the gland cistern proximally or the external environment distally (Fig. 1A,B). One simple supernumerary teat from a seven month-old ewe lamb exhibited an indented, or partially bifid, appearance (Fig. 1B). Twelve (48%) of the supernumerary teats examined from adult ewes had internal anatomy to some extent resembling that

of normal teats, including teat canal and/or teat cistern, and/or communication with the gland cistern (Fig. 1C,D,E,F). Hereafter we will refer to this group of supernumerary teats as anatomically complex.

Of the 11 ewes exhibiting bilateral supernumerary teats, four (36.4%) exhibited two simple supernumerary teats, whilst four ewes (36.4%) had a complex supernumerary teat associated with each mamma, and two ewes (18.2%) had one simple and one complex supernumerary teat. As already stated, one supernumerary teat from one ewe with bilateral supernumerary teats was lost at the time of sampling and was not further classified as simple or complex. Of the five ewes with unilateral supernumerary teats, the majority (4/5; 80%) exhibited simple structures (Fig. S1).

Anatomically complex supernumerary teats frequently macroscopically exhibited similar features to normal ovine teats. For example, we observed teat end hyperkeratosis, which has been described in dairy sheep, in one anatomically complex supernumerary teat (Fig. 1C). This condition involves hyperplasia of the teat canal stratum corneum, and may contribute to poor udder health when non-supernumerary teats are affected (Vouraki et al., 2018). There are likely to be many potential contributors to development of teat end hyperkeratosis, including environmental factors, tissue stress, and factors associated with milk production (Plummer and Plummer, 2012; Vouraki et al., 2018).

By assessment following dissection, it did not appear that anatomically complex teats in our study consistently drained a separate zone of mammary parenchyma but rather usually connected with the gland cistern of the main teat or had no apparent connection to the main gland cistern. In some instances the supernumerary teat, although independent, was present in very close proximity to the main teat, a condition which has been previously described as ‘cluster teats’ (Plummer and Plummer, 2012) (Fig. 1D,E). Not all anatomically complex teats exhibited all anatomical components of normal teats and this group was considered to represent a spectrum of development. For example, one anatomically complex teat exhibited a distinct teat cistern but no apparent teat canal and was therefore considered non-patent and blind (Fig. 1F). However, the majority of the anatomically complex teats

were considered functional, denoting the ability of the teat to produce and eject milk (Plummer and Plummer, 2012) and even those anatomically complex teats which were regarded as non-patent appeared to exhibit the capacity to accumulate milk in the teat cistern. In one supernumerary teat, there was clear evidence of milk accumulation within an enlarged supernumerary teat cistern (Fig. S2).

When the fixed tissue lengths and diameter at the barrel of supernumerary teats from adult ewes were compared, anatomically complex supernumerary teats were significantly longer and wider than those which did not exhibit internal anatomy resembling normal teats (Fig. 1G,H), indicating that size is likely to be a good predictor of level of anatomical complexity of supernumerary teats.

Anatomically complex supernumerary teats exhibit similar histological features to normal teats

Simple supernumerary teats had a 'bland' histological appearance comprising expansion of the dermis with bundles of collagen, together with dermal adnexal structures, and were considered to be non-functional (Plummer and Plummer, 2012) (Fig. 2A). By contrast, anatomically complex supernumerary teats generally exhibited keratin-lined teat canals, and teat cistern lined by a bilaminar epithelium, very similar to those of normal ruminant teats (Paulrud, 2005) (Fig. 2B,C).

In the anatomically complex supernumerary teats, glandular tissue was multifocally present in subepithelial foci adjacent to the teat cistern, again resembling the architecture of normal teats (Plummer and Plummer, 2012). Occasional corpora amylacea were noted (Fig. 2D). Corpora amylacea are concretions of amyloid and other proteins that have been previously reported to arise in the ruminant mammary gland (Nickerson and Sordillo, 1985; Ludewig, 1998). Infrequently observed in the mammary stroma, corpora amylacea are considered to arise principally in actively secreting mammary alveoli (Brooker, 1978; Claudon et al., 1998), therefore suggesting in our study that the glandular tissue adjacent to the teat cistern of anatomically complex supernumerary teats may be active.

The microenvironment of anatomically complex supernumerary teats is similar to that of normal teats

The microenvironment of the mammary gland and associated teat is characterised by complex interplay between the spectrum of cells present (Hughes and Watson, 2018a). As responses to mastitis pathogens are critically dependent on the mammary immune system (Sordillo, 2018) we wished to further explore the characteristics of the immune cell populations noted within the anatomically complex supernumerary teats and their relationship with the epithelial components.

Prominent aggregates of lymphocytes were multifocally apparent at the folds delineating the teat cistern – teat canal junction (Fig. 2B,C). These lymphocytic groupings were strikingly similar to the mucosa-associated lymphoid tissue which has been described at this location in the non-supernumerary teats of ewes (Mavrogianni et al., 2006; Mavrogianni et al., 2007; Mavrogianni and Fthenakis, 2007). These multifocal lymphocytic groupings included large numbers of CD3-positive T lymphocytes (Fig. 2E). Intraepithelial CD3-positive T lymphocytes, indicative of exocytosis, were also present in the bilaminar epithelial lining of the supernumerary teat cistern. This finding is consistent with other species: we have previously described intraepithelial CD3-positive T lymphocytes in the epithelial lining of sinus-like dilatations of the mammary ducts of rabbits and foci adjacent to the mammary ducts (Hughes and Watson, 2018b) and infiltration of lymphocytes also occurs in the bovine teat cistern (Nickerson and Pankey, 1983). Iba-1 positive cells, with morphology and location consistent with macrophages, were also present adjacent to the supernumerary teat cistern (Fig. 2F). CD68+ macrophages, and a population of MHCII+ cells have previously been described to be present in non-supernumerary teats at the junction of the teat cistern and duct, following experimental challenge with *Mannheimia haemolytica* (Fragkou et al., 2010), and thus the identification of macrophages in this location in supernumerary teats further suggests that there is a typical mammary immune microenvironment in these accessory structures.

The stromal compartment of the mammary microenvironment also includes fibroblasts and smooth muscle cells (Hughes and Watson, 2018a), constituents which are particularly important in the teat

(Fig. 3). Smooth muscle has previously been observed in supernumerary nipples in humans (Mercuri et al., 2019). In ruminants, smooth muscle cells encircle the teat cistern and teat canal with an inner longitudinal arrangement and outer circular orientation (Nickerson and Akers, 2011; Koyama et al., 2013) forming a sphincter that favours closure and thus maintenance of the teat canal innate immune barrier (Wagener et al., 2018). Simple supernumerary teats exhibited stromal deposition of smooth muscle in an apparently random pattern, similar to that of normal skin (Fig. 3A,B,E,F,I,J). By contrast, anatomically complex supernumerary teats exhibited dense bundles of smooth muscle similar to non-supernumerary teats (Fig. 3C,D,G,H,K,L). Interestingly, clear orientation was discernible in some foci where smooth muscle fibres were arranged in parallel with each other (Fig. 3K), although this was not a consistent feature and in many foci the smooth muscle cells lacked a clear or consistent arrangement.

Discussion

In this study we have demonstrated that the size of supernumerary teats in sheep correlates with complexity of the anatomy and microenvironment. Simple supernumerary teats generally have a ‘bland’ histological appearance resembling that of haired skin, whereas larger anatomically complex supernumerary teats exhibit internal anatomy and histology resembling that of normal teats. This finding has important implications for the removal of supernumerary teats. Whilst teat removal has attendant welfare implications, and evidence suggests that it may be avoided (Palacios and Abecia, 2014), it is a procedure which is undertaken occasionally (Sargison et al., 2018). If teat removal is contemplated, then evaluation of the size of the teat may inform this decision. For example, it has been suggested that the presence of supernumerary teats may be a minor risk factor for mastitis (Menzies and Ramanoon, 2001; Vasileiou et al., 2019) and several observations from this study may support this notion. Firstly, larger supernumerary teats which form ‘cluster teats’ with the main teat may prevent efficient suckling and increase the likelihood of teat trauma from lambs (Roger, 2009). In addition, many larger anatomically complex supernumerary teats exhibit teat canals which may facilitate pathogen ingress, particularly given that the smooth muscle surrounding the canal exhibits only multifocal foci of organisation of smooth muscle fibres in a parallel arrangement and may thus

constitute a compromised sphincter compared to that of a normal teat. The teat cistern of larger, anatomically complex, supernumerary teats may facilitate milk accumulation, forming a nidus for bacterial infection, particularly in a supernumerary teat which is not being efficiently suckled or in a non-patent anatomically complex teat with a teat cistern in which milk can build-up. Thus, anatomical proximity to the main teat, and size of the supernumerary teat, as a surrogate indicator of anatomical complexity, may constitute valid considerations when removal is contemplated.

Our study also provides evidence for the presence of lymphoid aggregates at the teat cistern – teat canal junction in supernumerary teats. The presence of such aggregates in non-supernumerary ovine teats has attracted considerable attention and the lymphoid accumulations are suggested to be induced in association with presence of bacteria (Mavrogianni et al., 2007). It is therefore tempting to speculatively attribute a similar association in the anatomically complex supernumerary teats exhibiting such structures. We also noted teat end hyperkeratosis in one anatomically complex supernumerary teat. Hyperplasia of the teat canal stratum corneum has been linked to an increased risk of mastitis in dairy cows (Besier et al., 2016) and this observation in an ovine supernumerary teat hints at potentially similar microenvironmental changes that may favour pathogen invasion.

There are several limitations to this study. A historical report examining sheep selectively bred for additional teats describes a complicated protocol of mounting udders from euthanased ewes on a wooden frame, prior to perfusion with coloured 5% formaldehyde, to delineate the mammary parenchyma drained by additional teats in terms of anatomy and volume (Phillips et al., 1946). The authors concluded that the additional teats drained anatomically separate regions of the udder that had a notably small capacity compared to the primary mammae. Thus, these teats drained a separate zone of parenchyma that was not connected to the primary teat. Our study was not specifically designed to assess the connections between the gland cistern and teats in this manner, as it would have compromised histological assessment of the supernumerary teats. However, our impression, based on dissection alone, was that the anatomically complex supernumerary teats in our study generally connected with the gland cistern of the main teat rather than a distinct and separate zone of mammary

parenchyma, although a connection was not consistently apparent. It is self-evident that any breeding programmes designed to increase teat number in sheep should be supported by anatomical and histological studies similar to those we have performed, both to delineate the anatomy of the additional teats and to facilitate understanding of the communication between the supernumerary teat and the underlying gland cistern(s).

A further limitation of the study is that the majority of the ewes examined were Welsh mountain ewes, although both hill and lowland breeds were represented in our population. No breed associations can be inferred from our data, which was heavily affected by the populations examined at our institutions. It would be desirable to examine supernumerary teats from a wider range of breeds including dairy sheep. Although animals from a range of stages of postnatal mammary gland development were examined, a more even distribution of developmental stages would be preferable. However, a strength of this study is that ewes from experimental, commercial, and smallholding flocks were examined. Therefore in spite of the considerations described above, it seems likely that the spectrum of anatomical variants of supernumerary teats we have described is likely to be applicable to other groups of sheep, although it is possible that differences in microenvironment may be observed in sheep managed in differing types of production system. That said, much of the work on inducible lymphoid follicles in ewe teats has been carried out in dairy breeds (Fragkou et al., 2007; Katsafadou et al., 2019) and yet we observe very similar follicle-like structures in a subset of the supernumerary teats from the suckler ewes detailed here, hinting that the teat microenvironment may be similar even in animals from different production systems.

Taken together, the data delineated in this study provide novel insights into the mammary microenvironment of supernumerary teats and provide anatomical evidence to aid clinical decision-making surrounding teat removal. Since the presence of anatomically complex supernumerary teats may favour intramammary infection, further investigations could inform data-driven management decisions on the selection of replacement breeding animals without specific anatomical traits as a longer-term strategy that is preferable to surgical removal in young animals. Similar investigations in

dairy sheep and cows are therefore warranted and will likely enhance understanding of the role of supernumerary teats in the pathogenesis of mastitis in these groups of animals.

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Author contributions

LJAH and CJP acquired and interpreted data, and critically revised the manuscript. ALF contributed to data acquisition and critically revised the manuscript. KH conceived and designed the study, acquired and interpreted data, and wrote the manuscript. All authors have approved the submitted article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- Bell AG** (1904) The Multi-Nippled Sheep of Beinn Bhreagh. *Science* **19**, 767-8.
- Besier J, Lind O, Bruckmaier RM** (2016) Dynamics of teat-end vacuum during machine milking: types, causes and impacts on teat condition and udder health - a literature review. *Journal of Applied Animal Research* **44**, 263-272.

- Bramis G, Gelasakis AI, Kiossis E, Banos G, Arsenos G** (2016) Predisposing factors and control of bacterial mastitis in dairy ewes. *Journal of the Hellenic Veterinary Medical Society* **67**, 211-222.
- Brooker BE** (1978) The origin, structure and occurrence of corpora amylacea in the bovine mammary gland and in milk. *Cell Tissue Res* **191**, 525-38.
- Claudon C, Francin M, Marchal E, et al.** (1998) Proteic composition of corpora amylacea in the bovine mammary gland. *Tissue Cell* **30**, 589-95.
- Crump RE, Cooper S, Smith EM, Grant C, Green LE** (2019) Heritability of phenotypic udder traits to improve resilience to mastitis in Texel ewes. *Animal* **13**, 1570-1575.
- Fragkou IA, Dagleish MP, Papaioannou N, et al.** (2010) The induction of lymphoid follicle-like structures in the ovine teat duct following experimental infection with Mannheimia haemolytica. *Vet J* **184**, 194-200.
- Fragkou IA, Mavrogianni VS, Papaioannou N, et al.** (2007) Presence of sub-epithelial lymphoid tissues in the teat of ewe-lambs and adult ewes. *Small Ruminant Research* **70**, 286-291.
- Guarin JF, Ruegg PL** (2016) Short communication: Pre- and postmilking anatomical characteristics of teats and their associations with risk of clinical mastitis in dairy cows. *J Dairy Sci* **99**, 8323-8329.
- Hughes K, Watson CJ** (2018a) The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: A One Health focus. *J Mammary Gland Biol Neoplasia* **23**, 27-41.
- Hughes K, Watson CJ** (2018b) Sinus-like dilatations of the mammary milk ducts, Ki67 expression, and CD3-positive T lymphocyte infiltration, in the mammary gland of wild European rabbits during pregnancy and lactation. *J Anat* **233**, 266-273.
- Katsafadou AI, Politis AP, Mavrogianni VS, et al.** (2019) Mammary Defences and Immunity against Mastitis in Sheep. *Animals (Basel)* **9**.
- Koyama S, Wu HJ, Easwaran T, Thopady S, Foley J** (2013) The nipple: a simple intersection of mammary gland and integument, but focal point of organ function. *J Mammary Gland Biol Neoplasia* **18**, 121-31.

380 **Ludewig T** (1998) [Histologic studies on the corpora amylacea in the mammary glands of goats,
381 horses, and ewes]. *Dtsch Tierarztl Wochenschr* **105**, 62-4.

382 **Martin P, Palhiere I, Tosser-Klopp G, Rupp R** (2016) Heritability and genome-wide association
383 mapping for supernumerary teats in French Alpine and Saanen dairy goats. *J Dairy Sci* **99**,
384 8891-8900.

385 **Mavrogianni VS, Cripps PJ, Brooks H, Taitzoglou IA, Fthenakis GC** (2007) Presence of
386 subepithelial lymphoid nodules in the teat of ewes. *Anat Histol Embryol* **36**, 168-71.

387 **Mavrogianni VS, Cripps PJ, Papaioannou N, Taitzoglou I, Fthenakis GC** (2006) Teat disorders
388 predispose ewes to clinical mastitis after challenge with Mannheimia haemolytica. *Vet Res*
389 **37**, 89-105.

390 **Mavrogianni VS, Fthenakis GC** (2007) Clinical, bacteriological, cytological and pathological
391 features of teat disorders in ewes. *J Vet Med A Physiol Pathol Clin Med* **54**, 219-23.

392 **Menzies PI, Ramanoon SZ** (2001) Mastitis of sheep and goats. *Vet Clin North Am Food Anim Pract*
393 **17**, 333-58, vii.

394 **Mercuri SR, Paolino G, Donati M, Brianti, P** (2019) Incidence of supernumerary nipples in a large
395 population, with relative dermoscopic and clinic-pathologic features: an update and a fast
396 approach. *G Ital Dermatol Venereol* **154**, 596-597.

397 **Nickerson SC, Akers RM** (2011). Mammary gland anatomy. In: *Encyclopedia of dairy sciences* (ed.
398 Fuquay JW), pp.328-337. San Diego: Elsevier Ltd.

399 **Nickerson SC, Pankey JW** (1983) Cytologic observations of the bovine teat end. *Am J Vet Res* **44**,
400 1433-41.

401 **Nickerson SC, Sordillo LM** (1985) Role of macrophages and multinucleate giant cells in the
402 resorption of corpora amylacea in the involuting bovine mammary gland. *Cell Tissue Res* **240**,
403 397-401.

404 **Palacios C, Abecia JA** (2014) Supernumerary teat removal can be avoided in dairy sheep. *J Appl*
405 *Anim Welf Sci* **17**, 178-82.

406 **Paulrud CO** (2005) Basic concepts of the bovine teat canal. *Vet Res Commun* **29**, 215-45.

- Peng WF, Xu SS, Ren X, et al.** (2017) A genome-wide association study reveals candidate genes for the supernumerary nipple phenotype in sheep (*Ovis aries*). *Anim Genet* **48**, 570-579.
- Phillips RW, Schott RG, Spencer DA** (1946) The multinipple trait in sheep. *J Hered* **37**, 19-26.
- Phythian CJ, Michalopoulou E, Duncan JS** (2019) Assessing the Validity of Animal-Based Indicators of Sheep Health and Welfare: Do Observers Agree? *Agriculture-Basel* **9**, 1-14.
- Phythian CJ, Michalopoulou E, Jones PH, et al.** (2011) Validating indicators of sheep welfare through a consensus of expert opinion. *Animal* **5**, 943-52.
- Plummer PJ, Plummer C** (2012). Diseases of the mammary gland. In: *Sheep and goat medicine*. (eds. Pugh DG, Baird AN), pp. 442-465. Missouri: Elsevier Saunders.
- Roberts J, Fishwick J** (2010) Teat surgery in dairy cattle. *In Practice* **32**, 388-396.
- Roger P** (2009) Problems of the periparturient ewe. *In Practice* **31**, 122-129.
- Rowson AR, Daniels KM, Ellis SE, Hovey RC** (2012) Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. *Semin Cell Dev Biol* **23**, 557-66.
- Santman-Berends IM, Olde Riekerink RG, Sampimon OC, Van Schaik G, Lam TJ** (2012) Incidence of subclinical mastitis in Dutch dairy heifers in the first 100 days in lactation and associated risk factors. *J Dairy Sci* **95**, 2476-84.
- Sargison N, Crilly JP, Hopker A** (2018). Legislation. In: *Practical Lambing and Lamb Care: A Veterinary Guide*, pp. 144. Chichester: John Wiley & Sons Inc.
- Sordillo LM** (2018) Mammary Gland Immunobiology and Resistance to Mastitis. *Vet Clin North Am Food Anim Pract* **34**, 507-523.
- Stone K, Wheeler A** (2015) A Review of Anatomy, Physiology, and Benign Pathology of the Nipple. *Ann Surg Oncol* **22**, 3236-40.
- Vasileiou NGC, Mavrogianni VS, Petinaki E, Fthenakis GC** (2019) Predisposing factors for bacterial mastitis in ewes. *Reprod Domest Anim* **54**, 1424-1431.
- Vaughan OR, De Blasio MJ, Fowden AL** (2018) Ovine uteroplacental and fetal metabolism during and after fetal cortisol overexposure in late gestation. *Am J Physiol Regul Integr Comp Physiol* **314**, R791-R801.

Vouraki S, Gelasakis AI, Rose IJ, Arsenos G (2018) Risk factors of teat-end hyperkeratosis and its association with udder health in dairy ewes. *J Dairy Res* **85**, 44-49.

Wagener MG, Leonhard-Marek S, Hager JD, Pfarrer C (2018) CD117- and vimentin-positive telocytes in the bovine teat sphincter. *Anat Histol Embryol* **47**, 268-270.

Supplementary material

Figure S1. The majority of ewes in the study population exhibited bilateral supernumerary teats. Flow chart detailing the numbers of ewes with bilateral and unilateral supernumerary teats, and the number of simple or complex teats in each group. Numerals represent number of ewes in each category.

Figure S2. Milk can accumulate in sinuses associated with anatomically complex supernumerary teats. Transverse section through a supernumerary teat (arrow) from a Texel ewe. Diamond indicates milk accumulation within a cistern associated with the supernumerary teat. Scale bar indicates 5 mm.

Figure legends

Figure 1. Size of supernumerary teats correlates with anatomical complexity. (A) Parasagittal section through a simple supernumerary teat (arrow), Hebridean ewe, dry previously parous. (B) Parasagittal section through a partially bifid simple supernumerary teat (arrow), seven month-old Welsh mountain ewe lamb. (C) Gross appearance of a complex supernumerary teat exhibiting hyperkeratosis (arrow), Welsh mountain ewe, 145 days gestation. (D) Gross appearance of a complex supernumerary teat (arrow) in very close proximity to the main teat, Welsh mountain ewe, 14 days involution. A parasagittal section from the same ewe (E) demonstrates that this anatomically complex supernumerary teat has a teat canal (arrow) and teat cistern (asterisk) that resemble that of the main teat. (F) Parasagittal section through an anatomically complex supernumerary teat that exhibits a teat cistern (asterisk) but lacks a discernible teat canal, Norsk Kvit Sau ewe, 10 weeks lactation. Diamonds

indicate gland cistern and arrowheads indicate teat canal of main teat (A, E, F). Scale bars indicate 10 mm (A), 5 mm (B), 5 mm (C, D), 10 mm (E, F). Scatter dot plots comparing the fixed tissue lengths (G) and teat diameter at the barrel (H) of simple and anatomically complex supernumerary teats demonstrate that anatomically complex teats are significantly longer and wider than simple supernumerary teats. Bars represent mean $\pm$ standard deviation; **** $p < 0.0001$, as determined by a two-tailed unpaired student's T-test

Figure 2. Simple supernumerary teats exhibit internal architecture similar to skin whereas anatomically complex supernumerary teats exhibit a microenvironment similar to non-supernumerary teats. (A) Sagittal section through a simple supernumerary teat. The dermis exhibits hair follicles (arrow) and adnexal structures and there is no teat canal. (B, C) Sagittal sections through two anatomically complex supernumerary teats which exhibit lymphoid follicles (arrows) present in folds of tissue at the junction between the proximal teat canal (arrowhead) and the teat cistern (diamond). (D) Corpora amylacea (arrow) are multifocally present within glandular tissue adjacent to the teat cistern (diamond) of anatomically complex supernumerary teats. (E) CD3-positive T lymphocytes (arrow) are present multifocally adjacent to the junction between the teat canal and the teat cistern. (F) Immunohistochemical staining for Iba-1 indicates cells consistent with activated macrophages surrounding bilayered ducts (double headed arrow) that branch from the teat cistern of anatomically complex supernumerary teats. Arrowhead indicates teat canal; diamond indicates teat cistern. Scale bars indicate 800 μ m (A), 400 μ m (B, C), 80 μ m (D, E, F). H&E stain (A, B, C, D) and immunohistochemical stain for CD3 (E) and Iba-1 (F) with haematoxylin counterstain (E, F). Images are representative from analysis of four (simple supernumerary teats) and six (complex supernumerary teats) biological repeats

Figure 3. Anatomically complex supernumerary teats exhibit dense, aligned, smooth muscle actin fibres adjacent to the teat canal. Sagittal sections through udder skin (A, E, I), the distal aspect of a simple supernumerary teat (B, F, J), a complex supernumerary teat (C, G, K) and a non supernumerary teat (D, H, L) respectively. The dermis of simple supernumerary teats (B, F, J) is

similar to that of udder skin (A, E, I), with usual dermal adnexal structures including sebaceous glands (diamonds). Adjacent to the teat canal (arrowheads) of complex supernumerary teats (C) and normal teats (D) there is paucicellular connective tissue expanded by smooth muscle fibres. In simple supernumerary teats (F, J) smooth muscle is present in the walls of blood vessels (black arrow) and apocrine glands, or is randomly arranged in the teat dermis similar to the arrangement in the dermis of udder skin (E, I) (purple arrow). In complex supernumerary teats (G, K), densely packed smooth muscle fibres are multifocally aligned in parallel (purple arrow) and are intermingled with collagen bundles similar to the arrangement found in non-supernumerary teats (H, L) (purple arrow). Smooth muscle actin is also present within the walls of large blood vessels (black arrows). H&E stain (A-D) and immunohistochemical stain for smooth muscle actin (brown) and E-cadherin (pink) with haematoxylin counterstain (E-L). Scale bars indicate 800 μm (A-D), 400 μm (E-H) and 80 μm (I-L). Images are representative of analysis of three biological repeats for each type of teat.

Figure 1

101x167mm (300 x 300 DPI)

Figure 2

166x155mm (300 x 300 DPI)

Figure 3

162x123mm (300 x 300 DPI)

Supplementary Figure 1

232x94mm (300 x 300 DPI)

Supplementary Figure 2

99x132mm (300 x 300 DPI)