University of Reading

The human follicular mites *Demodex folliculorum* and *D. brevis* (Acari, Demodicidae) biology and molecular studies

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School of Biological Sciences

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Declaration

To our knowledge, this photic response has not been previously noted

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

EDHAH SALEM M. ALSAEEDI

October 2019

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Abstract

Demodex mites (Acari) are highly specialised arthropods which infest the hair follicles of most mammals, including humans. Two species have been detected in human skin, *Demodex folliculorum* Simon, 1842 and *D. brevis* Akbulatova, 1963. They are common, intracutaneous parasites of the pilosebaceous complex and meibomian glands and can be found on the face, forehead, chest, neck, eyelids, eyebrows, scalp and the ear canal. The pathogenesis of human *Demodex* mites is far from well understood, but skin diseases such as gland dysfunction, dermatitis, rosacea and even follicular basal cell carcinoma may be caused by them. This study contributes to our knowledge of human *Demodex* biology with an evaluation of, and improvements on, existing methods for their microscopic identification. No method is currently available to sustainably rear these mites *in vitro*, so research continues to rely on *de novo* collection from willing hosts, a laborious and unreliable process. We investigate some published rearing media and propose new formulations for artificial media that may lead to success in this important area. We analysed the prevalence of *Demodex* mites in samples from human subjects of different ages, ethnicities, host's sex, birth modes and postnatal feeding regimes, and found that age and postnatal feeding were influential on rates of infestation. Protocols and primer combinations are developed for a new, single, multiplex PCR reaction for DNA-based identification of mite species hosted by humans, which also includes the commonly carried dog mite, *D. canis.* The multiplex PCR successfully discriminated the three species, based on DNA fragment sizes and was also tested successfully on mites from several host ethnic groups. Finally, we assessed various proprietary kits and protocols for extracting, purifying and quantifying genomic DNA from *D. brevis*, since full nuclear sequences is not yet available for this

species. The quality of resultant genomic DNA samples and the limitations of the kits are discussed. We make recommendations for further research and methodological improvements that may take forward our important contributions to the biology and DNA based identification and characterisation of these mites.

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List of Abbreviations

AGMB	Agarose Gelatine Based Medium
AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
ARBM	Agarose RPMI Based Medium
COI	*Cytochrome Oxidase* Subunit I
DIN	DNA Integrity Number
ESEM	Environment Scanning Electron Microscopy
gDNA	Genomic DNA
GLM	Generalised Linear Model
IVLSCM	*In Vivo* Scanning Confocal Microscopy
MASM	Melanoma Cell with Human Artificial Sebum
MRSA	Methicillin Resistant *Staphylococcus aureus*
mtDNA	Mitochondrial DNA
NCBI	National Centre of Biotechnology Information
NGS	Next Generation Sequencing
PBS	Phosphate Buffer Saline
RCM	Reflectance Confocal Microscopy
RPMI	Roswell-Park Memorial Institute
SSSP	Standardised Skin Surface Biopsy
ZANB	Zero Altered Negative Binomial
ZINB	Zero Inflated Negative Binomial
ZIP	Zero Inflated Poisson

Chapter 1: Introduction

Follicular mites are classified as members of the Arthropoda, class Arachnida, subclass Acari (Krantz and Walter, 2009). According to Coston (1967), there are more than 1 million species of mites, and more than 1600 different species have been described in the United Kingdom alone. Species of Acari are either free living or parasitic. Free-living Acari might be found in various environments, including soil, caves, birds' nests and water (Krantz and Walter, 2009). Parasitic mites can have an impact on human beings, causing diseases, for example *Sarcoptes scabiei* is responsible for scabies, but most often because they serve as a vector for transmitting diseases to either humans or domestic animals, for example *Dermanyssus gallinae* both is the cause of anaemia in birds and is likely responsible for transmitting other diseases such as Salmonellosis, and *Cheyletiella yasguri* (= *Cheyletiella dermatitis*) which causes Dermatitis in dogs (Hughes, 1959; Wayman, 1969; Walton and Currie, 2007).

Follicular mites belong to the family Demodicidae in the order Trombidiformes. Members of this family infest the hair follicles of various mammals, including both wild and domestic animals (Nutting, 1976a). Humans are no exception, two species of follicular mites have been detected in human skin, namely *Demodex folliculorum* (Simon, 1842) and *Demodex brevis* (Akbulatova, 1963). Many studies have been conducted worldwide and a vast number of books and articles have been published by acarologists and dermatologists on follicular mites, however, many of their findings regarding the roles of these mites on human skin remain controversial and the conclusions lack sufficient evidential justification (Gmeiner, 1908; Ayres and Mihan, 1967; Izdebska, 2004). Moreover, researchers often have divergent points of view, Owen (1844), for example, stated that *D. folliculorum* is usually found in humans, but that it does not seem

to have a harmful effect on the host, however, dermatologists have frequently linked some skin diseases and hair loss to follicular mites, in 1932, Ayers and Anderson did so by reporting that the cause of a rosacea-like disease in 17 out of 28 of their patients was *D. folliculorum* (Ayres, 1932). Other examples of this disagreement are the lack of a consistent terminology for describing the life stages in *Demodex* and the different numbers of segments of the legs and palpi that have been recorded (Akbulatova, 1963).

1.1 The history of human follicular mites

Follicular mites were identified for the first time in 1841 by the French scientist Berger, who discovered a microorganism in human earwax (Berger, 1845). The Swiss scientist Henle independently discovered the same organism in ear canals when studying vertebrates, but he was not sure how to classify his new finding (Henle, 1845). In 1842, the German physician Gustav Simon isolated the organism from hair follicles and determined it to be a mite, he called it *Acarus folliculorum*, and described it in detail, including its life cycle (Simon, 1842). However, his description of the order of the developmental stages has not been upheld (Desch, 2009). In 1844, the English dermatologist Erasmus Wilson published research on human follicle mites describing their morphology and pointing to the occurrence of a type of mite living on human skin, which he characterised as a parasite and to which he gave the name *Entozoon folliculorum.* Wilson found that Simon's description of the anatomical structure was wrong, because there is no separation between head and thorax and it has four pairs of legs, characters that differentiate it from other species of *Acarus*. Subsequently, the English scientist Owen gave it the name *Demodex folliculorum, Demodex* comes from the Greek words *δημος* meaning fat (which he termed 'lard', a reference to the sebaceous glands which are its habitat) and *δηξ*, meaning boring worm (Owen, 1843, p. 252). Later, Hirst (1919) described five life stages in relation to *Demodex*, the same stages were

described by Fuss (1935), but he gave them different names. Leydig's work on Demodicidae and Sarcoptidae led to the description of more follicular mite species, such as *D. phyllostomatis* from bats and *D. canis* from dogs (Leydig, 1859). Seven more species of follicle mites were described by Owen (Owen, 1844; Hirst, 1919) and 13 *Demodex* species in wild and domestic mammals were found by Gmeiner in 1908 (Desch and Nutting, 1972). Another 32 mites were identified between that date and 1960 (Desch, 2009). In 1963, in Russia, Akbulatova noticed morphological differences in human follicle mites and concluded that there are two distinct types, which she named *D. folliculorum longus* and *D. folliculorum brevis* (Akbulatova, 1963). In 1972, Desch and Nutting renamed these mites as *D. folliculorum* (Simon, 1842) and *D. brevis* (Desch and Nutting, 1972). Most recently, in 2009, Desch calculated that there were more than 86 known species of follicle mites, which he claimed inhabit almost all mammalian species (Desch, 2009). A list of some common *Demodex* spp. and their mammalian hosts can be found in Appendix 1.

1.2 The taxonomy of follicular mites

Arthropoda is one of the largest phyla in the kingdom Animalia and it is incredibly diverse (Hickman *et al.*, 2008), consisting of four subphyla, including: Hexapoda, Crustacea, Myriapoda and Chelicerata (Brusca *et al.*, 2016). Arachnida is a significant class of the subphylum Chelicerata because it comprises a variety of terrestrial assemblages (Pearse *et al.*, 1987; Hickman *et al.*, 2008), such as spiders, scorpions and mites (Acari) (Brusca *et al.*, 2016). According to Brusca *et al.* (2016) there are more than 110,000 recognised species of mite, classified in 16 orders. As with the phylum Arthropoda in general (Krantz and Walter, 2009), and other Arachnids, mites have a chitinous exoskeleton, jointed legs, striated muscle and an open circulatory system (Hickman *et al.*, 2008). In addition, they have two fused body segments, with or without eyes (Brusca *et al.*, 2016).

Morphological and physiological differences, such as the fused head and thorax (cephalothorax), four pairs of legs, and absences of antennae and mandibles distinguish the Arachnida from other Arthropods (Pearse *et al.*, 1987). Mites and ticks are classified in the subclass Acari, which comprises approximately 54,600 described species (Robinson, 2005). They are considered the smallest organisms in the Arthropoda (Brusca *et al.*, 2016), most species are microscopic (excluding the ticks and Opilioacarida) and have a two-section segmented body. There are two main lineages in the Acari, the Acariformes and the Parasitiformes, these major superorders are closer to each other than to other arachnids and each contains a variety of orders. Parasitiformes is divided into four orders, including Ixodida and Holothyrida, while Acariformes has two orders, separated primarily by presence or absence of stigmata and their location, Trombidiformes and Sarcoptiformes (Krantz and Walter, 2009). Trombidiformes has two suborders, namely Sphaerolichida and Prostigmata (Krantz and Walter, 2009). Prostigmata can be classified into four supercohorts each of them is divided into several superfamilies, including superfamily Cheyletoidea (Krantz and Walter, 2009).

The superfamily Cheyletoidea is one of the most diverse groups of mite, it includes 130 lineages and more than 600 described species; it contains seven families. About 75 % of the described species have a parasitic relationship with mammals, birds or reptiles (Pearse *et al.*, 1987). Krantz and Walter (2009) place human follicular mites in the family Demodicidae, these are some of the smallest known arthropods. Morphological features which are important in this family include the shapes and distance between the supracoxal spines of the setae, the presence and shape of epimeral plates and the presence or absence of a proctodeum (Akbulatova, 1963; Desch and Nutting, 1972; Pearse *et al.*, 1987; Izdebska, 2009). In general, they are worm-like parasites that infest mammalian hair follicles, sebaceous glands or epidermis and may cause hyperplasia, epithelial destruction

or dermatitis (Krantz and Walter, 2009). Most mammals may serve as natural hosts for various of the *Demodex* species, including bats and aquatic and terrestrial mammals (Pearse *et al.*, 1987). However, the nature of the mite-host association may vary, for instance, *Demodex canis* (Leydig, 1859) causes severe mange in young dogs or those with a compromised immune system (Krantz and Walter, 2009; Fondati *et al.*, 2010), while in horses, *D. equi* causes pruritus and alopecia (Nutting, 1976a). Humans are infected by two *Demodex* species, *D. folliculorum* and *D. brevis* (Figure 1.1).

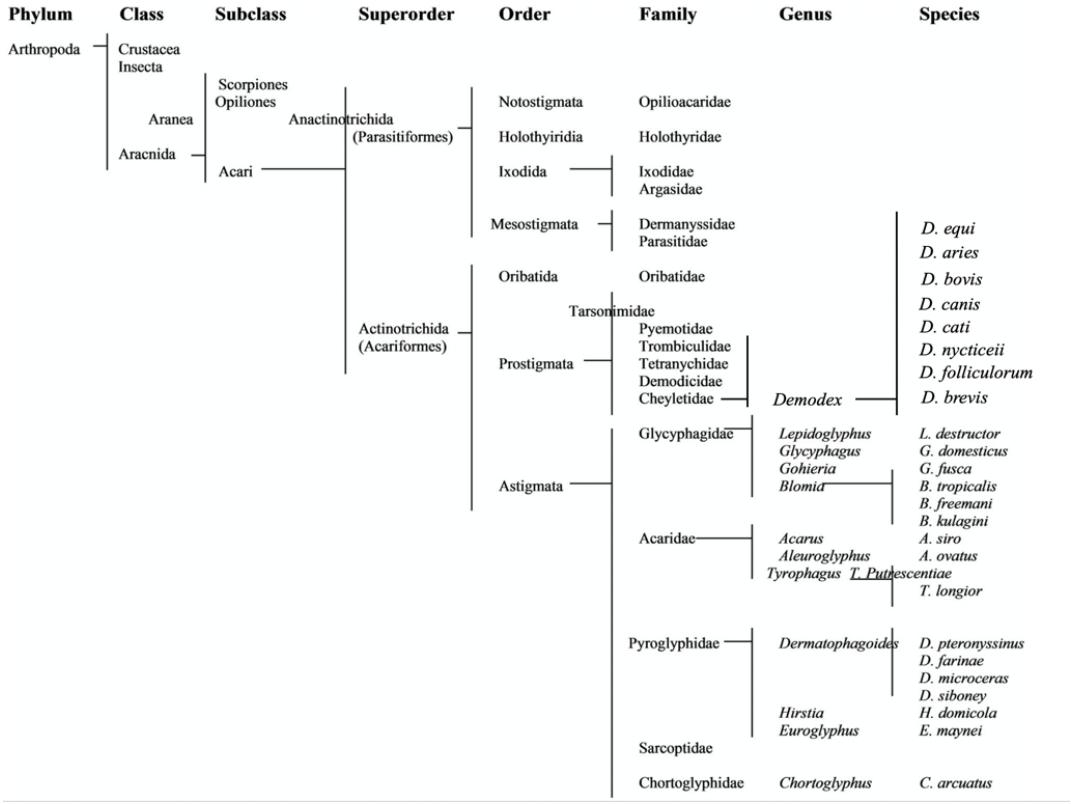

Figure 1.1 Taxonomic distribution of *Demodex* mites.
Adapted figure from (Sanchez-Borges *et al.*, 2017)

1.3 The biology of *Demodex* mites of humans

Demodex species are some of the most specialised arthropod parasites of mammals, they are obligate ecto-parasites (Desch and Nutting, 1972). In humans, *Demodex folliculorum*

and *D. brevis* are common intracutaneous parasites of the hair follicles and sebaceous and meibomian glands. They can be found on the face, forehead, chest, neck, eyelids, eyebrows and scalp, as well as in the ear canal (Akbulatova, 1963), nipples (Chambers, 1925; Borrel, 1909), knees (Vance, 1981), sebaceous glands of the tongue (Trodahl, 1967) and foreskins (Breckenridge, 1953). Moreover, they can spread to the bloodstream through the lymph nodes and reach interior organs, such as the liver, intestinal wall and lungs (Akbulatova, 1963). They are a cause for concern in particular as they are linked to skin disorders, however, most people with healthy skin carry either one or both species (Gmeiner, 1908; Ayres and Mihan, 1967; Perotti *et al.*, 2009).

1.3.1 The ecological niche of *Demodex*

Demodex folliculorum usually inhabits the area above the sebaceous gland next to the hair shaft and its dorsum is towards the hair follicle wall, its body may be half protruding from the follicle. In contrast, *D. brevis* resides fully in the sebaceous gland, with mouthparts pointed towards the fundus (Desch and Nutting, 1972). These niche separations, despite both species being found in the same pilosebaceous complex (hair, hair follicle, arrector pili muscle and sebaceous gland), help to distinguish them (Akbulatova, 1963; Desch and Nutting, 1972; Desch, 2009; Carly and Dirk, 2014).

1.3.2 Description of *Demodex folliculorum*

Demodex folliculorum are larger than *D. brevis* and are more likely to be extracted from the skin because half of the body remains outside of the hair follicle (Desch and Nutting, 1972). In contrast, *D. brevis* live deep in the sebaceous gland (Perotti *et al.*, 2009) so are less accessible for extraction. The body of the adult *D. folliculorum* has a long, rectangular lumbriciform shape with a small gnathosoma and long opisthosoma (Akbulatova, 1963) comprising 70 % of the body (Desch and Nutting, 1972). The two

sexes are almost identical (Akbulatova, 1963), however, females' average length is 436.5 μm, while that of males is about 279.7 μm. The body width of the female averages circa 118.7 μm, while the male body averages circa 101.7 μm (Desch and Nutting, 1972).

The gnathosoma has a trapezoidal shape with a rounded distal end (Akbulatova, 1963). Its average length in the female is 21.3 μm and 19.5 μm in the male; its width is greater than its basal length. The pharyngeal bulb opening is on the posterior side and has a horseshoe-like shape (Desch and Nutting, 1972). There are five or more rod-shaped papillae on the distal segment of each palp (Akbulatova, 1963). The supracoxal spines have minute lateral projections, the middle one is largest (Desch and Nutting, 1972). Four pairs of short legs are present in the adult and nymph stages, each of which has a pair of tarsal claws (Desch and Nutting, 1972). The vulva opening of the female is located on the ventral side between the fourth pair of legs (Izdebska, 2009) and is about 8.5 μm long. The well-developed aedeagus of the male is located dorsally in the centre of the podosoma (Izdebska, 2004). Ova are transparent and sagittiform with a narrow end measuring 81.8 μm (Desch and Nutting, 1972).

1.3.3 Description of *Demodex brevis*

The morphology of *D. brevis* is similar to that of *D. folliculorum*. The female is also longer than the male; the total length of the female is 208.5 μm, while males measure approximately 165.8 μm (Desch and Nutting, 1972). Both have a short opisthosoma with a cone-like dorsal end (Akbulatova, 1963), which makes up about two-thirds of the body length (Desch and Nutting, 1972).

The gnathosoma is shortly trapezoid and 14.5 μm and 16.3 μm long in the male and female, respectively (Desch and Nutting, 1972), it also has two basal transverse striations (Akbulatova, 1963). The pharyngeal bulb opens from the posterior side and is horseshoe

shaped. The supracoxal spines are cone shaped and there are minute setae on the subgnathosoma (Desch and Nutting, 1972). The palpi have bristles dorsally that are barely visible (Akbulatova, 1963), and the setae have three claws (Desch and Nutting, 1972). No proctodaeum is present in either sex (Desch and Nutting, 1972). In contrast to *D. folliculorum*, *D. brevis* have wide podosoma (Akbulatova, 1963). Four pairs of legs are connected to the podosoma, and each leg has a pair of claws (Desch and Nutting, 1972). The vulva has a basic slit structure, is located posteriorly behind the fourth pair of legs and is 6.9 µm long (Desch and Nutting, 1972). The male aedeagus is located dorsally between the second pair of legs and measures about 17.6 µm (Desch and Nutting, 1972). Eggs are oval-shaped, 60.1 µm (Desch and Nutting, 1972) and dark brown with small grains (Akbulatova, 1963).

1.3.4 *Demodex* life cycle

In both species the life cycle is identical, the copulation of *D. folliculorum* occurs at the follicle opening at night because the mites have a negative phototactic reaction (Spickett, 1961). After insemination, the females travel deep inside the hair follicle or to the sebaceous gland, where they deposit eggs (Desch and Nutting, 1972). Spickett (1961) stated that females lay eggs after about 12 hours of copulation, the eggs hatch and give rise to larvae approximately 60 hours later. The average length of a *D. folliculorum* larva is 282.7 µm, and its width is about 33.8 µm; in *D. brevis*, the larva is approximately 105.4 µm long and 33.8 µm wide (Desch and Nutting, 1972). The larva devolves to a protonymph after 40 hours (Spickett, 1961). The main difference between these stages is that the larva has three pairs of legs that have tubercles while the protonymph has four pairs of legs. The length and width of larva are different between the two species; they are about 364.9 µm long and 36.3 µm wide in *D. folliculorum* and 147.6 µm long and 34.4 µm wide in *D. brevis* (Desch and Nutting, 1972). The protonymph develops to a

deutonymph after 42 hours (Spickett, 1961), which is about 41 µm wide in both species but in *D. folliculorum* it is 392 µm, long and in *D. brevis* 165 µm long. The deutonymph develops into an adult after approximately 60 hours and lives for five days. According to Spickett (1961) the life cycle of *Demodex* spp. is approximately two weeks, however, this has not been confirmed and Wilson (1844) reported live *Demodex* mites from human corpses at 14 days from death, at room temperature conditions (Figure 1.2).

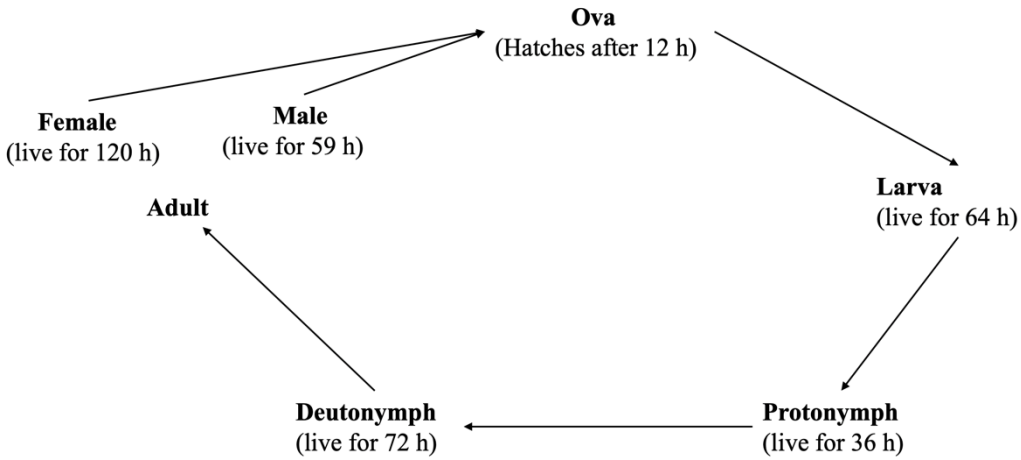

Figure 1.2 The life cycle of human *Demodex* mites. Approximately 14 days according to (Spickett,1961).

1.4 Feeding

From the larval to the adult stages, *Demodex* spp. consume hair follicular epithelium cells and the sebum from the sebaceous gland (Nutting, 1963). In a study conducted on the

digestive system of *D. folliculorum*, Desch (1998) observed that the chelicerae puncture the host epithelial cells, allowing the cytoplasm to drip into the preoral cavity. Saliva is secreted onto the cytoplasm and a liquid mixture is produced. This mixture is transferred by the pharyngeal pump through the narrow lines of the oesophagus towards the gut cells. Desch (1998) presumed that the anterior-most cells (Type 1 cells) absorb the mixture and process it and that lipids may be reserved and stored in elongated (Type 2 cells) to be used when energy is needed. He surmised that nitrogenous waste products were converted to non-toxic, guanine-based granules and then stored in Type 1 cells and that other digestive products were stored as lipid droplets in the cytoplasm of Type 2 cells and then used to build other body structures. A surprising finding in Desch and Nutting (1978) study was that there is no mid-gut or anus opening in the digestive system of *Demodex* mites.

1.5 Human *Demodex* mites and skin disorders

A large number of diseases affecting hair follicles have been reported. For instance, androgenetic alopecia and hypertrichosis may be linked to hormonal disorders or to autoimmune diseases, such as lupus erythematosus in the case of inflammatory alopecia (Paus and Cotsarelis, 1999). However, it has been suggested that the development of some skin diseases, including dermatitis, rosacea and pityriasis folliculorum may be caused by *Demodex* mites (Ayres, 1932; Morrás *et al.*, 2003; Crawford *et al.*, 2004). Scabies-like eruptions, facial pigmentation, gland dysfunctions, hair loss from the scalp and even follicular basal cell carcinoma have all, also been attributed to follicular mites (Erbagci *et al.*, 2003). It has been confirmed that *D. folliculorum* and *D. brevis* are the cause of blepharitis (Liu *et al.*, 2010; Carly and Dirk, 2014). They may be carried by almost everyone (Gmeiner, 1908; Ayres and Mihan, 1967; Lacey *et al.*, 2009; Perotti *et al.*, 2009), including healthy individuals, without any apparent harmful effects, so their

presence has an unclear pathogenesis (Akbulatova, 1963; Owen, 1844; Simon, 1842). In their comprehensive literature review, Kligman *et al.* (2011) cast doubt on the widely held view that *Demodex* are responsible for skin disorders (Kligman and Christensen, 2011).

There is evidence that a high density of *Demodex* infesting the hair follicles is correlated with some skin diseases, such as papulopustular rosacea and blepharitis (Lacey *et al.*, 2009). Individuals suffering from rosacea have an average density of *Demodex*, from skin biopsies, of 10.8 cm^2 whereas in healthy individuals it is < 5 cm^2 (Forton and Seys, 1993) and mite densities in rosacea patients are 5.7 times higher than in healthy individuals (Casas *et al.*, 2012). Gao *et al.* (2005) suggest that it is the high populations of the mites with their commensurate waste and debris which block the hair follicles which ultimately causes the follicles to dysfunction, leading to cylindrical dandruff and the rosacea.

Proliferation of the mites may stimulate microbial diseases and inflammatory responses by acting as a vector, carrying bacteria such as *Streptococcus* spp. or methicillin-resistant *Staphylococcus aureus* (MRSA) (Wolf *et al.*, 1987). However, the involvement of *Demodex* spp. in bacterial infections which cause skin diseases lacks conclusive research, hence is unclear and requires further investigation (Casas *et al.*, 2012; Jarmuda *et al.*, 2012; Cheng *et al.*, 2015).

1.6 Transmission

It has been reported that adult mites moving from follicle to follicle and climbing hairs is a possible process by which transfer between hosts occurs (Daniel *et al.*, 1973). Spickett (1961) reported seeing *D. folliculorum* in the deutonymph stage crawling on the skin surface and speculated on this evidence that adults and nymphs are most likely to be the transmission stages. According to Desch and Nutting (1972), *D. folliculorum* is usually

found infesting the area above the sebaceous gland with part of the opisthosoma outside the hair follicle, therefore, it can be easily spread from individual to individual via skin-to-skin contact (Carly and Dirk, 2014). There is no direct evidence that *Demodex* transmits horizontally in humans (Perotti *et al.*, 2009) and it has been reported that no transmission took place between partners despite living together for long time (Fisher, 1973; Bukva, 1990), hence it is widely accepted that transmission between humans does not occur. Molecular DNA evidence produced recently has shown, however, that some transmission does take place where there is close contact, Palopoli *et al.* (2015), for example, demonstrated greater similarity between haplotypes of *D. folliculorum* within families and spouses than in unrelated people. While Ugras *et al.* (2009) examined the healthy skin of scrotums and perineum areas of 100 male subjects aged between 20 and 70 and did not find *D. folliculorum* or *D. brevis* in any samples, Breckenridge (1953), however, did report the occurrence of *D. folliculorum* in foreskins, so that transmission via sexual contact may be taking place.

Follicular mites have never been studied in new-born babies (Czepita, 2004; Ozdemir *et al.*, 2005; Carly and Dirk, 2014). Both *D. folliculorum* and *D. brevis* have, however, been reported to survive in nipples (Borrel, 1909; Chambers, 1925; Yokoyama *et al.*, 2014), therefore, it is possible that *D. folliculorum* and *D. brevis* are acquired by vertical transmission during infancy (Fisher, 1973; Czepita, 2004; Carly and Dirk, 2014), via the close contact between the mother and her child during breast feeding and nursing (Izdebska and Fryderyk, 2012; Carly and Dirk, 2014). The difference between the mother's and child's temperatures during breast feeding and caressing may play a significant role in enhancing the transmission process (Perotti *et al.*, 2009).

1.7 Collection and study of human *Demodex* mites

There are various techniques for obtaining *Demodex* from human skin (Ozdemir *et al.*, 2003; Karaman *et al.*, 2010) such as hair epilation, usually from the eyelashes (Kojima *et al.*, 2011; de Rojas *et al.*, 2012a; Thoemmes *et al.*, 2014; Cheng *et al.*, 2015), scraping or squeezing the skin (Zhao *et al.*, 2007; Izdebska, 2009; Isa *et al.*, 2011), the cellophane tape method (Ozdemir *et al.*, 2003; Izdebska, 2009; Isa *et al.*, 2011; de Rojas *et al.*, 2012a) and standardised skin surface biopsy (SSSB) (Ozdemir *et al.*, 2003; Lacey *et al.*, 2007; Ugras *et al.*, 2009; Isa *et al.*, 2011; de Rojas *et al.*, 2012a).

To examine the mites collected from humans, various methods have been widely employed (Lacey *et al.*, 2009; Carly and Dirk, 2014), such as light microscopy (Ozdemir *et al.*, 2003; Dong and Duncan, 2006; Karaman *et al.*, 2010; Isa *et al.*, 2011; Kojima *et al.*, 2011; de Rojas *et al.*, 2012a; Weyrich *et al.*, 2015), environment scanning electron microscopy (ESEM) (Tang, 2000; Matsuo and Takahashi, 2002; Kumar and Bate, 2004; Jing *et al.*, 2005), reflectance confocal microscopy (RCM) (Cheng *et al.*, 2015) and *in vivo* scanning confocal microscopy (IVLSCM) (Kojima *et al.*, 2011).

These techniques are described, where appropriate, in detail in Chapter 2.

1.8 Molecular studies on human *Demodex* mites

Mitochondrial DNA (mtDNA) has been employed in most of the research so far conducted on *Demodex* mites of humans (de Rojas *et al.*, 2012a; Zhao *et al.*, 2013a; Palopoli *et al.*, 2014), because it is suitable for distinguishing between closely related mite populations. A high degree of resolution is achieved because mitochondrial genes evolve rapidly, more so than many nuclear genes (de Rojas *et al.*, 2012a). A high percentage of adenine/thymine (AT) in mtDNA genomes seems to be a common feature of Acariform mites, it averages circa 74.9 % in all 15 Acariformes species available on

GenBank ® (www.ncbi.nlm.nih.gov/genbank/) including *D. brevis* (69 %) and *D. folliculorum* (71.1 %) (Palopoli *et al.*, 2014). Palopoli *et al.* (2014) considered that the same selective forces and collections of mutation pressures which give rise to this effect must be operating on *Demodex* lineages as on other species of Acariformes. mtDNA in both species of human follicular mites has 2 ribosomal RNA, 13 protein-coding RNA and 22 transfer RNA genes (Boore, 1999; Smith, 2015), which are identically arranged in both *D. brevis* and *D. folliculorum* genomes, but not found in other Acariform species (Shao *et al.*, 2006; Yuan *et al.*, 2010). Moreover, mitochondrial transfer RNA genes in both species are markedly shorter than in other ancestral Acariforms (Yuan *et al.*, 2010).

1.9 Aims

The principal overall objective of this study was to better understand the biology of the two human *Demodex* spp. and its significance for their association with human beings. Specifically, the research objectives were:

- to assess the microscopic identification methods of the two human follicular mites frequently used in the literature;
- to investigate the prevalence of *Demodex* mites in a small human population;
- to design a molecular test for distinguishing the two species of human follicular mites via a single multiplex PCR;
- to formulate an effective artificial medium for rearing *D. folliculorum*; and
- to evaluate two commercial genomic DNA extraction kits for extraction of genomic DNA from *D. brevis*.

The following significant questions were addressed in this thesis:

- what are the diagnostic morphological characters of the two *Demodex* mite species of humans, suitable for microscopic identification?
- Do factors such as age, host's sex, host's ethnicity, type of childbirth and method of postnatal feeding affect infestations of *Demodex* mites in humans?
- Can the two human follicular mites be differentiated by a single PCR test using a specific molecular marker?
- What are the best methods for extraction of genomic DNA from *Demodex brevis*?
- Can *Demodex* mites be successfully reared on artificial media and how long can they be kept alive outside their natural habitat?

1.10 Overview of the chapters in this thesis

Chapter 1, the introduction, briefly highlights most of the research that has been done (to the date of writing this thesis) in the field of *Demodex* mites of humans, detailing some of the scientific controversies, divergent views and gaps in our knowledge regarding their biology and pathogenicity.

Chapter 2 describes techniques for collecting mites from humans and their microscopic identification. The aim of this chapter is to collect mites from the nose and forehead of the experiment participants, using two different collection methods, and to evaluate the methods based on the total number of mites collected. The morphological characteristics of the two *Demodex* spp. which affect the numbers of mites collected are assessed.

Chapter 3 assesses the prevalence of the human follicular mites, *Demodex folliculorum* and *D. brevis*, statistically. The data collected in Chapter 2 is used to compare numbers of mites based on factors including host's sex, age group and ethnicity of the participants.

Chapter 4 investigates a reliable molecular identification test for distinguishing three *Demodex* spp. (*D. canis, D. brevis and D. folliculorum*) using a multiplex PCR method. A method was successfully developed which allows a dermatologist to easily and accurately diagnose and identify *Demodex* species in samples using just a single multiplex PCR reaction.

Chapter 5 aims to formulate an artificial rearing medium for *D. folliculorum*, suitable to keep mites alive to at least the first generation. Various media are tested in order to achieve this objective which has implications for the ease of conducting future research.

Chapter 6 tests a variety of methods and materials to extract genomic DNA from *D. brevis*, for the purpose of generating samples suitable for sequencing its whole genome.

Chapter 7 contains the overarching conclusions and some final discussion of the key results from previous chapters and makes recommendations for further research to fill gaps in our knowledge highlighted by this study. Methodological issues which have impacted on the results of the study and ways to address them to improve the outcomes for future research are presented.

Chapter 2: Human *Demodex* mites: a review of microscopic identification and assessment of collection methods

2.1 Introduction

Experience of using the morphological character keys for the two human *Demodex* species (Izdebska, 2004) and of sampling techniques for collecting the mites from human subjects (Zhao *et al.*, 2011) are crucial to achieve accurate and reliable results. *Demodex* spp. are difficult subjects for study because they are minute (200 μm - 400 μm) and are only visible under a microscope (Nutting, 1976a).

Adult mites are worm-like, with two fused segments covered by a thin transparent exoskeleton (Desch and Nutting, 1978). There are three distinguishable body structures (Izdebska, 2004): 1) a small-rectangular gnathosoma, which includes the mouthpart and supracoxal spines; 2) the podosoma, to which four pairs of short legs are attached (larvae have 3 pairs of legs), in some cases, each leg has a pair of claws (Desch and Nutting, 1972); and 3) the tubular shaped opisthosoma which is the largest part (80 % in some species) of the body (Nutting, 1976b). These external features have been studied using various microscopy methods (Lacey *et al.*, 2009; Carly and Dirk, 2014). Nevertheless, despite using modern microscopes, there remain many incorrect identifications in the literature, as is evident from the photomicrographs that are presented in some studies. For instance, Yucel and Yilmaz (2013, p. 197) in their Figure 2: a young adult is given as *D. brevis*, however, it actually shows *D. folliculorum,* and Carly and Dirk (2014, p. 740) have an incorrectly identified photomicrograph of *D. brevis*.

Disagreement exists amongst researchers concerning the description of *Demodex* species, in particular there are conflicting views on the number of palpal segments connected to

the gnathosoma, the terminology that should be used to describe their life stages and their copulation and reproduction strategies (Akbulatova, 1963) (Table 2.1). This lack of consistency may contribute to some of the mistaken identifications in the literature.

Table 2.1 Different terminologies used to describe the life stages of human *Demodex* mites

	Hirst (1919)	**Fuss (1935)**	**Akbulatova (1963)**	**Desch and Nutting (1972)**
Mite Life Stages	Ovum	Ovum	Egg	Ovum
		Hexapod larva		
	Larva	Octopod larva	Larva	Larva
	Protonymph		Nymph I	Protonymph
	Deutonymph	Nymph	Nymph II	Deutonymph
	Adult	Adult	Adult	Adult

To assess the ease of distinguishing individuals based on these characters, and at different life cycle stages, we compared two microscope techniques, a stereomicroscope and a phase contrast microscope which allows examination at higher magnitudes (up to 100 ×).

Microhabitats, such as the hair follicle, play significant roles in accessing *Demodex* mites (Izdebska, 2004) found on humans, since they affect access to the body parts needed for identification. As *Demodex* mites live wholly or partially inside the sebaceous glands (Spickett, 1961; Akbulatova, 1963; Desch and Nutting, 1972) and so are difficult to access, various techniques have been described to extract them from the human skin (Ozdemir *et al.*, 2003; Karaman *et al.*, 2010).

- Skin scraping: the skin must be cleaned with sterile water, then carefully scraped with a blunt implement, such as the back edge of a surgical implement, or a blackhead remover, the scrapings then transferred to a microscope slide and mounted following standard oil immersion practice (Isa *et al.*, 2011).

- Skin squeezing: the skin is pressed firmly using the thumb and index finger after first cleaning the area with distilled water, the resulting exudate is extracted on to a clean microscope and oil mounted as for skin scraping (Izdebska, 2004; Zhao *et al.*, 2007; Isa *et al.*, 2011).
- Epilating hair: the eyelashes or eyebrows are usually used as source materials in this technique. A suitable hair is pulled out and placed on a clean slide and mounted as for skin scraping (Kojima *et al.*, 2011; Isa *et al.*, 2011; Thoemmes *et al.*, 2014; Cheng *et al.*, 2015).
- Cellophane tape: this is the most widely used technique as it is easy to perform and is gentle on the human subject. Cellophane tape's adhesive side is placed on the cleaned skin area, for approximately two to eight hours (often overnight while the subject is sleeping). The tape is removed and replaced on a clean slide and mounted as for skin scraping (Ozdemir *et al.*, 2003; Ozdemir *et al.*, 2005; Izdebska, 2009; Isa *et al.*, 2011; Thoemmes *et al.*, 2014).
- Standardised skin surface biopsy (SSSB): is usually deployed when measuring the density of mites on the skin of an individual, since it provides readily comparable measurements. An area of 1 cm^2 (1 cm $\times$1 cm) is drawn on a clean, dry slide and cyanoacrylate glue is dropped onto that area, the slide is pressed against the ether-cleaned skin surface of a human subject and removed slowly after approximately one minute, then mounted as for skin scraping (Ozdemir *et al.*, 2003; Ozdemir *et al.*, 2005; Lacey *et al.*, 2007; Lacey *et al.*, 2009; Ugras *et al.*, 2009).

The two techniques being compared in this study are skin scraping and cellophane tape. We used published and self-determined variations of these techniques in our assessment of their efficacy.

The principal objectives of this Chapter were as follows.

- Using well established morphological characteristics, to accurately and reliably identify the two species of human follicular mites in their various life cycle stages, comparing results from stereo and phase contrast microscopy.

- To assess the efficiency of the skin scraping and cellophane tape techniques for collecting *Demodex* mites from human subjects.
- To highlight the limitations of the microscopic identification methods for the human *Demodex* mites.
- To locate large populations of mites of both species on willing subjects, which are suitable to provide source mites for the testing of rearing *in vivo* in an artificial medium and for genomic DNA extraction.

2.2 Materials and Methods

2.2.1 Human subjects

Human experimental subjects were recruited via a leaflet campaign, a copy of a flier designed for this purpose is in Appendix 2. Copies of the flier were hung in the most popular places at the University of Reading, including the Student Union, the library, the Saudi Society and the main bus stops on the university campus. An announcement was also sent that included the flier and the project information sheet (Appendix 2) using university email lists. During a Postgraduate Symposium, in 2016, the audience was invited participate. The information sheet for participants of this human follicular mites' project (Appendix 2) was approved by the Ethics Committee of the University and it contained a brief explanation of the processes involved in the project, which had previously been reviewed and approved by the School of Biological Sciences Ethics Research Committee in 2013, before the project started.

For this project, male or female donors aged between 18 to +60 years old were sought. Each volunteer was required to read the project sheet (Information for Participants) and to agree to mite extraction. Participants completed a short questionnaire (Appendix 2) as part of the project process, that contained five questions to determine: name, age, host's

sex, childbirth, breastfeeding and ethnic group. The questionnaires containing volunteers' personal information are retained in a secure, confidential file for future reference and only the researcher and his supervisor have access to this data. Participants' data have been fully anonymised before use in this study.

2.2.2 Collection methods

Health and safety standards were observed to a high standard during mite collection from the volunteers. Therefore, precise instructions regarding how to collect mites were provided to each participant. Each volunteer could choose the method that he or she believed would be most comfortable.

Instructions for the cellophane tape (Figure 2.2) comprised: thoroughly washing the face with warm water to remove all dust and sebum and then drying the skin with a clean towel or tissue paper to ensure good adhesion. Before sleeping, about 3 cm of the provided tape is placed on the tip of the nose and another 3 cm strip on the forehead, to be left in place overnight. In the morning, the tapes are removed and glued to each of the provided slides, one tape per slide, and placed in a bag labelled with the volunteer's name and promptly returned to the researcher.

Instructions for the skin scraping method comprised: thoroughly washing and drying the face. Using the blackhead remover (Figure 2.1), one end is carefully pressed to scrape the skin on the nose, and the other end is used for the forehead, care was urged to avoid cross-contamination. The ends were clearly marked: (N) for the nose and (F) for the forehead. After scraping, the whole blackhead remover is placed in the bag labelled with the participant's name and promptly returned to the researcher.

Figure 2.1 Images for the cellophane tape and blackhead remover used in skin scraping for collecting mites from human subjects.

2.2.3 Mite specimen preservation

Skin scraping: the accumulated sebum collected on the blackhead remover were extracted as soon as possible after receipt using a pin and spread on a clean microscope slide by the researcher. Mites could be found moving between the epithelial cells and the sebum and extracted immediately with a pin and placed away on a clean space on the slide, then mounted by the researcher. Photomicrographs of the mites were taken using a Leica MC120 HD camera connected to a stereo microscope (LEICA M125) magnification × 10. The photos were saved and printed. The mites were then carefully

removed using a pin, placed in watch glasses and washed in 100 % ethanol for 30 minutes. Finally, under the stereo microscope, a 1-20 µl pipette was used to collect the mites from the watch glasses and to transfer them to a 1.5 mL Eppendorf tube containing about 0.5 mL absolute ethanol where they were preserved at room temperature for further investigation.

Cellophane: mites found during examination of the cellophane tape were circled using a permanent marker, the glue on the tape prevented the mites from being moved without being damaged. A phase contrast microscope (Nikon Optiphot Number 173478) was used to examine the mites at magnification × 100. Photomicrographs of the mites were taken with a (Moticam 10.0 MP) camera connected to the microscope, using. Motic Images (Plus 3.0 ML) software. Images were saved and printed.

Two methods were used to extract the mites from the cellophane tape to prevent damaging the mites, the widely used Xylene and Alcohol (95 % ethanol) method and a new method as described below.

Xylene and Alcohol method: the purpose of his approach is to dissolve the glue from the tape, thereby releasing the mite. A solution consisting of 1:1 Xylene – Ethanol was prepared inside a safety cabinet to prevent eye and skin damage (Kandyala *et al.*, 2010). Scissors were used to excise a small piece of tape containing a single mite which was placed in a 15 mL tube with 5 mL of the solution. The tube was incubated at room temperature for about 15 minutes then centrifuged for 3 minutes at 3000 rpm and the solution poured into a watch glass and re-checked under a stereo microscope as previously. The mites were then transferred to 100 % ethanol for washing, for 15 minutes. Finally, the mites were taken from the alcohol using a 10 µl pipette and stored in a 1.5 mL Eppendorf tube, which contained 0.5 mL absolute ethanol, for further analyses. Tubes

were labelled with the human subject number, the date of extraction and the count and name of *Demodex* species.

New method: this approach was developed after losing some mites using the Xylene-Ethanol method. The mites were located and marked as previously, then, the tape containing the mite was excised from the slide by cutting around the mark using a sharp blade and held and flipped upside-down placing the glue side uppermost, using tweezer-forceps. Under the stereo microscope the excised pieces were transferred to a clean watch glass which was filled with approximately 2 mL of ethanol for about 5 minutes. Finally, under the microscope, a pin was used to remove the mite from the tape and to place it in the solution. The mite and solution were transferred to a 1.5 mL Eppendorf tube with 0.5 mL ethanol and stored at room temperature. All mites extracted using these methods were kept in alcohol for further analyses, such as DNA extraction and permanent mounting. Tubes were labelled with the human subject number, the date of extraction and the count and name of *Demodex* species.

2.2.4 Microscopic identification

Samples from both collection methods were promptly examined under a stereo microscope (Leica M12, magnification × 10), calibrated using software driven scaling. Each slide was intensively investigated to find mites in all their life cycle stages. When a mite was found, its location was circled using a permanent marker so that it could be easily re-located. At this stage, slides were not covered by slips, to avoid affecting the mite's body shape. Based on the morphological descriptions by Desch and Nutting (1972) and Izdebska (2009), the mites were determined as *Demodex brevis* or *D. folliculorum*. The characters used for identifications are summarised in Table 2.1. Measurements were repeated on ~20 mites per species and averaged.

Table 2.2 Morphological differences between the two *Demodex* mites of humans.
Characters are taken from the morphological descriptions in Desch and Nutting (1972).

Character		***D. folliculorum***	***D. brevis***
Average body length	Female	294.0 ± 58.1 μm	208.3 ± 26.5 μm
	Male	279.7 ± 52.0 μm	165. 8 ± 18.5 μm
Opisthosomal shape		Round	Pointed
Opisthosoma length to whole body length		2/3	1/2
Egg shape		Arrow	Oval
Epimeral plates		Separate, four pairs of legs	Separate, three pairs of legs

2.2.5 Statistical analyses

Statistical analyses were performed using R in RStudio version 1.0.135 (RStudio Team. *RStudio: Integrated Development for R*. Boston, MA: RStudio Inc; 2016. http://www.rstudio.com/) for Mac. The total number of mites collected from the participants was used for comparing the efficiency of the two collection techniques. The number of mites collected by the two methods was tested using a Kolmogorov-Smirnov test and both were found to have highly non-normal distributions, as is the case with many types of ecological data (Zuur *et al.*, 2009). Subsequent statistical analysis is, therefore, of necessity non-parametric. The 95 % confidence level ($p \leq 0.05$) for statistical significance was used in all tests.

Summary statistics are presented comparing the efficiency of both collection methods, based on the percentage of negative results (mites not found) and positive results (mites found) for each sampling technique. The results were considered positive if either *Demodex* spp. or both was found. A Mann-Whitney U test (Dytham, 2011) was used to assess the efficiency of the two collecting techniques, in terms of the total number of mites collected by each. Box and whisker plots are used to illustrate the results, as there are a large number of outliers in the data, the boxplot only shows the interquartile range

(25th – 75th percentiles) and the proximal outliers, for readability. Median values are shown as the mean is heavily skewed by the zero inflated data.

2.3 Results

2.3.1 Morphological comparison

Body length: microscopic examination of the mites confirmed that *Demodex. folliculorum* and *D. brevis* have spindle like bodies with four pairs of stamped legs. *Demodex folliculorum* mean body length is approximately 290 ± 58.1 µm, *D. brevis* is about 200 ± 43.1 µm. Dorsal and posterior views of the same mite were visualised with the phase contrast microscope, *D. brevis* was consistently more transparent than *D. folliculorum* (Figure 2.2).

Epimeral plates: the epimeral plates are extended and separate all four legs pairs of *D. folliculorum,* while they only separate the first three pairs of legs in *D. brevis*. The separation lines between the legs arising from these plates can be clearly seen in both species (Figure 2.3).

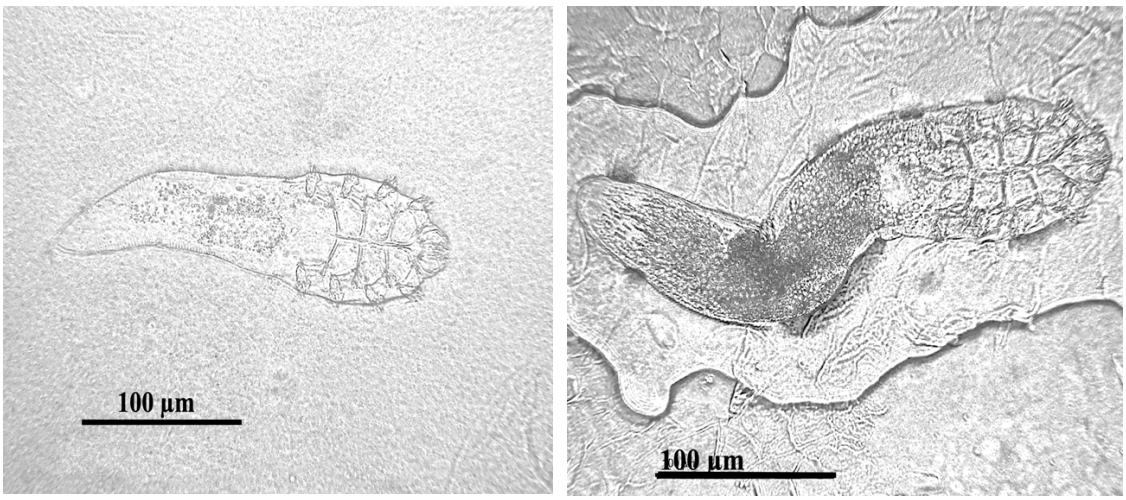

Figure 2.2 Posterior view of the two human *Demodex* mite species
Left, *D. brevis.* Right *D. folliculorum*. Collected on cellophane tape.

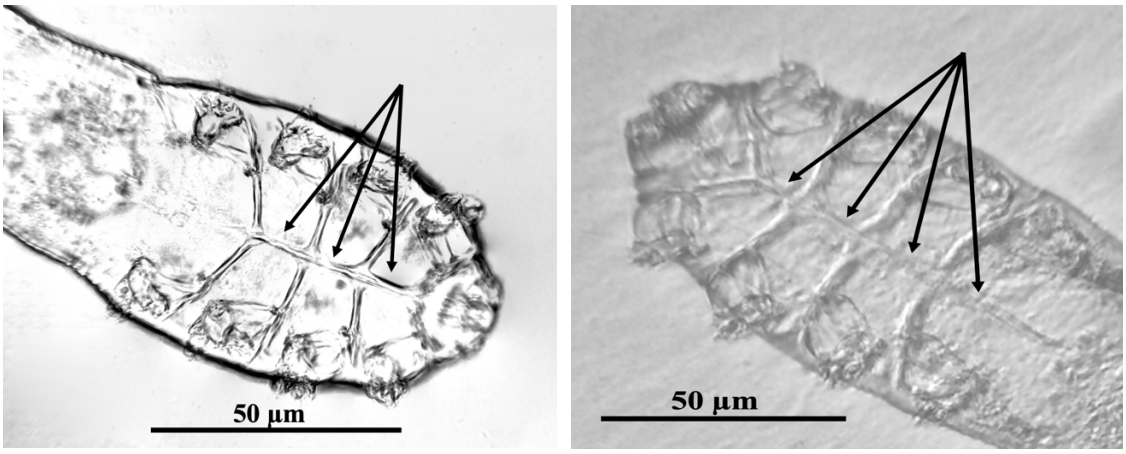

Figure 2.3 Highly magnified view of the epimeral plate (podosoma) of the two human *Demodex* mite species

Left, *D. brevis* - epimeral plates separate three pairs of legs. Right *D. folliculorum* - epimeral plates separate all four pairs of legs. Collected on cellophane tape.

Egg shape: in *D. brevis* is oval, whereas, in *D. folliculorum* eggs are sagittiform (arrowhead shaped) (Figure 2.4).

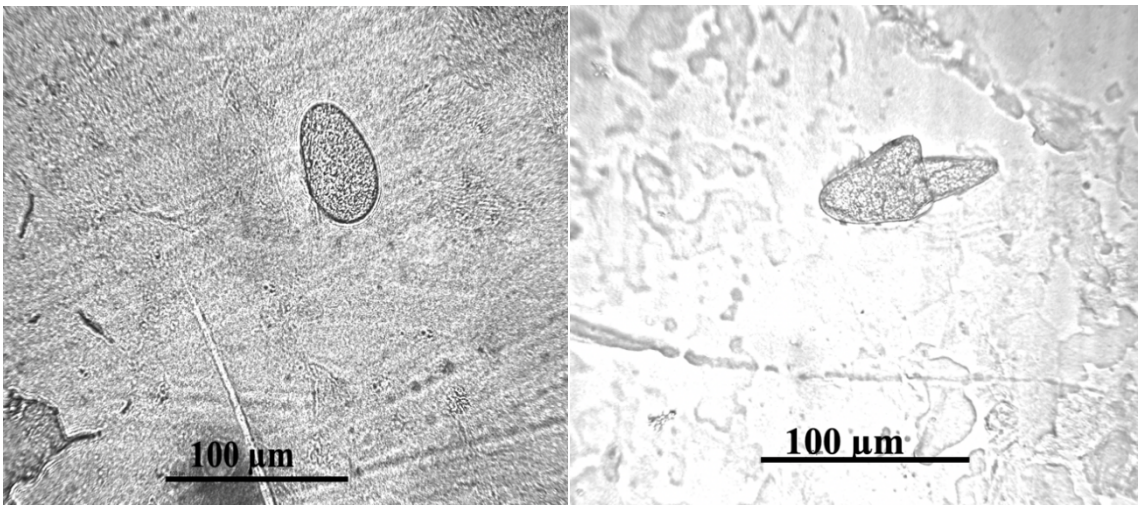

Figure 2.4 The eggs of the human *Demodex* mites

Left, *D. brevis* – egg oval. Right, *D. folliculorum* – sagittiform (arrowhead shape). Collected on cellophane tape.

Opisthosoma shape: *D. brevis* has an elongate opisthosoma, in *D. folliculorum* it is rounded. In both species it has striations and appears to contain a crystalline structure, perhaps involved in storing waste products. These structures are clear in *D. folliculorum* and make its body less transparent than *D. brevis*. The opisthosoma comprises >70 % of body length in *D. folliculorum* and approx. 60 % in *D. brevis* (Figure 2.5).

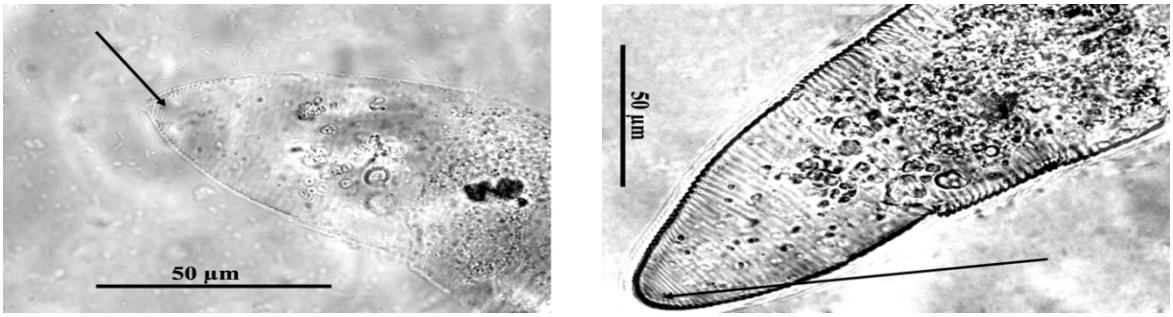

Figure 2.5 Highly magnified view of the opisthosoma of the two human *Demodex* mites, showing crystalline structures causing opacity

Left, *D. brevis* opisthosoma is pointed. Right *D. folliculorum* – opisthosoma is rounded, striations are clearly visible. Collected on cellophane tape.

2.3.2 Distinguishing males from females in *Demodex* mites

Although the two sexes have similar body structures in both *Demodex* spp., females are longer and wider compared to males (Figure 2.6). The genital organs are located in the podosoma. The male genital orifice is a small slit located on the dorsal side at the second leg-pair level of the body and is a well sheathed aedeagus. The female opening is found posteriorly behind the fourth leg-pair and is covered by epimeral plates. These tiny genitals are difficult to observe under a stereo microscope; but phase contrast showed what is clearly the female genital opening in both species. (Figure 2.6, A and B, arrows).

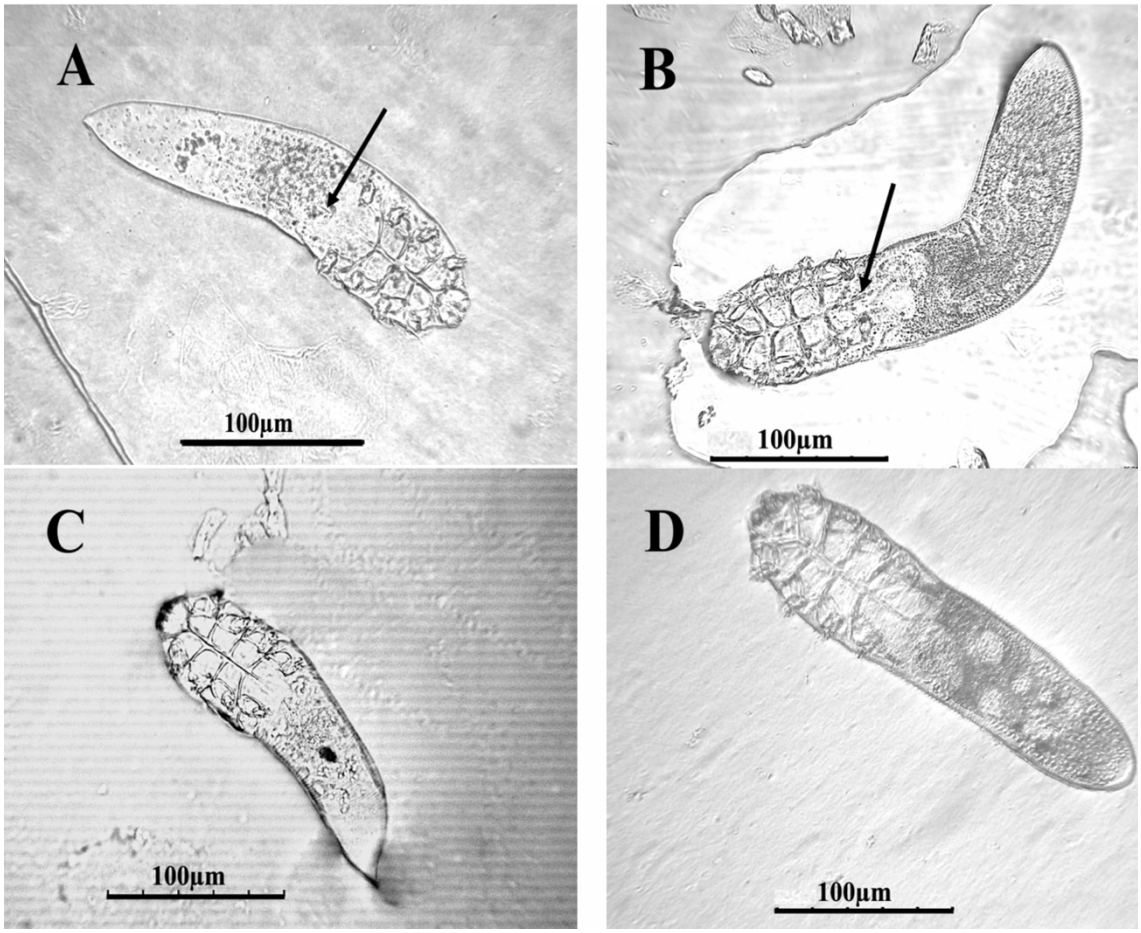

Figure 2.6 Morphological comparison the two sexes of human *Demodex* spp.
A - *D. brevis* female. B:- *D. folliculorum* female. C: *D. brevis* male. D: *D. folliculorum* male. To the same scale for size comparisons. Collected on cellophane tape

2.3.3 Life cycle stages of *Demodex* mites of humans

The life cycle is identical for both human *Demodex* spp. (Figures 2.7 and 2.8). An image appearing to show mating between two adult *D. folliculorum* mites was captured by both microscopic methods (Figure 2.7, 1 and 2). In addition, an egg can be seen formed inside a *D. folliculorum* female (Figure 2.7, 3).

Other life cycle stages can be seen in Figure 2.7 (4, 5 and 6).

In *D. brevis* it was observed that adults and nymphs were usually found in isolation (Figure 2.8, 5 and 4). However, a photomicrograph was captured with the phase contrast

microscope showing both sexes in one view (Figure 8, 1). In addition, an egg formed inside s female's body and on the tape was seen (Figure 8, 2 and 3).

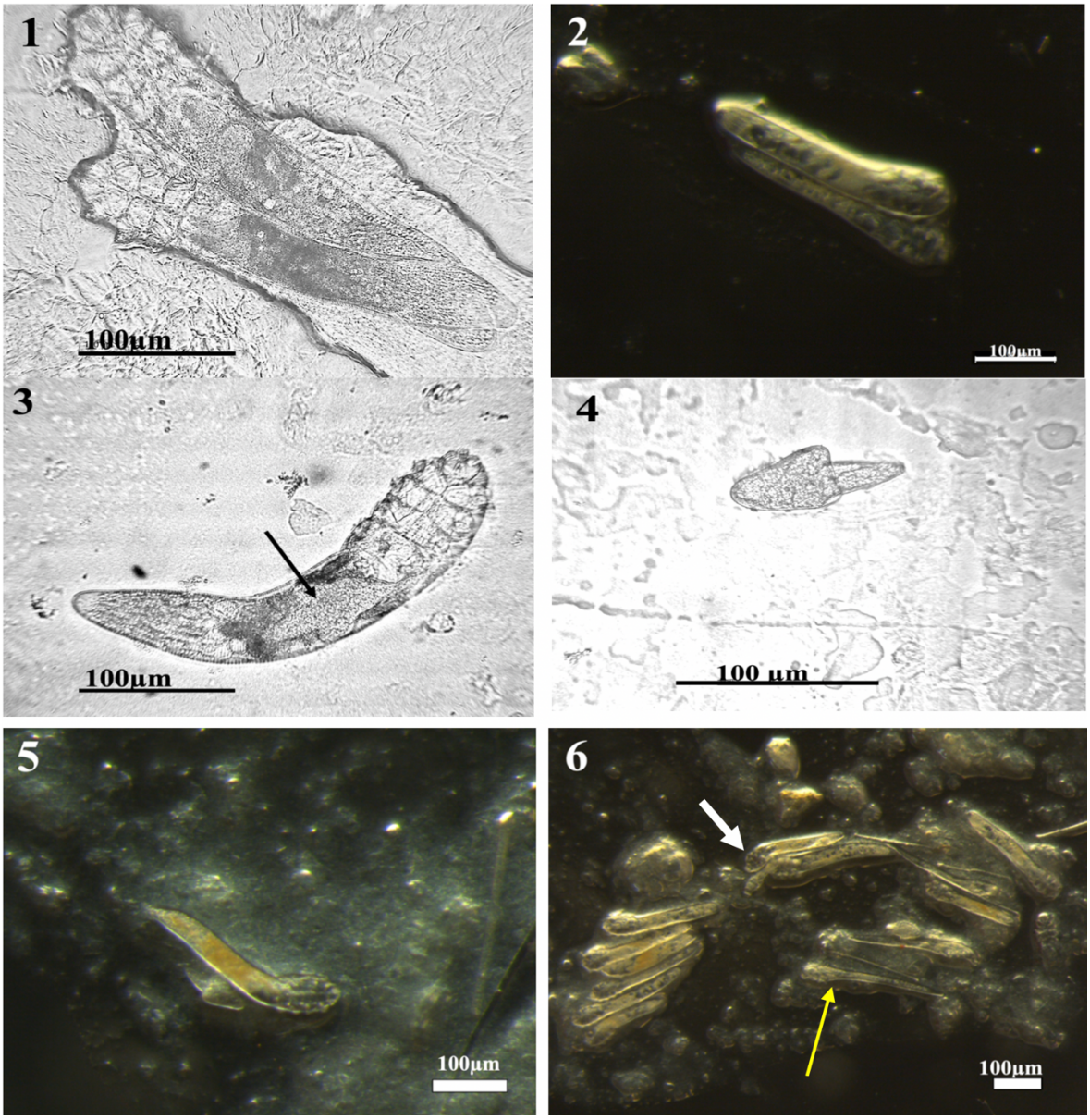

Figure 2.7 Photomicrographs of *D. folliculorum* showing different life cycle stages.
1 and 2: male and female mating; 3: female (the black arrow is pointing at an egg; C: sagittiform egg. D: female with an egg beside it (after deposition), stereo microscope; 6: mixed life cycle stages, the white arrow is pointing at a larva and the yellow is pointing at a nymph, stereo microscope.

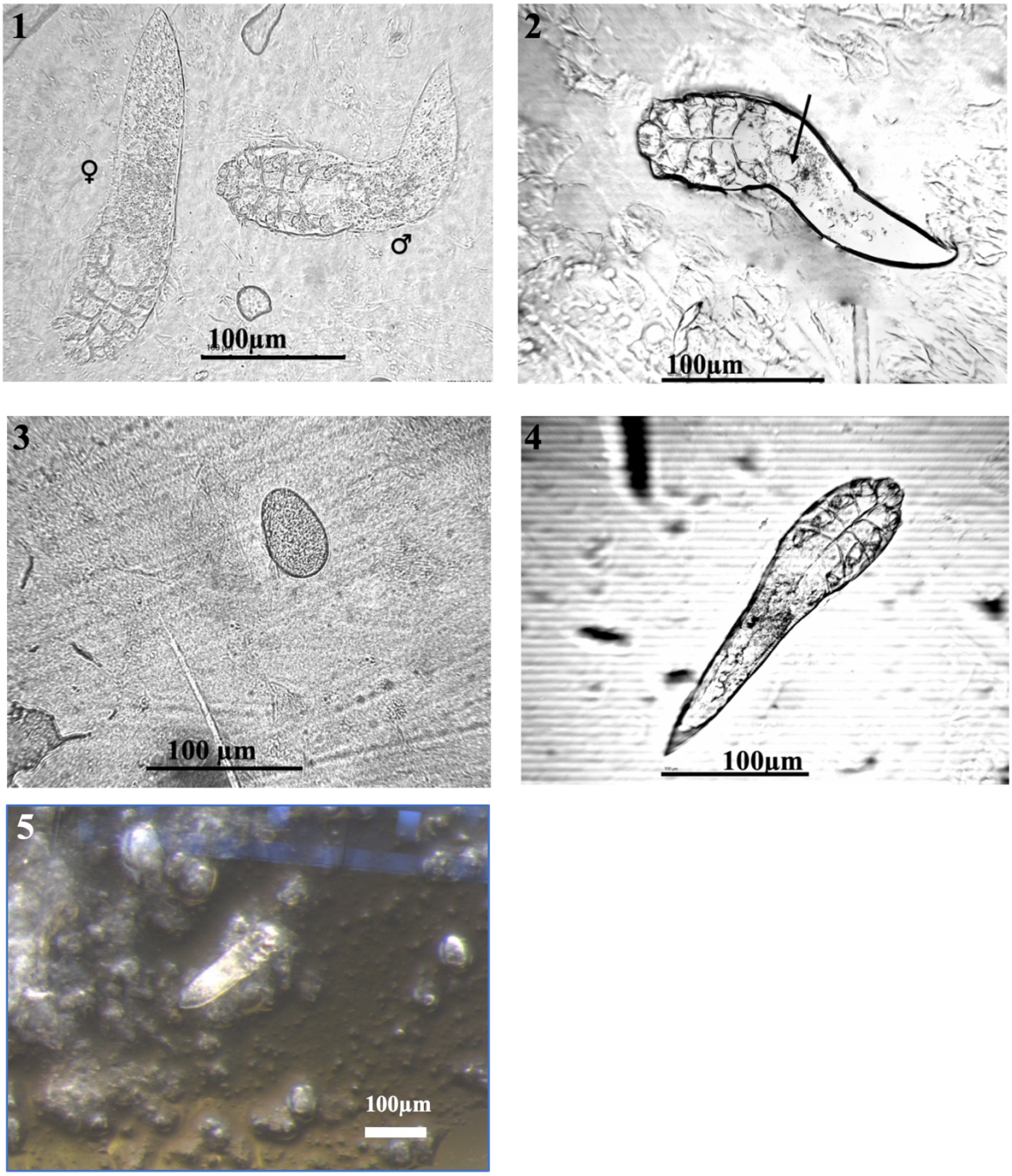

Figure 2.8 Photomicrographs of *D. brevis* showing different life cycle stages.

1: male and female. 2: female (the black arrow is pointing at an egg). 3: deposited egg; phase contrast microscope. 4: nymph, 5: adult; stereo microscope.

2.3.4 Limitations of microscopic identifications

Visualisation using microscopy is an easy method for distinguishing adult and egg life cycle stages in both species of mite. However, differentiation of the immature stages such

as nymphs and larva, or of the mites when desiccated, especially when confirmatory adults or eggs of the relevant species are absent, was not possible. For instance, Figure 2.9 A shows a dried egg and a *Demodex* mite next to it, from the arrow-shape of the egg it is highly likely that this egg belongs to *D. folliculorum* and the mite beside it is presumed also, on that basis, to be *D. folliculorum*, however, these identifications must be regarded as provisional, given the lack of diagnostic features apparent in the material. Photomicrograph C, in the same figure, shows the opisthosoma is rounded so the mite is clearly *D. folliculorum,* however in Figure 2.9 B the mite is dry so it cannot be identified. In D, an immature stage of one species found by the cellophane tape method is solitary and therefore lacks sufficient information to be identifiable.

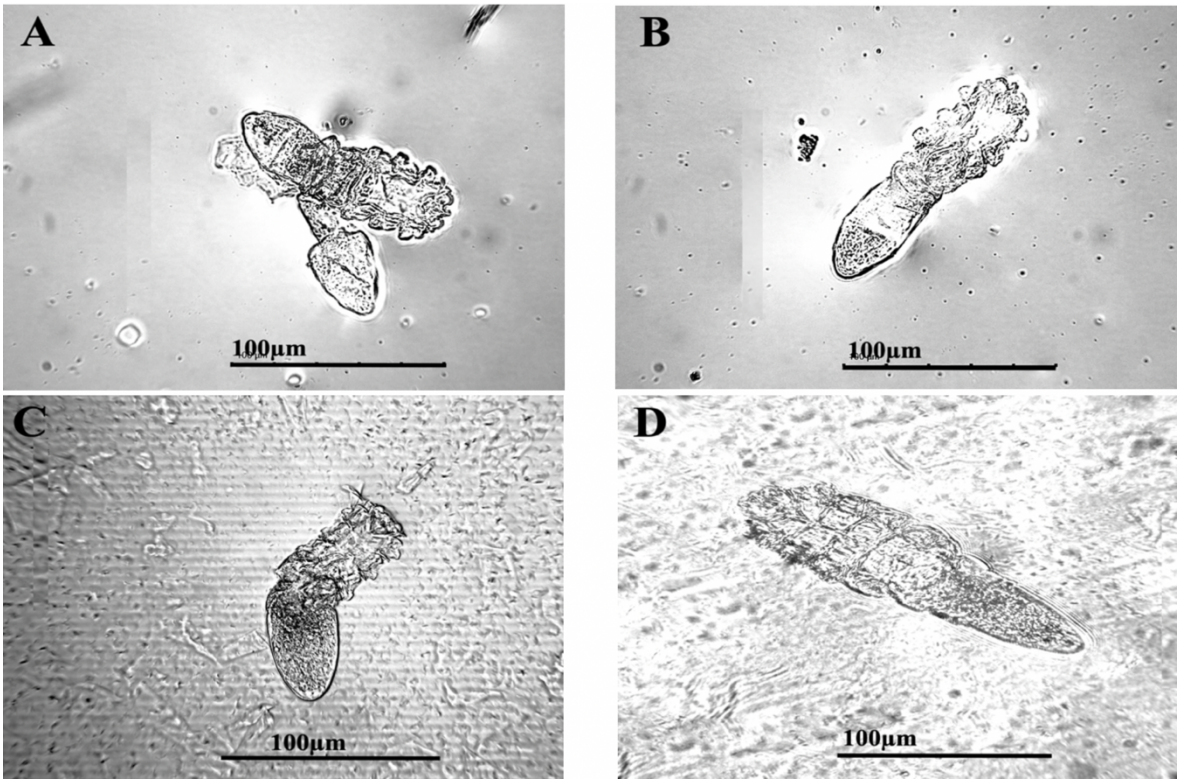

Figure 2.9 Photomicrographs of *Demodex* mites from a single sample, showing some of the issues preventing identification.

A: adult *D. folliculorum* mite with an egg, both are dried out. B and C: *Demodex* mite dried out on the cellophane tape. D: isolated *Demodex* mite at an early life stage that cannot be reliably identified.

2.3.5 Comparison of the two sampling methods used in this study

The relative efficiencies of collecting human mites using the two selected techniques, skin scraping and cellophane tape, has not been reported in the literature. Figure 2.10 shows the clearly non-normal distribution of the results which are highly skewed with a high level of kurtosis. Of the samples, 64 and 32, respectively, yielded zero-mites, figures are summarized in Table 2.2. A boxplot (Figure 2.11) confirms the left-side skew and high kurtosis present in the data, notably all of the outliers are above the third quartile, as however, the range of values, as seen in Figure 2.10, is so great, the extreme outliers are omitted. The third quartile is clearly larger for the cellophane tape method, indicating more results were above the median value compared to the skin scraping method. The median number of mites per sample is 1 for both methods, but the distributions of sample sizes are similar (Figure 2.10 and 2.11). The Mann-Whitney U test showed a significant difference ($U = 2627$, $p < 0.05$) between the two sampling methods (Table 2.2) and that the cellophane tape method is more efficient than skin scraping when collecting *Demodex* mites from humans, as shown by the higher median number of mites per sample, 1.9 compared to 1.3 mites respectively.

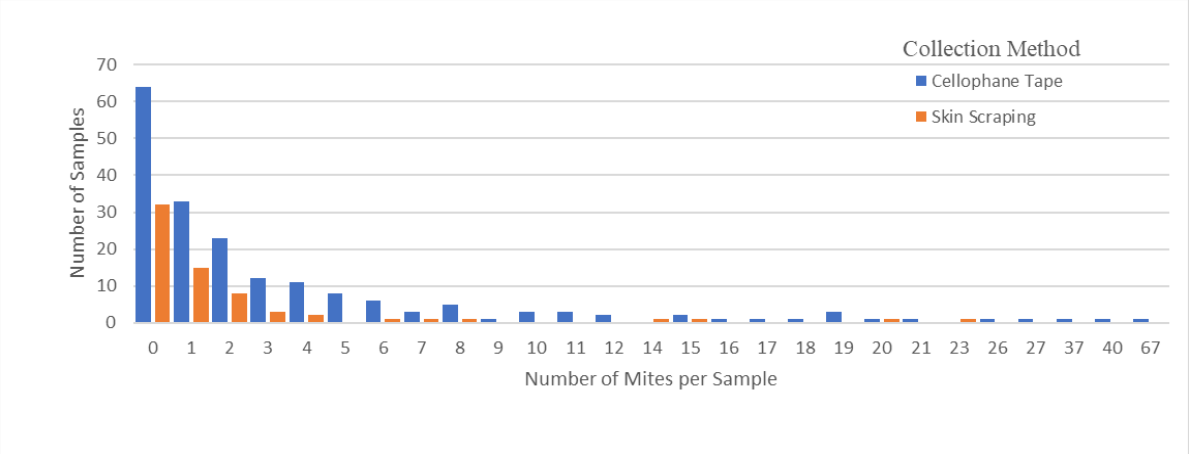

Figure 2.10. Distribution of sample sizes for each collection method
Figure produced in Microsoft Excel (Microsoft Office 365 ProPlus)

Table 2.3 Comparing the two sampling methods used for collecting the human follicular mites in this study.

Sampling technique	-ve Samples (mites not found)		+ve Samples (mites found)		Total Number of Mites	Total number of samples	Mann Whitney U Statistics
	count	%	count	%			
Cellophane tape	64	33.8	126	66.2	768	190	U = 2627,
Skin scraping	32	48.4	34	51.6	141	66	$p < 0.05$

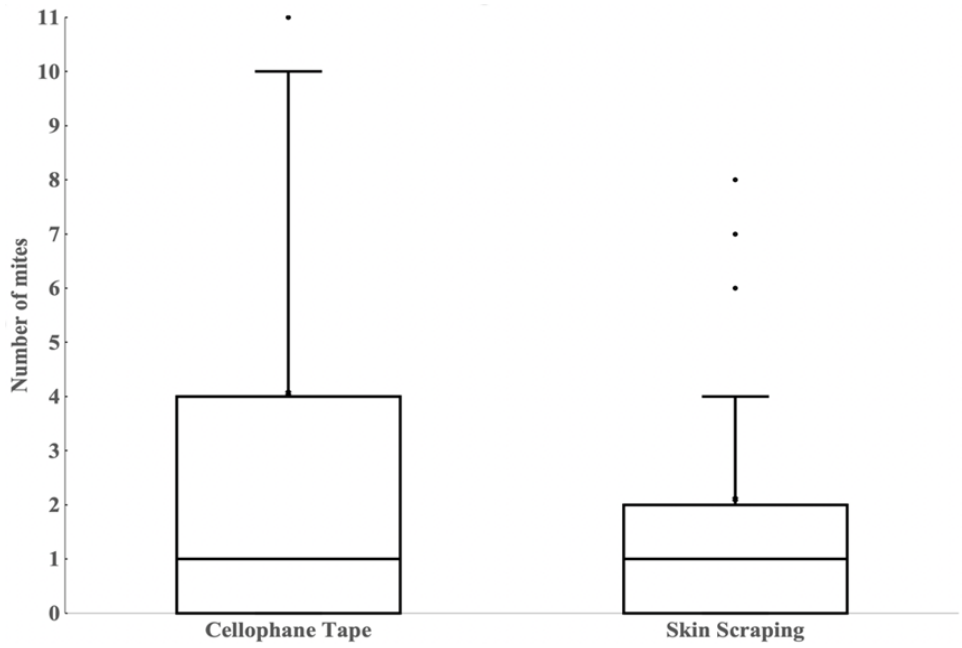

Figure 2.11 Boxplot showing a comparison between the number of *Demodex* mites collected by two skin scraping and cellophane tape sampling methods.

The total number of mites collected by skin scraping was 141 (median 1.0, range 0-23). Total number of mites collected by cellophane tape was 768 (median 1.0, range 0-67). The figure only shows the proximal outliers.

2.4 Discussion

Several collection methods have been used and are widely reported. For this study, Standardised Skin Surface Biopsy (SSSB) was not selected, because it has limitations (Forton and Song, 1998) such as, the requirement for repeated sampling for the same individual. We found that most volunteers were not willing to repeat the extraction procedure as many times as this method needed, and notably the use of powerful adhesive

materials might lead to scouring of the skin (Forton and Song, 1998), which might have further reduced the number of willing participants. Furthermore, the purpose of this method is primarily to measure *Demodex* densities, which was not an objective of this study. The skin squeezing and epilating hair (mostly from the eyelashes or eyebrows) sampling methods are also potentially painful, intrusive processes hence were not considered farther for similar reasons of wishing to retain a large cohort of willing participants and insufficient time to replace those that were overly deterred, as well as being cognisant of the pain or discomfort that such procedures might inflict on the subject, Isa *et al.* (2011) came to a similar conclusion. Cellophane tape and skin scrapings are instead relatively painless and unintrusive techniques, easy to perform and tolerant on the participants' skin, hence were considered appropriate sampling options for this research.

Cellophane tape and skin scraping methods have been employed to collect *Demodex* mites from humans many times, for example Ozdemir *et al.*, 2005, Zhao *et al.*, 2007, Izdebska, 2009, and Isa *et al.*, 2011, but their efficiency was not reported in any study we found. In this study, their efficiency was evaluated by comparing the total number of mites collected by each technique, per sample. The results showed that about 66 % of the cellophane tape samples contained one or both human *Demodex* spp. compared to skin scraping for which approximately 50 % of the samples had mites (Table 2.2). The boxplot (Figure 2.11) shows the median is about 1 for both methods with various outliers in each group, which are all samples with larger numbers of mites (Figure 2.10). A Mann-Whitney U test showed that there was a significant difference ($U = 2627$ $p < 0.05$) between the two sampling methods and that the cellophane tape is the more effective of the two collecting methods. This outcome may be due to the tape being in place on the skin overnight, i.e. for a much longer period, and covered a larger area than was likely to be sampled by skin scraping. The participants preferred the tape method, because they

found it more comfortable and less painful than the skin scraping, which also raises the possibility, albeit without evidence at this time, that in some cases skin scraping was not undertaken with full vigour.

The collected human *Demodex* mites and their life cycle stages, where possible, were identified to species based on the description of their morphology reported by Desch and Nutting (1972) and Akbulatova (1963) (Table 2.1). Overall body length was found to be a reliable character for distinguishing these species, as was reported previously by Desch and Nutting (1972), *D. folliculorum* averaged 290 µm long, while *D. brevis* about 200 µm (Figure 2.2). Cover slips were not applied to our slides before inspection, to avoid affecting the mites, for example by compressing and changing their body length or by causing a fold in the abdomen, also affecting the observed length. Some minor variations in measurements may have arisen from limitations in the calibration software, however, adjustments were made by manual re-measurement against fixed length scale bars. The epimeral plates are extended and separate all four pairs of legs of *D. folliculorum* while they only spread the three pairs of legs of *D. brevis* (Figure 2.3). Opisthosoma shape is another key character described by Desch and Nutting (1972) which was found to be reliable for differentiating the two species, *D. brevis* has a conical-like opisthosoma and *D. folliculorum* has a rounded opisthosoma. The opisthosoma of both species has striations and internal crystalline structures, the latter may be for storing waste products (Desch and Nutting, 1978). The crystalline structures can be seen very clearly in *D. folliculorum* which make its body appear less transparent than *D. brevis* (Figure 2.4) under stereoscopic and phase contrast microscope lighting. The eggs of the two *Demodex* mite species have different shapes. In *D. folliculorum* they are sagittiform, whereas in *D. brevis* they are oval-shaped, confirming Desch and Nutting's (1972) findings (Figure 2.5). The bodies of adult males and females of the these *Demodex* mites are both spindle

shaped and similarly structured, but females are longer and wider than males in both species (Figure 2.6), this confirms the previous observations of Coston, 1967 and Desch and Nutting (1972).

Mating between a male and a female has not been observed in these species, according to Izdebska (2009), however, she assumed that the male slides underneath the female during copulation, as is the case in some other species. Figure 2.7 has two images of adult *D. folliculorum* in what is strongly believed to be a copulation process, this is considered to be the first image of human *Demodex* mating reported in the literature.

The human follicular mite life cycle, in both species, is about 15 days (Spickett, 1961). When dying, their body starts to shrink and becomes dry, starting with the podosoma. Dried *Demodex* mites were often found in cellophane tape samples (Figure 2.10), according to Spickett (1961) they die shortly after leaving the human body because they are deprived of nutrition or their requisite high humidity environment (Spickett, 1961). As a result, significant changes happened to the body shape, which made it very difficult to distinguish between the two *Demodex* species in this state. Further, immature mites in early life stages can be hard to classify as well (Figure 10).

Very few juvenile life stage mites were recovered by either collection method, ~5 % of samples contained eggs and ~15 % contained larvae and nymphs. This may be due to either methodological limitations, or biological factors, such as the life cycle strategies of these species.

Two microscopy methods were compared, which differed largely in the scale of the magnifications possible. Using a stereo microscope, it was found that adult mites could readily be discriminated to species on gross morphology, however, to adequately

visualise fine morphological detail, phase contrast microscopy was necessary. For example, to distinguish male and female mites, or to reliably interpret the epimeral plate separation of the legs. So, for comprehensive morphological studies, both techniques are necessary tools.

In conclusion, collecting *Demodex* mites from humans is a process that must be performed using the appropriate techniques. The methods selected for research should abide by the appropriate health and safety standards. *Demodex* mites of humans are identified based on their morphology which, as our results show, can be effective in the adult and eggs stages, if applied rigorously and appropriately. However, it is a time-consuming and laborious procedure, which does not fully guarantee conclusive results, particularly when the mites are in their larval and nymph stages, and may be unreliable, even when undertaken by experienced practitioners, Therefore, a reliable molecular identification technique is also needed to enhance accurate and reliable identifications of the two human *Demodex* species.

Chapter 3: Factors affecting the prevalence of human follicular mites in a sample population

3.1 Introduction

Infestation of humans by follicular mites has been studied in several countries, in specific locations and on small populations. Only a few of these studies focused on the factors which may affect the levels of infestation found in any particular population. Sengbusch and Hauswirth (1986) investigated the effects of gender, ethnicity and age on the total number of *Demodex* mites obtained from humans using a skin squeezing method on the noses of a selected population in western New York City. However, due to the small number of different ethnicities and unequal sample sizes for age groups in their sample, they were unable to find any significant differences, nor were there significant differences between host genders in this study. Isa *et al.* (2011) studied the prevalence of the human *Demodex* spp. on the facial skin of medical students aged between 20 to 25 at the University of Kebangsaan, Malaysia. They also found that sex, age, ethnicity and acne lesions had no discernible effects on the number of *Demodex* mites. However, they did find that using skin moisturisers significantly increased the numbers of mites. In a sample population in Turkey, Kaplan *et al.* (2012) studied similar factors but included antibiotic usage and hygiene behaviour such as frequency of showering and hand and face washing, to investigate the prevalence of *Demodex* spp. in people suffering from infections and healthy individuals. They concluded that the prevalence of *Demodex* spp. increased with age and more frequent showering, whereas, gender, cosmetic application usage and hand and face washing had no significant influence. In another such study at Chiang Mai University in Thailand, Manoyana *et al.* (2014) found that mite densities in men were significantly higher than in women amongst young adult students, but that there were no

differences between three facial sampling locations, nose, cheek and forehead. Some studies have concluded that men are more frequently infested by *Demodex* spp. than women, such as Andrews, 1982 and Ozdemir *et al.*, 2005.

A great number of researchers have investigated the prevalence of human follicular mites without controlling for some common, widely considered factors, such as host's sex or age, and the results of such studies are variable. These outcomes may also be linked to different extraction techniques and isolation methods (Perotti *et al.*, 2009). For instance, Akbulatova (1966) found that 36 % of the follicles in subjects in their study group had *D. folliculorum,* 23 % had *D. brevis* and both species were found in about 41 % of nasal samples. *Demodex folliculorum* and *D. brevis* were found in 20–80 % of a healthy sample population (Andrews, 1982; Ozdemir *et al.*, 2005), but other studies have found lower densities, for example, about 3-55 % in a healthy human population (Carly and Dirk, 2014). Most reported results fall in the approximate range 10-20 % infestation (Ozdemir *et al.*, 2003; Kemal *et al.*, 2005; Ozcan and Cetinkaya, 2007).

There is insufficient published data, so far as we have been able to determine, to draw conclusions about the prevalence human follicle mites in the United Kingdom or, probably, in western Europe generally. The main objective of this research was to investigate both *Demodex* spp. of human follicles in respect of their prevalence in a small human population at the University of Reading, UK, it is likely the first research ever carried out among the academic sector in the UK to do so, and also unusual because we test the hypothesis that host's sex, age , ethnicity, and facial skin area are potentially influential factors in this cohort. Based on the evidence that says *Demodex* mites have extremely limited motility and die shortly after leaving the host's follicles, it has been hypothesised that human *Demodex* mites transmit to other humans during childhood, by

very close skin to skin contact between a mother and her offspring (Nutting and Green, 1976). Thus, type of childbirth (normal vaginal delivery or caesarean section) and postnatal feeding mode (breastfeeding or bottle) were employed as factors to test this second hypothesis.

The principal objectives of this Chapter were as follows.

- To investigate the relative abundances of the two species of human follicular *Demodex* mites in a small population of staff and students, considering age, host's sex and ethnic group as potentially influential factors.
- To investigate inter-human-host transmission of mites using childbirth mode and breastfeeding mode as potentially influential factors.

3.2 Materials and Methods

3.2.1 Research ethics

The collection of the human follicular mites for this study has been subjected to an ethical review, according to the procedures specified by the University of Reading School of Biological Sciences Research Ethics Committee and given a favourable ethical opinion for conducting this research since 2013 (Appendix 2). This research involved studying neither humans nor human tissues. The collected human mites were stored and are preserved in the Acarology Laboratory collection, School of Biological Science, University of Reading.

Volunteers were given full explanations of the procedures of the sample collection and they provided informed consent to participate in this project. All participants answered the five questions on the project questionnaire (Appendix 2), concerning age, sex, ethnicity, breastfeeding and childbirth. All samples and data are fully anonymised.

3.2.2 Mite sampling

Samples were collected from > 250 participants, staff and students of the University of Reading within a 3-year period. Sampling techniques and identification of the *Demodex* mites were as described in Chapter 2. Data collected from each participant contains host's sex (male/female), age group (18-30, 31-40, 41-60 and 60+), ethnic group, mode of childbirth (vaginal delivery or caesarean section) and postnatal feeding mode (breastfeeding, yes or no). Facial area for the sample collection (nose or forehead), sampling method (cellophane tape or skin scraping) and, after microscopic examination, the number of mites and their species, were anonymously recorded for each participant in a Microsoft Excel (Microsoft Corp., WA, USA) spreadsheet.

3.2.3 Statistical analyses

Statistical analyses were performed using R studio software on Mac (Version1.0.153 – © 2009-2017 R Studio, Inc.), which was also used to prepare the boxplots. The 95% confidence level ($p \leq 0.05$) for statistical significance was used in all tests. The total number of mites collected from the participants per sample was used for comparisons between groups: host's sex, age range and ethnicity. A Kolmogorov-Smirnov test was initially used to check for a normal data distribution, which showed that the total number of mites per sample was not normally distributed, see section 2.3.5. Summary statistics were also prepared using R.

Boxplots were selected as suitable to illustrate summary comparisons between categories of data for the factors analysed in this study. For clearer presentation of the results, and because there are a large number of outliers in the data, boxplots only show the interquartile range and a small number of the most proximal outliners.

Mite counts per sample were analysed by host's sex and age group, summary statistics are presented, and two Mann Whitney U tests were conducted to test for differences in sample counts related to host's sex and then to facial area. Mann-Whitney U test (Dytham, 2011) is a non-parametric analysis which tests the hypothesis that two groups of samples derive from the same population without making any assumptions about normality of the data, therefore it is appropriate given our data is highly skewed and non-normal.

Age groups were then analysed, and summary statistics are presented. Homogeneity of the variances between the four groups (18-30, 31-40, 41-60 and >60) was assessed using Levene's test (Levene, 1960), results were significant ($p < 0.05$), indicating heterogeneity of variances, so a Kruskal-Wallis test (Kruskal and Wallis, 1952) was needed in preference to ANOVA, to compare the four age groups. A post hoc Dunn test (Dunn, 1964) was conducted to make a pairwise comparison of the groups, following a significant result from the Kruskal-Wallis test, as the Kruskal-Wallace test does not show where the differences are amongst the groups.

Summary statistics for mode of childbirth and postnatal feeding mode are presented. A Mann Whitney U test was conducted for each factor, as above.

Summary statistics for ethnic groups are presented. A Levene's test indicated homogeneity of variance ($p > 0.05$) between the ethnic groups, so a Kruskal–Wallis test was used to test if ethnic group was influential on the mite sample sizes.

Count data is widely recognised as presenting particular issues with regard to model selection for ANOVA type analysis. As a visualisation shows the data is apparently both heavily zero inflated and skewed (Figure 2.10) an analysis was conducted using the function rootogram of the r latticeExtra package (Sarkar and Andrews, 2016). This

function compares frequencies of empirical distributions and fitted probability models and has been extended to regression models, it is particularly designed for diagnosing and treating issues such as overdispersion and/or excess zeros in count data models (Zeileis and Kleiber, 2016). It can treat both zero inflated poisson (ZIP) fitted data, the most commonly used distribution for count data, and zero-inflated negative binomial (ZINB) fitted data.

Other models were also considered, the Zero Altered Negative Binomial model (ZANB) and Hurdle models were rejected because they do not discriminate between true or false zeros in the count data. In contrast, ZINB has two modelling parts for measuring the probability of positive counts, calculating the counts that are zeros first, and, then identifying false zeros in a binomial model in which the probability of measuring a false positive is compared to count and true zeros (Tukey, 1972; Zuur *et al.*, 2009). Subjects with no mites retrieved from their skin were common, thus, ZINB was selected instead of ZANB. However, GLMs using all three models, ZINB, ZANB and ZIP were built for a statistical comparison. The three models were built with four factors as predictors, age, host's sex, postnatal feeding and birth modes. All three models showed significant ($p \leq 0.05$) outcomes for the influence of age and postnatal feeding on mites per sample, however the ZIP model ($X2 = 676.8$, $df = 1$, $p < 0.001$, AIC = 1714) resulted in a higher AIC than the ZANB model (AIC = 1040) and the ZINB model (AIC = 1035), indicating that the ZINB model best fitted the data distribution. The rootogram comparison of fit also clearly shows that ZINB is a better fit for the mite count data than a ZIP model of distribution (Figure 3.1), since, particularly at counts of 1-2 mites per sample, the overprediction is considerably lower. Consequently, results for the ZINB model are included.

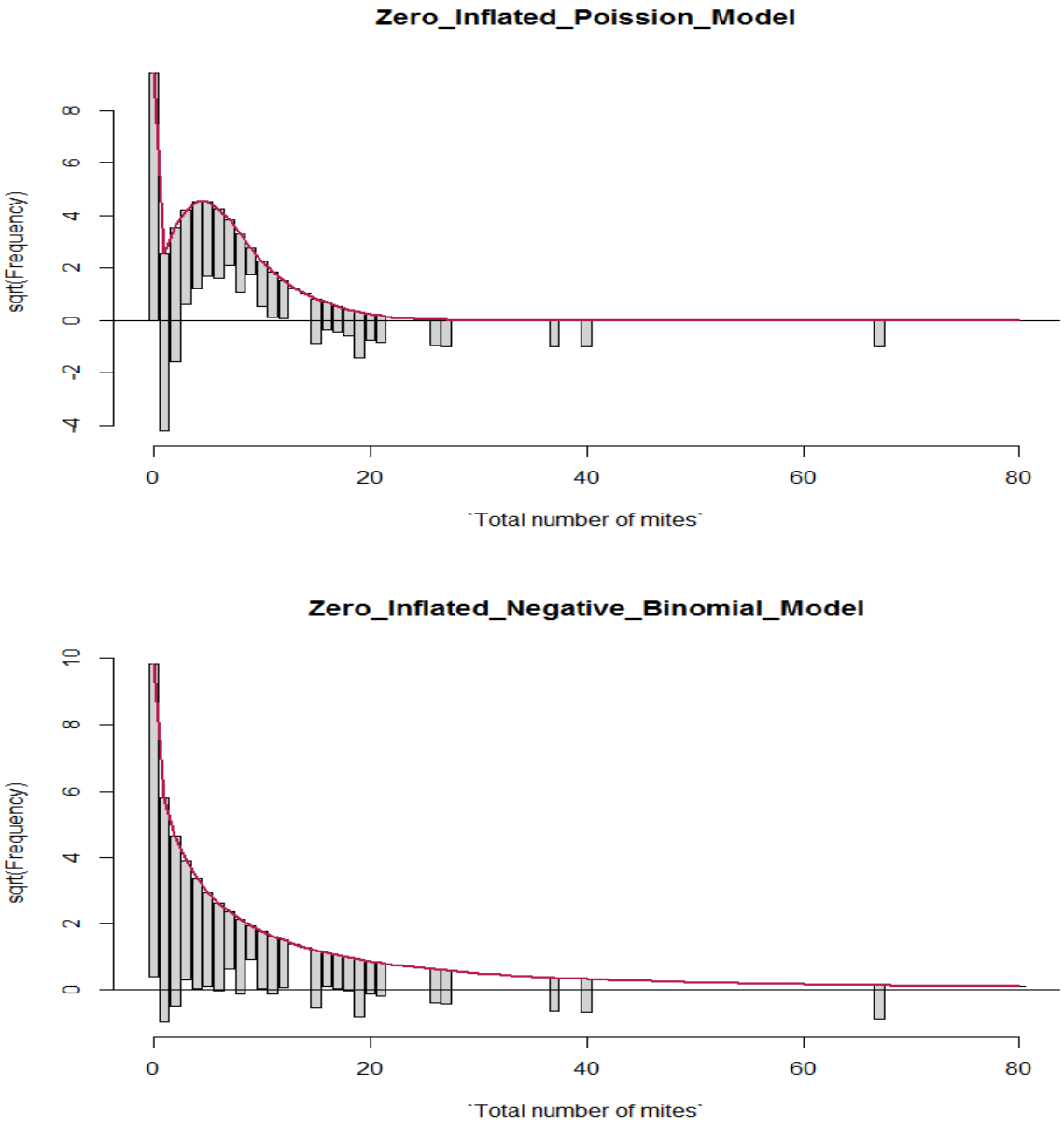

Figure 3.1 Rootogram visualisations of Zero-Inflated Poisson and Zero-inflated Negative Binomial models of distribution from the mite count per sample data

Expected counts are shown by the fitted curve (red line), actual counts are shown by the vertical bars, If a bar doesn't reach the zero line then the model over predicts a particular mite count value, and if the bar exceeds the zero line it under predicts the mite count value. Overall the Zero-inflated Negative Binomial model is a better fit to the data because there is less under prediction of the data, particularly when counts were approx. 1-2.

3.3 Results

3.3.1 Host's sex and Age Group

Of the 256 volunteers who agreed to participate in this study, 142 were male and 114 females, ages ranged from 18 to over 60 years old. The human follicular mites' infestation in men was measured at approximately 64% of samples containing mites, compared to 60% in women. A Chi^2 test showed no significant difference between the numbers of mites on the skin of men and women ($Chi^2 = 392.3$, df = 2, p-value > 0.05). The infestation increased with age of the host, *Demodex* mites were found in 51 % of samples from younger participants (aged 18 – 30) compared to 88 % of samples found on older participants (aged 60+) (Table 3.1).

Table 3.1 Prevalence of human follicular mites based on host's sex and age group

Factor	*Demodex* spp. samples				Total number of participants
	-ve (no mites found)		+ve (mites found)		
	n	%	n	%	
Host's sex					
Male	51	36	91	64	142
Female	45	40	69	60	114
Age groups					
18 - 30	53	49	56	51	109
31 - 40	28	46	33	54	61
41 - 60	13	19	57	81	70
60 +	2	12	14	88	16

Table 3.2 also shows age related differences by the host's sex. The pattern is less clear and may be affected by the smaller sample size of the oldest age group. There may be a small increase in the mean number of positive (mite containing) samples with age for both sexes, but it is not certain, because of limitations in interpretation of such highly non-normally distributed data. By comparison, the median number of mites collected

from men increases slightly with age, as does the median number of mites collected from women.

Table 3.2 Summary statistics for the human *Demodex* mites by the participants' sex and age.

Sex	*Age*	*n*	*%*	$\bar{x}$	*Median*	*SD*	*1Q*	*3Q*	*Min.*	*Max.*
Male	18-30	63	24.2	1.30	1	2.30	0	2	0	20
	31-40	37	15.9	2.89	1	2.89	0	3	0	19
	41-60	32	12.4	7.93	4	7.93	1	10	0	37
	61-100	10	3.4	5.12	2	5.13	1	4	1	17
Female	18-30	46	17.2	3.15	1	10.7	0	3	0	67
	31-40	24	9.9	3.52	1	8.43	0	4	0	37
	41-60	38	15.0	3.54	2	4.87	1	5	0	21
	61-100	6	1.7	5.5	3	4.43	3	7	0	10

The Mann-Whitney U test showed no significant difference ($U = 6321$, $p > 0.47$) between the numbers of mites collected from men and women, this is illustrated by a boxplot (Figure 3.2), the median value is 2 in both groups. There are numerous outliers in each group. Results are also presented for age vs. host's sex in another boxplot (Figure 3.3, B).

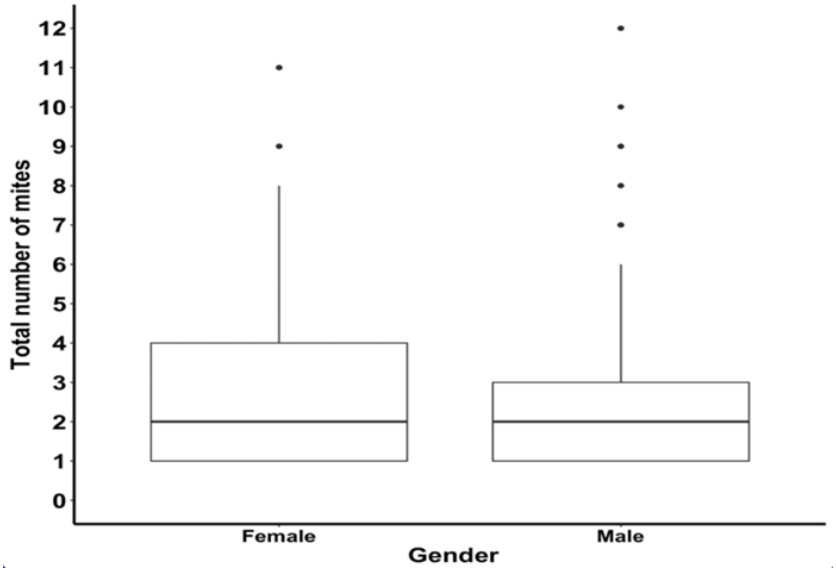

Figure 3.2 Boxplot diagram showing the total number of human *Demodex* mites by the host's sex of the host.

The thick horizontal line shows the median value, thin horizontal lines represent first and third quartiles, and vertical whiskers show the ranges excluding outliers. The total number of mites in females (women) (n = 114; median 2.0, range 0-67) and the total number of mites in males (men) (n = 142; median 2.0, range 0-40). Only proximal outliers are shown.

The total number of human follicular mites collected per sample from each age group was analysed. Levene's test for homogeneity of variances between the four age groups showed significant differences between them (F value = 2.2, df = 3, $p < 0.05$). A non-normal distribution with a positive skew of 2.5 for the male host group and 5.9 for female group. A Kruskal-Wallis non-parametric test was used, therefore, to compare age groups. It showed a significant difference between the number of mites in the different age groups ($Chi^2 = 27.7$, df = 3, $p < 0.0001$). Therefore a Dunn post hoc test was performed (Table 3.3), to find out which group or groups were different and the youngest age group, 18-30, was shown to differ significantly from the two older participant age groups, 41-60 and 60+ ($p = 0.01$ and $p = 0.01$, respectively), but not from the mid-aged age group, 31-40 ($p = 1.0$). The mid-aged age group, 31-40 also differed significantly from the higher-aged age group, 41-60 ($p = 0.01$). However, there were no significant differences between close age groups, such as 18 -30 and 31- 40 ($p = 1.00$). The number of mites seems to generally increase by age (Figure 3.3, A).

Table 3.3 Dunn test results, showing comparisons between the age groups

Age Group Comparison	Z -score	Unadjusted p- value	Adjusted p- value
18-30 - 31-40	-0.82	0.41	0.9000
18-30 - 41-60	-4.58	0.00	0.0002
31-40 - 41-60	-3.33	0.00	0.0005
18-30 – 61+	-3.12	0.00	0.0001
31-40 – 61+	-2.58	0.01	0.0005
41-60 - 61-100	-0.70	0.049	0.9800

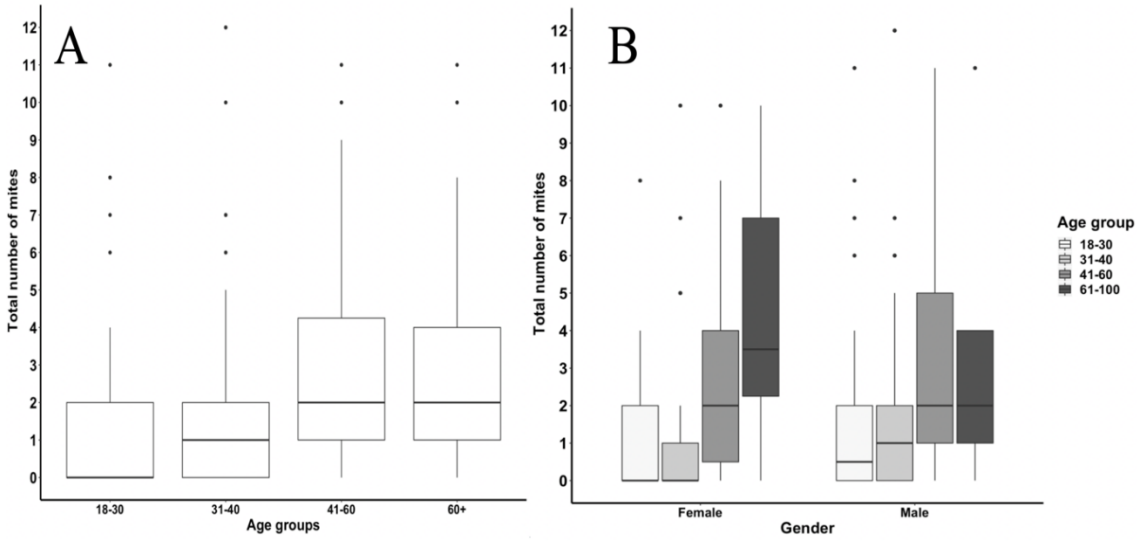

Figure 3.3 Boxplots of host age groups and host's sex

A: Boxplot showing the total number of human *Demodex* mites collected per sample from each host by age groups.

B: Boxplot comparing the total number of human *Demodex* mites per sample from each age group for each host's sex.

The thick horizontal line shows the median value, thin horizontal lines represent first and third quartiles, and vertical whiskers show the ranges excluding outliers. The total number of mites in age group 18-30 is 292 (n = 109; median 0.0, range 0-67), the total number of mites in age group 31-40 is 192 (n = 61; median 1.0, range 0-37), the total number of mites in age group 41-60 is 385 (n = 70; median 2.0, range 0-37) and the total number of mites in age group 61-100 is 73 (n = 16; median 2.0, range 0-17). Only proximal outliers are shown

3.3.2 *Demodex* species

Demodex folliculorum infestations were slightly more frequent in number (618) compared to those of *D. brevis*, (291) collected from both host sexes combined. The number of *D. folliculorum* collected from men was 379 compared to 239 from women, a Mann Whitney U test, however, showed the difference was not statistically significant ($U = 6392$, $p > 0.05$). Although, the number of *D. brevis* collected from men was lower, 116 compared to 175 from women, a Mann Whitney U test also showed no statistically significant difference ($U = 6658$, $p > 0.05$) between them. The data is non-normally distributed, with a positive skew and with numerous outliers in each group. Thus, the

median value is 0 for the number of *D. brevis* and 1 for the number of *D. folliculorum* collected from both sexes. This result is illustrated by a boxplot (Figure 3.4)

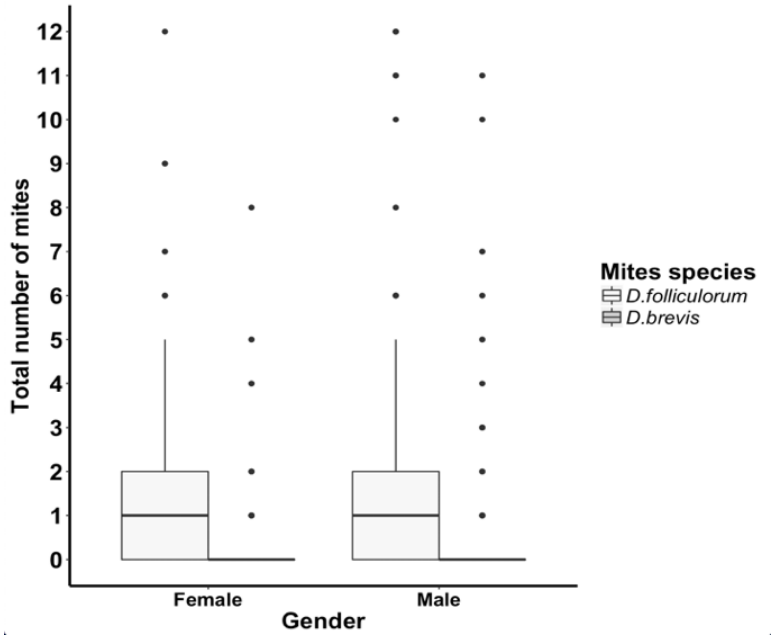

Figure 3.4 Boxplot showing the distribution of the two human *Demodex* species by host's sex.
In females (women), the total number of *D. folliculorum* mites is 239 (median = 1.0, range 0-21) and of *D. brevis* is 175 (median = 0.0, range 0-68). In males (men), the total number of *D. folliculorum* mites is 379 (median = 1.0, range 0-40) and of *D. brevis* is 116 (median = 0.0, range 0-15). Only proximal outliers are shown.

3.3.3 Birth and postnatal feeding modes

Demodex mites were found in about 61 % of the samples from participants who declared to have been breast fed, and about 72 % of those who declared to have been bottle fed. About 62 % of participants who declared to have been born by vaginal delivery had samples containing mites, similar in proportion to the 64 % of samples from participants who declared to have been born by caesarean section which contained them (Table 3.4).

Table 3.4 Prevalence of human follicular mites based on birth and postnatal feeding modes

	Demodex spp.				Total number of participants
	-ve (no mites found)		+ve (mites found)		
	n	%	n	%	
Postnatal Feeding					
Breast feds	86	39	134	61	220
Bottle fed	10	28	26	72	36
Birth					
Vaginal	88	38	146	62	234
Caesarean	8	36	14	64	22

Most participants in the study were born via a normal vaginal delivery and were breastfed (208 participants), which is 81 % of the total number of participants. Only 4 % of participants were bottle fed and born by caesarean section. The summary statistics shows that the median number of mites per sample collected from both is between 1 and 3 (Table 3.5). However, there were two remote outliers in the participant data, 40 mites were recovered from one participant and 67 from another, considerably higher than the median (or mean) values), however, both declared to have been bottle fed.

Table 3.5 Summary statistics for the total number of human *Demodex* mites based on the birth and postnatal feeding modes of the hosts

Birth mode	Postnatal Feeding	Number of participants	Total Mite count	Median	1st Quartile	3rd Quartile	Mite Count Range	
							from	to
Vaginal	**Breast fed**	208	664	2	0	3	0	40
	Bottle fed	26	176	3	0	7	0	67
Caesarean	**Breast fed**	12	31	1	0	2	0	18
	Bottle fed	10	38	2	1	2	0	23

The number of mites per sample was compared for the two birth modes (vaginal and caesarean) using a Mann Whitney test which showed no statistical differences between them ($U = 2502$, $p > 0.05$), in contrast, another Mann Whitney test, which used a Wilcoxon rank sum test with continuity correction, showed did show a significant

difference (W = 4744, p = 0.04956), albeit marginal, in mean mites per sample between participants who were breast or bottle fed. The differences in the distributions of results for each variable are compared in boxplots, Figure 3.5 A and Figure 3.5 B, and for the two variables combined in Figure 3.5 C.

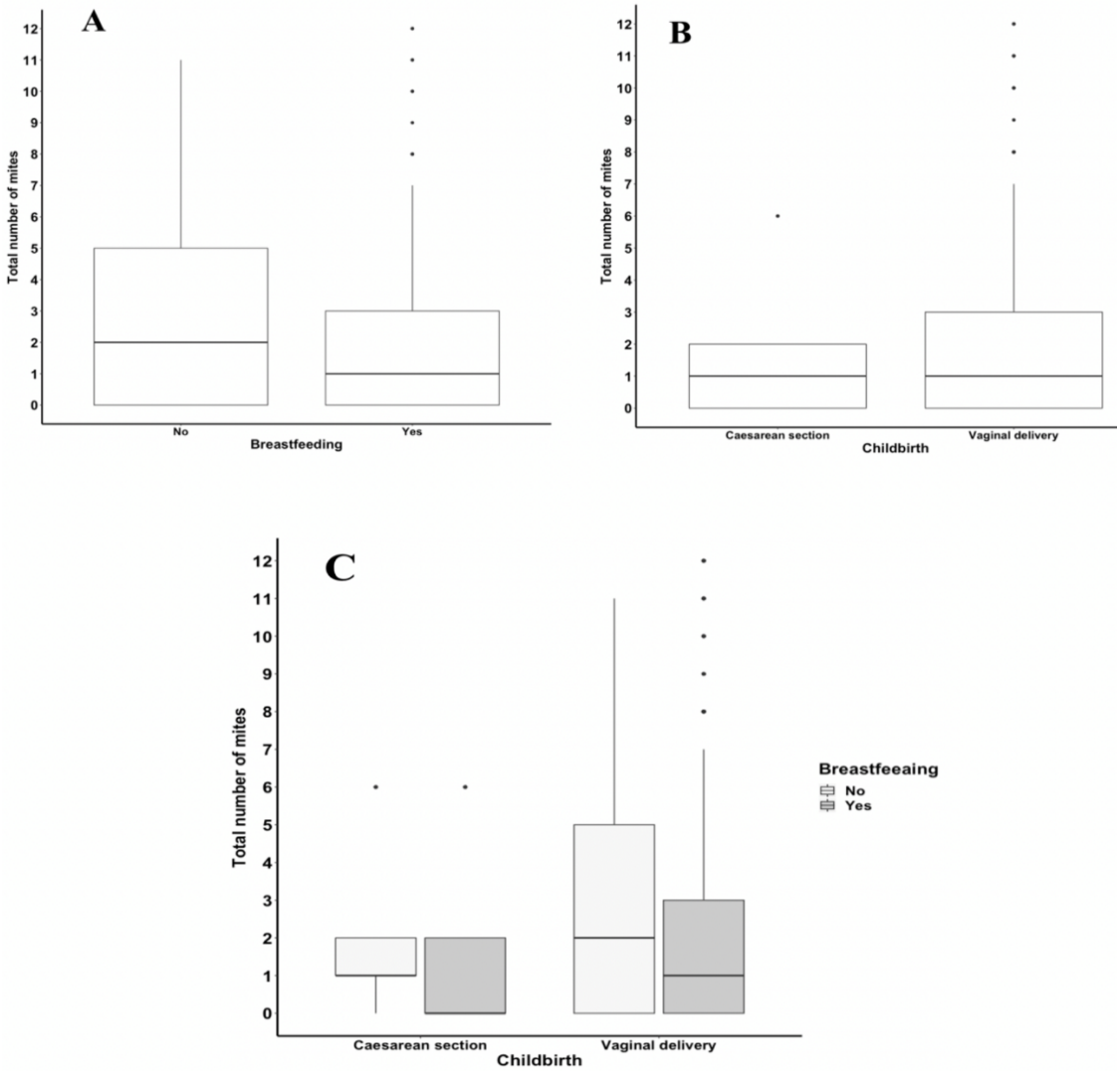

Figure 3.5 Boxplot diagrams showing comparisons between the two modes of childbirth and breastfeeding, based on the total number of human follicular mites collected per sample.

Total number of mites per sample collected from the participants based on: **A:** postnatal feeding: breast fed 695 (median = 1, range 0-40), bottle fed 214 (median 2, range 0-67). **B:** birth mode: vaginal delivery 840 (median = 1, range 0-67). caesarean section 69 (median = 1, range 0-40). **C**: breast fed and vaginal delivery 664 (median = 1, range 0-40), bottle fed and vaginal delivery 176 (median = 2, range 0-67), breast fed and caesarean delivery 31 (median = 0, range 0-18), bottle fed and caesarean delivery 38 (median = 1, range 0-23). The thick horizontal line shows the median value, thin horizontal lines represent first and third quartiles, and vertical whiskers show the range excluding outliers. Only proximal outliers are shown.

3.3.4 Ethnic groups

On the basis of declarations by the participants, 11 different human ethnic backgrounds were recognised in this study. The largest number of participants were British White, 76 individuals, comprising about 30% of the total and the lowest were East-Black Caribbean and Japanese ethnicities, with 4 participants each, representing 1.6 % each of the total (Table 3.6). The prevalence of human follicular mites per sample among the ethnic groups varied between 50 % in participants identifying as Black African up to about 75.5 % in those identifying as Japanese (Table 3.6). Summary statistics show the median of the total number of mites collected from all the different ethnicities varies between 1 and 6 and a boxplot illustrates the differences between the groups (Figure 3.6).

Based on a Levene's test, the variances were equal in all the ethnic groups ($F = 1.5$, $df = 10$, $p > 0.05$). Thus, a Kruskal-Wallis test was used and showed no significant differences ($Chi^2 = 16.5$, $df = 10$, $p > 0.05$) in the number of mites per sample between the ethnic groups. This test is appropriate because the data is non-normally distributed with a positive skew and numerous outliers.

Table 3.6 *Demodex* mite prevalence in samples, based on ethnic group of the participants in this study

Ethnicity	*Demodex* spp.				Total of participants
	-ive (no mites found)		+ve (mites found)		
	Number of participants	%	Number of participants	%	
Chinese	9	31.0	20	68.9	29
Japanese	1	25.0	3	75.5	4
Asian Indian	9	31.0	20	68.9	29
Pakistani	6	31.5	13	68.5	19
Other Asian	25	49.0	26	51.0	51
Mediterranean White	6	30.0	14	70.0	20
Black African	10	50.0	10	50.0	20
East-Black Caribbean	1	25.0	3	75.0	4
South American	5	38.4	8	61.6	13
British White	25	32.8	51	67.2	76
Central Europe	3	30.0	7	70.0	10

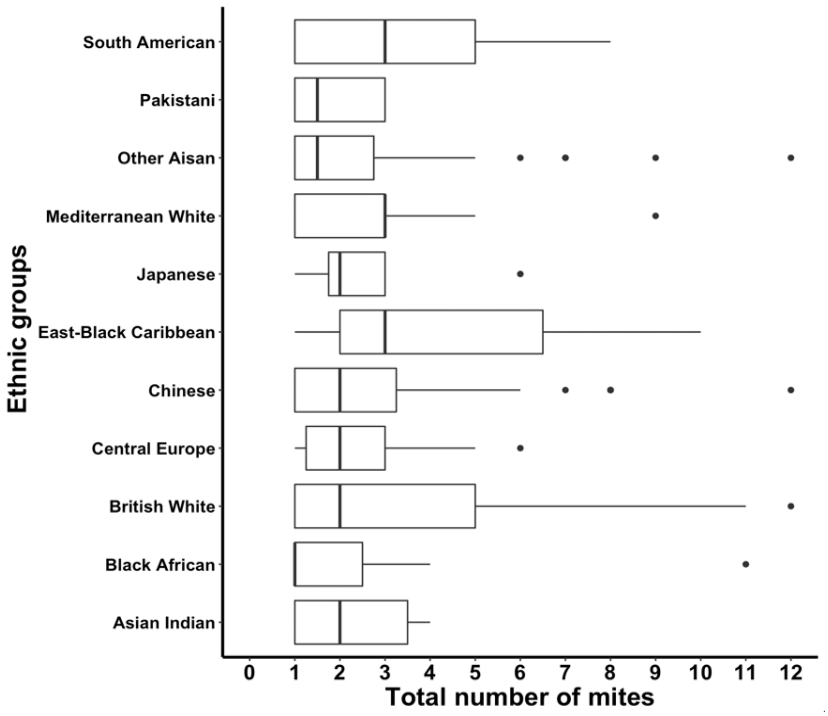

Figure 3.6 Boxplot diagram showing the total number of human *Demodex* mites in 11 human ethnic groups.

The total number of mites per group and other statistics are shown in Table 3.7. The thick horizontal line shows the median value, thin horizontal lines represent first and third quartiles, and vertical whiskers show the range excluding outliers. Only proximal outliers are shown.

3.3.5 Host's sex, age, postnatal feeding and childbirth birth modes

The GLM built to analyse these four on the number of mites per sample collected from the study population of University of Reading students and staff was based on a Zero Inflated Negative Binomial Model (ZINB). The GLM ZINB model was built with four factors, host's sex, age, childbirth mode and postnatal feeding modes as predictors of mites per sample. The result of a ZINB model is in two parts, the first compares all the counts in the data, treating each value (0, 1, 2, etc) as separate. The second compares the probabilities of finding *Demodex* mites in the samples, again based on all the factors included in the model, but treating counts as binomial, either 0 or >0. It showed no significant differences in the number of *Demodex* mites found in the participants' samples between male and female hosts nor between birth modes (Table 3.7). Age and breastfeeding, however, were shown to have significant effects on the number of *Demodex* mites. They are apparently significant for the count model but, not for the probability of measuring the zero-inflation model. This result is clear when comparing the exponential of the coefficient of the two models, the participants who had been breast-fed have are significantly likely to have approximately two times the number mites on their skin compared to bottle fed children. The model also shows a significant likelihood that the probability of having mites generally increases with age. For instance, participants in the age group 41-60 have about two times the probably of having *Demodex* mites than younger participants. The model shows the likelihood of a small decrease in the probability of having mites in participants over 60 years old; which may be explained by a small sample size, only 12 participants from this age group were sampled.

Table 3.7 Zero Inflated Negative Binomial Model (ZINB)

Model with host's sex, age, birth and postnatal feeding modes as factors effecting the count of the number of *Demodex* spp. mites per sample from the human study participants

ZIMP Model – Part 1 - Count data coefficients (negbin with log link)

* indicates a significant result

	Estimate	Std. Error	z value	Pr (>|z|)
Host's sex - Male	-0.05	0.24	-0.22	0.8234
Childbirth - Vaginal delivery	0.30	0.42	0.72	0.4736
Breastfeeding - Yes	-0.85	0.35	-2.41	0.0005*
Age 31-40	0.76	0.34	2.22	0.0266*
Age 41-60	1.09	0.31	3.48	0.0005*
Age 61-100	0.90	0.49	1.85	0.0063943*
Log (theta)	-0.67	0.17	-3.98	0.0005*

ZIMP Model Part 2 - Zero-inflated coefficients (binomial with logit link)

	Estimate	Std. Error	z value	Pr(>|z|)
Host's sex - Male	-0.95	1.69	-0.57	0.572
Childbirth - Vaginal delivery	0.55	3.88	0.14	0.888
Breastfeeding - Yes	0.23	2.13	0.11	0.915
Age 31-40	1.12	2.30	0.49	0.625
Age 41-60	-12.55	483.02	-0.03	0.979
Age 61+	-10.20	201.68	-0.05	0.960

Theta = 0.5113; Number of iterations in BFGS optimization: 80; Log-likelihood: -506.5 on 11 Df

Exponential values and 95 % Confidence interval of the coefficient of the Part 1 ZINB model.

Values are shown for the count part of the model only. Adjusted coefficients showing the variation in mite counts per sample and confidence limits.

	Coefficients	95 % CI	
		2.5 %	97.5 %
Count Host's sex - Male	0.94	0.59	1.50
Count Childbirth - Vaginal delivery	1.34	0.58	3.05
Count Breastfeeding - Yes	0.43	1.09	0.854
Count Age 31-40	2.14	1.09	4.21
Count Age 41-60	2.98	1.61	5.52
Count Age 61+	2.46	0.94	6.40

3.4 Discussion

The objective of this Chapter was to investigate the abundance and distribution of human follicular mites in a small population of people at the University of Reading, and to present novel findings from this not previously studied sector of society in the UK. Several studies have been conducted worldwide for similar purposes but scrutinising different subjects and methods have consequently reported related but quite distinct independent findings. The lowest prevalence rate of human follicular mite infestation reported was 10-20% (Ozdemir *et al.*, 2003; Kemal *et al.*, 2005; Ozcan and Cetinkaya, 2007; Ozdemir *et al.*, 2005), whereas the highest was 100% from a sample population of the over 70s age group (Fuss, 1935; Rufli and Mumcuoglu, 1981; Okyay *et al.*, 2006). Sengbusch and Hauswirth (1986) attributed the variation in frequencies in these reports to different methodologies, the levels and qualifications of the investigators, the size of the human population samples and the area of the skin that was examined. Different collection methods were also considered to play a significant role in the reporting of prevalence of human *Demodex* mites (Sengbusch and Hauswirth, 1986; Perotti *et al.*, 2009). The current study involved taking just a single sample per participant, as have other authors, for example Perotti *et al.* (2009), which affects the conclusions about the prevalence of human follicular mites in the target population, many studies re-sample, leading to higher rates of incidence, for example Spickett (1961). We adopted a single-sample approach partly for methodological reasons, given that participants showed great antipathy to re-sampling.

The prevalence of human follicular mites is approximately 64% in men and 60% in women (Table 3.1) and this result was not significantly different when analysed by a Mann Whitney test ($U = 6321$, $p > 0.05$), boxplot illustration (Figure 3.2) illustrates this finding. These results were similar to the rates of prevalence reported by Sengbusch and

Hauswirth (1986), Isa *et al.* (2011) Kaplan *et al.* (2012) and other authors. This finding shows that other factors than just the host's sex may be involved in determining prevalence, since several other studies, drawing subjects from different sources and with different methodologies have found instead that men are more frequently infested by *Demodex* spp. than women (Andrews, 1982; Ozdemir *et al.*, 2005).

The results show that age has a significant role in increasing the number of *Demodex* mites found on humans. Desch and Nutting (1972), Czepita *et al.* (2004) and Carly and Dirk (2014) all stated that the prevalence of hair follicles infested by *Demodex* mites in humans increases during an individual's lifetime. Our study shows that the people who have a high number of *Demodex* mites in their sample, in general, are older participants (Figure 3.3 and Table 3.2). Riechers and Kopf (1969) noted that elderly people have higher sebum secretions due to the more numerous sebaceous glands on their skin and because their follicular orifices reach the maximum width, especially on the face. This might be the reason for collecting higher numbers of *Demodex* mites from older compared to younger people. Comparing age groups in this study, the results showed that there are statistically significant differences between the number of mites in different age groups. A Dunn Test indicated a significant difference between older age groups (41 to 60+) and younger groups (18 to 40 combined). However, there is no significant differences between closer age groups, such as 18 -30 and 31- 40 (Table 3.3). Although our results confirm those of Riechers and Kopf (1969), we are unable to confirm the contributory factors as this was outsied the scope of this study.

Despite the fact that there were no significant differences in the number of mites that infested different both host's sex in this study, the number of mites seems to increase generally by age in both host's sex (Figure 3.3, B). Czepita *et al.* (2004), in a study of

Demodex infestations in blepharitis sufferers and others, using eyelash epilation collection, also found no host's sex differences while prevalence was higher in older participants. However, other factors were also reported to be significant, such as wearing glasses, which might also be associated with age. This study, using a different collection method, found, as did we, that *D. folliculorum* occurs far more commonly than *D. brevis*.

We found that the two *Demodex* spp. were similarly distributed in both host's sex, albeit with a slightly higher number of *D. folliculorum* collected from both, compared to *D. brevis* (Figure 3.4). There was no statistical difference when comparing the numbers of *D. folliculorum* collected from men and women, nor between the numbers of *D. brevis* collected from both sexes. These results concur with those reported by Akbulatova (1963), in which *D. folliculorum* was found in 36 % in the participants and *D. brevis* in 23%, the differences were not reported to be statistically significant. They also agree with Isa *et al.* (2011), who reported the highest percentages of *D. folliculorum* infestation (81 %) in any study we found, compared to 31% infestation of *D. brevis*, but no statistical difference between host's sex, and with a recent study with similar findings by Zhong *et al.* (2019). However, our result is incongruent with Sengbusch and Hauswirth (1986), they found that 44% of participants studied had *D. brevis* and just 24% had *D. folliculorum*, but again no differences between host's sex. According to Akbulatova (1963), Desch and Nutting (1972) and Carly and Dirk (2014) and others, human *Demodex* mites are found in the same pilosebaceous complex, *D. folliculorum* usually inhabits the area above the sebaceous gland whereas *D. brevis* lives deep inside it. This may explain why we collected higher numbers of *D. folliculorum* in this study, given that participants were known to sometimes be reluctant to undertake overly intrusive skin scraping or cellophane tape collection.

Two facial areas (nose and forehead) of both hosts were compared (See Appendix 3 (1) for results) and the median of number of *Demodex* spp. collected from both areas for both sexes is 0, because of the number of zero-mite collections. A Mann Whitney test showed no statistically significant differences in the number of mites collected from the nose ($U = 6131$, $p > 0.05$) and from the forehead ($U = 6584$, $p > 0.05$) between the two sexes. Similar results were reported by Manoyana *et al.* (2014) who found no significant differences between three facial areas, cheek, forehead and nose, in a study specifically and, therefore, more comprehensively targeting facial differences.

The prevalence of *Demodex* mites found on participants who declared to have been breast fed was about 61%, while in participants who had not been breast fed (assumed to have been bottle fed) it was about 72%. However, summary statistics show that the median is 1 or 2 respectively, the Mann Whitney test for comparison of the two postnatal feeding types was significant ($p < 0.05$), so, therefore, this analysis appears to show that mites were more frequently found on participants who had been bottle fed. However, there are two notable outliers in the data, both participants declared to have been bottle fed but both bore large numbers of mites, it is likely that these samples have distorted this test. The prevalence of *Demodex* mites is approximately 60% and the median is also the same for participants who reported to be borne by normal vaginal delivery or by caesarean delivery (Table 3.4 and 3.5) and there was no significant difference between childbirth modes, Mann Whitney $p > 0.05$ (Figure 3.6).

Kaplan *et al.* (2012) found that the prevalence of *Demodex* mites is the same in humans from different ethnic backgrounds, likewise, our results showed that there were no significant differences between the median number of mites collected from the 11 ethnic groups in this study. The prevalence of the human *Demodex* mites is over 50% in all

ethnicities (Table 3.7, 3.8 and 3.9), the median is also quite varied for all the ethnic groups. A Kruskal -Wallis test showed no statistical differences ($Chi^2 = 16.5$, df = 10, $p > 0.8$) in the number of mites between the human ethnic groups, in this study.

The prevalence of *Demodex* mites in humans has been analysed based on factors such as age, ethnic background and host's sex in many of the studies conducted in the literature using classic ANOVA-based statistical tests, for example, Isa *et al.* (2011), Kaplan *et al.* (2012), Manoyana *et al.* (2014), Enginyurt *et al.* (2015) and Zeytun and Karakurt (2018). In this study, the analyses are in the context of the results, which were tested extensively and showed highly non-normally distributed data with extreme kurtosis and left skew. These characteristics of the data preclude the reliable use of ANOVA-based analysis, since they violate some of its fundamental assumptions (Tukey, 1977). Consequently, a Zero-Inflated Negative Binomial Generalised Linear Model (ZINB) was adopted as a more appropriate and statistically justifiable approach.

A ZINB was built to measure the influence of four factors: host's sex, birth mode, postnatal feeding mode and age on the number of mites collected from our study group and showed host's sex and the childbirth as predictors had no effect on the number of *Demodex* mites in a sample, so that so far as our cohort is concerned, host's sex, whether male or female and birth by normal virginal delivery or by caesarean section make no significant difference. This result is similar to reports in the literature, for example Sengbusch and Hauswirth (1986), Isa *et al.* (2011) and Kaplan *et al.* (2012). Age and postnatal feeding, however, have significant effects on the number mites found per sample on the participants. They are significant for the part 1 count model but not for the part 2 probability of measuring zero or not model (Table 3.8). This result is made clearer by the exponential coefficient of the two factors (Table 3:8), which shows that the

participants who had been breast fed were twice as likely to have mites on their skin than participants who hadn't been breast fed. Sengbusch and Hauswirth (1986) studied the effect of postnatal feeding on the number of mites infesting humans (cited in Sengbusch and Hauswirth (1986)), without reporting results or conclusions, however no published results regarding postnatal feeding were found for this study. Breast feeding is considered as direct skin to skin contact for prolonged periods between the between the mother and her child (Fisher, 1973; Izdebska and Fryderyk, 2012). Fisher *et al.* (1980) calculated the transmission time of *D. bovis* in cattle and found that the mites can transmit from an infested cow to her calf in just two days, which suggests that transmission by this form of contact may also occur in humans. However, although our Mann Whitney test did not find significantly more mites per sample on breastfed participants, the ZINB, which we consider a more robust test, showed highly significantly ($p = 0.0005$) that there was a likelihood that breastfed participants had about 0.4 mites more per sample than non-breast fed. It has been hypothesised that close skin to skin contact with the mother during critical times, such as delivery and during breast feeding, may increase the prevalence of *Demodex* mites (Fisher, 1973; Nutting and Green, 1976; Czepita *et al.*, 2004), while birth mode was found to have no effect on our mite counts per sample, in either this model, or the Mann Whitney analyses, so that whether born via normal or caesarean delivery is immaterial, the results for postnatal feeding are more complex. Mites numbers were significantly higher in samples of participants who had not been breast fed according to the Mann Whitney test, combined with a higher median value for assumed bottle fed participants, however, the ZIMB. This leads to the conclusion that mites may be transmitting to humans during early life facilitated by close contact, perhaps hugging, holding and kissing (Perotti *et al.*, 2009; Izdebska and Fryderyk, 2012; Carly and Dirk,

2014), which are also forms of close skin to skin contact between the child and his mother and that breast feeding is not *per se* a significant event in the context of mite transmission.

The ZINB also predicts a general increase in the probability of older participants having mites, compared with young participants. For instance, participants in age groups between 41 and 60 have about twice the probably of having *Demodex* mites compared to younger participants. Nevertheless, the model shows a small decrease in the probability of having mites in participants over 60 years old, compared to age groups between 41 and 60, this may be explained by limitations in the analysis due to some small sample sizes, there are, for example, only 16 participants in the 60+ age group in this study. Many reports support this finding such Sengbusch and Hauswirth (1986), Isa *et al.* (2011), Zeytun and Karakurt (2018) and Zhong *et al.* (2019). While Manoyana *et al.* (2014) found instead that young adult male between 20 to 22 have more mites than elderly participants.

In conclusion, *Demodex* follicular mites can be found in men and women from different age groups and from different ethnic backgrounds. Host age (older) and postnatal feeding (breast fed) seem to be significant factors in the prevalence of human follicular mites. Whereas, host's sex, ethnicity and mode of birth were not found to have any significant effects. To draw reliable conclusions regarding these complex factors, larger studies on bigger populations, with more balanced and representatives from defined ethnic groups are highly recommended. Our study did not differentiate between ethnic origin and country of residence, for example, and so any of our participants may have had extensive contacts with participants in different ethnic groups and we did not test for differences in transmission of mites dependant on ethnic group. An interesting study might be to investigate whether there are indeed differences in mite populations between ethnic groups and whether the differences arise from an inability of the mites to transfer for

biological reasons or because of lack of opportunity through less physical contact. Chapter 4 investigates general differences and similarities in DNA fragment lengths for selected genes in the same ethnic groups and, therefore, takes aims to provide more information in this regard. More extensive and focussed studies may, however, build upon our results with more detailed findings regarding the subtleties and influences on our permanent skin-resident mites.

Chapter 4: Multiplex PCR identification of the two human *Demodex* mites and *D. canis* (Dog mites)

4.1 Introduction

Although the pathogenesis of human *Demodex* mites is far from well understood (Gmeiner, 1908; Ayres and Mihan, 1967; Dong and Duncan, 2006; Lacey *et al.*, 2009), and cutaneous infection and skin disruptions are considered to have various causative factors (Gilhar and Kalish, 2006; Grice and Segre, 2011; Lacey *et al.*, 2011; Talghini *et al.*, 2014; Weyrich *et al.*, 2015), human follicular mites have often been implicated as etiologic agents of skin lesions. These include serious conditions such as follicular basal cell carcinoma (Erbagci and Erkiliç, 2000; Erbagci *et al.*, 2003; Sun *et al.*, 2005), scabies-like eruptions, pigmentation on the face, gland dysfunction, rosacea and alopecia (Lacey *et al.*, 2009; Chen and Plewig, 2015). The pathogenetic role of *Demodex* mites in these and other skin conditions has, however, long been disputed (Ayres and Mihan, 1967; Pena and Andrade Filho, 2000; Perotti *et al.*, 2009).

Demodex canis has been confirmed as a cause of severe mange in young dogs or those with a compromised immune system (Peikes *et al.*, 2001; Krantz and Walter, 2009; Fondati *et al.*, 2010). It is also believed that *D. canis* occurs on and may play a role in causing skin disorders in other mammals, and it has been established to be the cause of types of mange in cats, captive bats and domestic ferrets (Thoemmes *et al.*, 2014; Sastre *et al.*, 2016). Sastre *et al.* (2016) noted that it is likely to be the most infectious amongst several species of mite they studied. As dogs make up 31 % of the pets owned in the UK (Murray *et al.*, 2010), and physical contact between pet and owner is a common feature of pet ownership, unsurprisingly, cross of this mite infection between dogs and humans

has frequently been reported (Morsy *et al.*, 1995; Wang *et al.*, 1998) and it is possible that some or all skin lesions in humans are caused by *D. canis* and not by the human *Demodex folliculorum* or *D. brevis*. The methodology developed here includes molecular differentiation of *D. canis* from the human mite species, as, clearly mites of this species could potentially be recovered from human hosts and contaminate research or medical investigation samples, and because, if their pathogenicity in humans is confirmed, this will be an important contribution to the diagnosis of skin disorders in future research.

Mite research, including medical diagnosis of skin disorders associated with *Demodex* mites, has to date relied entirely on microscopic examination as the only technique available to confirm the presence and identities of the mites collected from human hosts. The morphological characteristics of *D. brevis*, *D. folliculorum* and *D. canis* are similar Sastre *et al.* (2016), particularly in the nymph and larval life cycle stages (Desch and Nutting, 1972; Izdebska and Fryderyk, 2011) so that using microscopy identification mistakes can be made (Section 2.2) even by experienced researchers or medical practitioners. The possibility of also finding *D. canis* where only the human species are expected, can only increase the likelihood of misidentifications occurring and this may have led to under-recording of *D. canis* mites on human hosts in the past. It can be difficult to discriminate between these species even for most expert investigators (Izdebska, 2009), thus, a reliable diagnosis technique, such as reliable molecular identification, is both a highly important and highly desirable objective.

The whole mitochondrial genomes of both human *Demodex* mites have been sequenced and made available in GenBank® (www.ncbi.nlm.nih.gov/genbank/) (accession number KM114225 and KM114226) for *D. folliculorum and D. brevis*, respectively (Palopoli *et al.*, 2014). Mitochondrial DNA (mtDNA) genes have been employed in most molecular

research on mites conducted to date (de Rojas *et al.*, 2012a; Zhao *et al.*, 2013b; Palopoli *et al.*, 2014) because they can be highly suited to distinguishing between closely related mite species, a result of these genes often evolving more rapidly compared with nuclear genes (de Rojas *et al.*, 2012a). Thus, mitochondrial cytochrome oxidase subunit I (mt*COI)* and *16S* ribosomal RNA (*16S* rRNA) were selected for molecular discrimination between *D. brevis*, *D. folliculorum* and *D. canis* in this study.

The main objective of this study is to produce an easy, reliable and inexpensive diagnostic protocol for physicians, medical laboratory technicians and mite researchers in other disciplines to correctly identify the *Demodex* mite species in clinical and research samples from humans. To address this aim, a special molecular technique that allows for amplifying of more than one DNA fragment in a single PCR reaction was needed, since potentially *D. brevis*, *D. folliculorum* and *D. canis* are present. Multiplex PCR has been used to discriminate between species in mites families such house dust Dermatophagoidinae using *mtCOI* (Thet et al., 2012) and spider mites Tetranychidae using *COI, ITS1, ITS2* and *18S* (Gómez et al., 2019, Sinaie et al., 2018, Zélé et al., 2018). Multiplex PCR assay is the best option for this approach because it can amplify several DNA fragments in the same reaction tube. That means, that more than one pair of primers can be used, which decreases the expense and time required (Sambrook and Fritsch, 1989) for the PCR process. However, multiplexing methods are complex and have some disadvantages, because several elements must be carefully optimised first before it can be conducted. For instance, the interactions between various sets of primers must be minimised, melting temperatures must be optimised to be approximately the same for all primers in use and PCR products with readily distinguishable lengths must be generated (Sambrook and Fritsch, 1989).

The principal objectives of this Chapter were as follows.

- To design a multiplex PCR protocol to achieve a molecular characterisation of human follicular mites using mt*COI*, *16S* rRNA and *28S* rRNA gene regions.
- To design primers to amplify the *28S* rRNA gene region of *D. canis*.
- To successfully distinguish *D. brevis, D. folliculorum* and *D. canis* on the basis of molecular DNA polymorphisms, using the multiplex PCR protocol.

4.2 Materials and Methods

To achieve the experimental objectives the following experimental process was followed.

1) Mites were sampled and DNA extracted.
2) Primers to amplify mt*COI* and *16S* rRNA were selected and prepared for *Demodex folliculorum* and *D. brevis*.
3) Primers to amplify mt*COI* and *28S* rRNA were evaluated and prepared for *D. canis.*
4) Single mite species DNA PCR amplifications were conducted establish the most suitable conditions to amplify all the included primers, specifically to establish the most appropriate annealing temperature, the specificity of the primers; to determine that PCR products are distinguishable, and to ensure that no interactions between primers takes place.
5) Evaluative single species PCRs were conducted to compare mites sampled from hosts of different ethnic backgrounds to establish the universality of the new protocols.
6) Multiplex PCR amplification were conducted to determine the best combination of primers which amplified distinguishable electrophoretic bands for species and primers and in which there were no interactions between primers; to test samples at lower DNA concentrations; and to test QIAGEN proprietary Q-solution.
7) Following each PCR, amplification fragments were resolved by agarose gel electrophoresis.

4.2.1 Mite sampling and DNA extraction

The two human *Demodex* spp. were collected from two facial skin regions (nose and forehead) of participants, using cellophane tape and skin scraping techniques. After collection, all samples were immediately preserved in 100% ethanol, then poured into a watch glass, species identities were confirmed according to the morphological characteristics described in Section 2.2.4. Dog mites (*D. canis*) were provided by Dr. MA. Perotti, University of Reading, Reading, UK and were obtained from a veterinary clinic in Spain, their identity was confirmed against the descriptions in Izdebska and Fryderyk (2011) and de Rojas *et al.* (2012a), by Dr. MA. Perotti. For each species, two mites were transferred to a sterile 1.5 mL Eppendorf tube kept open to allow the alcohol to evaporate and DNeasy Blood & Tissue Kits (QIAGEN) used to extract genomic DNA from the mites, precisely following the manufacturer's recommended procedure, except the final step in which the elution volume was decreased to 50 µl.

Genomic DNA was extracted from *D. folliculorum* mites collected and identities confirmed from each of the 11 ethnic groups (Section 3.3.4) included in this study and from *D. brevis* for 10 ethnic groups (mites of this species were not recovered from participants of Pakistani background), following the procedures described here; for comparison.

Genomic DNA was extracted from all three species using a single mite sample, also following the procedures described, here to test the sensitivity of the test to low DNA concentractions.

4.2.2 *Demodex folliculorum* and *D. brevis* primers preparation

Primer sequences (unpublished) were supplied by Dr. MA Perotti, University of Reading, Reading, UK and Dr. HR Braig University of Bangor, Bangor, UK for the two human

Demodex species, to amplify mt*COI* and *16S* rRNA. All primers were synthesised by Eurofins (Ebersberg, Germany) and stock solutions for forward and reverse primer sequences prepared individually by adding the exact volume of sterile, double-distilled water (ddH_2O) recommended by the supplier to attain a concentration of 100 pmol/µl. Forward and reverse primers for PCRs were prepared from 10 µl of stock primer solution with 90 µl of ddH_2O, to obtain a final concentration of 10 µM/µl, which was used in every PCR assay.

Table 4.1 Primer Sequences to amplify mt*COI* and *16S* rRNA for *Demodex folliculorum* and *D. brevis*

Some primer sequences are omitted as they were, following the tests described below, not used further in this study.

***Demodex* spp.**	**Primer name (as supplied)**	**Sequence Label**	**Sequence (5'-'3)**	**Expected amplicon length**
D. brevis	*Db COI* 350	*Db COI* 63F	TACCCTAGCCTTCTACACAGGT	~350 bp
		Db COI 411R	ATAGTAGGTATCGTGGAGGGTGAT	
D. folliculorum	*Df COI* 250	*Df COI* 144F	RGGATTTATTGTATGAGCCCACCA	~250 bp
		Df COI 396R	GAGTGTTACGTCTAGGGAAGAGTTA	
D. brevis	*Db 16S* 300	*Db 16S* 80F	GTATGAGGGGATTTTTTGAGAAAT	~300 bp
		Db 16S 357R	ATCGAGGTAACAAACTCAATCTC	
D. folliculorum	*Df 16S* 200	*Df 16S* 78F	GTATGAGTGGAG TAATAGGTGC	~200 bp
		Df 16S 296R	CAAAGTATCTAT CCATACACCCTA	
D. brevis	*Dbm COI* 200	Dbm *COI* 1318F	TACCCAGACGCATTCACCCCTTG	~200 bp
		Dbm *COI* 1517R	ACGGTTTGGAGGTGGGAGTGGT	
D. folliculorum	*Dfm COI* 320	Dfm *COI* 363F	TGGCTGAACATTCTACCCACCCCT	~320 bp
		Dfm *COI* 678R	ACGGTTTGGAGGTGGGAGTGGT	

4.2.3 *Demodex canis* primers evaluation and preparation

Various primer sets have been used to amplify the mt*COI*, and *28S* and *16S* rRNA gene regions of *Demodex canis*. We tested the primers for mt*COI* used for *D. canis*, other dog mites and *D. folliculorum* by De Rojas *et al.* (2012b), they also used *16S* rRNA, and found both markers useful to establish the phylogenetic relationships between species in these mites. The primers they used were designed by Navajas *et al.* (1994) and de Rojas *et al.* (2002), respectively. Zhao *et al.* (2012b) sequenced nearly the entire rRNA and designed

28S primers for *D. folliculorum* and *D. brevis* but could only partially recover *28S* from *D. canis* so excluded it from their sequence alignments and phylogenetic analysis. Dr. H. R. Braig, University of Bangor, Bangor, UK (unpublished) has designed several primers sets which amplify mt*COI* and 16*S* rRNA from *D. canis*. A range of primers from these sources were evaluated and, after several trials, only one primer set successfully amplified *28S* in *D. canis*, a sequence which was designed originally to amplify *28S* of *D. folliculorum, as shown:*

DFM 28SF ACGAATACGGACCGCGAAAGCG

DFM 28SR TAGCATCGCGCCATTGATCGGT

In order to obtain a *D. canis* specific primer, the PCR product from this amplification was purified and, expecting that the quantity of the DNA was likely to be low, as has been previously reported (Zhao *et al.*, 2009a cited in Zhao *et al.*, 2012b) and, therefore, too poor to be sequenced directly, PCR product was increased by cloning, to obtain more DNA. Cloning was conducted using StrataClone PCR Cloning kits (Agilent Technologies, USA), according to the manufacturer's instructions. The resultant nucleotide fragments were sequenced externally (Eurofins Genomics, Germany) and the Primer-BLAST software tool developed at NCBI, which generates target-specific primer pairs (Ye *et al.*, 2012; http://www.ncbi.nlm.nih.gov/tools/primer-blast), was used to design three sets of primers based on the resulting sequences (Table 4.2).

Table 4.2 Three primer sets designed to amplify *28S* rRNA in *D. canis* using Primer-BLAST (NCBI)

Sequenced *28S* fragment (5'-'3)	Primer name	Forward / Reverse	Primer sequence (5'-'3)	Expected Product length
GCTCAGAGGTGTCAGAAAAGTTACCACAGG GATAACTGGCTTGTGGCGGCTCAGCGTTCA TGGCGAGGTCGATTTTTGATCCTTCGGCTC TTTCTATCATTGTGAAGCAAAATTCGCCAA ATGTTAAATTGTTCACCCACTAATAGGAAA CGTGAGCTGAGTTTAGACGGTCGTGAAACA GGTTAGTTTTAACCTACCGATATTATTGTC CTTAAAGTGGAAATCCTACTCAGTTCGAGA GGAACAGCAGGTTTGTATAATTGGTACATG CACTTGATCGACCGATCAATGGCGCGATGC TAATGCTAAGCGCCT	*Dc 28S* 277	F	CCACAGGGATAACTGGCTTGTGGC	~277 bp
		R	GCATCGCGCCATTGATCGGTCG	
	Dc 28S 237	F	GGTCGATTTTTGATCCTTCGGC	~237 bp
		R	TTAGCATCGCGCCATTGATCG	
	Dc 28S 110	F	GTATGAGTGGAG TAATAGGTGC	~110 bp
		R	ACCTGCTGTTCCTCTCGAA	

Primer sets Dc *28S* 277 and Dc *28S* 237 were excluded from the multiplex PCR assays as the amplified products were of similar length those from other species, and, therefore, gel generated bands were indistinguishable from other bands with similar lengths; except for one further test of Dc *28S* 237 specificity. Therefore, only Dc 28S 110 was carried forward to the multiplex assays.

4.2.4 Single mite species PCR amplifications

PCR assays were performed for each species and primer pair separately, to optimise annealing temperatures for primer pairs, to evaluate the specificity of the primers and to choose primer pairs that amplify clearly distinct amplicon sizes, as shown by gel electrophoresis. The results of these assays to be used to baseline subsequent multiplex PCR amplifications. Each PCR contained a suitable positive control and a negative control, ddH_2O.

PROMEGA PCR kits (Promega, USA) were used for the PCRs and implemented according to the manufacturer's instructions. The formulation of the PCR mix is shown in Table 4.3. Primer mixtures comprised 1 µl of 10× primer stock solution, of either the forward or reverse primer for mt*COI* or the forward and reverse primer for *16S* rRNA (details as shown in Table 4.3), with 9 µl ddH_2O, to obtain a final concentration of 10 µM/µl.

Table 4.3 Single genome assays, PCR mixture formulation

Component	**Final volume**		**Final concentration**
2× GoTaq Green Master Mix		12.5 µl	1× (1.5 mM $MgCl_2$)
RNase-free double-distilled water		15.87 µl	
Forward primer *COI* or *16S*		1.0 µl	0.5 mM
Reverse Primer *COI* or *16S*		1.0 µl	0.5 mM
DNA template, one of:	*D. folliculorum*	1 µl	
	D. brevis	1 µl	
	D. canis	1 µl	

A PCR was performed which comprised: activation hot start at 95 ˚C for 30 seconds followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing (Gradient) for 30 seconds and extension at 72 ˚C for 1 minute, then a final extension at 72 ˚C for 10 minutes and storage at 4 ˚C until the sample was required.

Three conditions were investigated: 1) an optimal, initial melting temperature suitable for all the primer sets if used in combination; 2) suitability of the resultant PCR products to correctly identify the three target species, which is dependent on the specificity of the primers; and 3) the interaction between the primers, to ensure appropriate gene-specific amplification was taking place.

Optimal annealing temperature. Regular PCRs were used to find the annealing temperature for all the primer sets being tested. The thermocycler (PEQSTAT, Universal

Gradient) was set to test a range of temperatures from 52 ˚C to 61 ˚C, which were all under the published melting temperature (T_m) of the primers.

Specificity of the primers. Primers sets designed for specific *Demodex* species were used to amplify the DNA template for the other two species, in regular PCR assays, as described above, all combinations of species and primers were tested, with the optimal annealing temperature as selected above.

Gene amplification interactions. The selected primer combinations were subject to evaluation using the Sequence Multipulation Suite, Bioinformatics.org (Stothard, 2000), a collection of applications to generate, format, and analyse short DNA and protein sequences which is is commonly used by molecular biologists. The output files produced by the Genetic Analyzer were input to the software. Specifically, two tests were applied, PCR Products Analysis which accepts one or more DNA templates and two primer sequences and returns matching primer annealing sites that generate PCR products and their sizes, and PCR Primer Stats which accepts and evaluates a list of PCR primer sequences, returning melting temperature, percent GC content, and PCR suitability for each primer. These tests evaluate the suilabilty of the primers such as self-annealing, presence of hairpins and the fragments' expected length amplified by each primer set in the reaction.

4.2.5 Multiplex PCR primers and optimisation

From an initial >25 different primer sets designed to amplify the three target genes in the three *Demodex* spp., at least one suitable primer was sought for each species. Three primer sets were selected for amplifying the target genes in each human *Demodex* spp. and one primer set was chosen for amplifying the *28S* gene in *D. cani*s, based on successful amplifications in single PCR assays. Based on the gel band lengths produced

by individual PCR assays, and the requirement to generate gel bands of clearly distinguishable lengths, four different primer combinations (A-D) were devised for multiplexing, each containing one set per species of mite. (Table 4.4).

Table 4.4 Four different primer set combinations used in the multiplex PCR assay

Primer sequences are shown in Table 4.1 - *D. brevis*, *D. folliculorum* and 4.2 – *D. canis*

Primer Combination	*Demodex* spp.	Primer Name	Forward / Reverse Sequence Labels	Amplicon Product length
A	*D. brevis*	*Db 16S* 300	*Db 16S* 80 F	~300 bp
			Db 16S 357 R	
	D. folliculorum	*Df 16S* 200	*Df 16S* 78 F	~200 bp
			Df 16S 296 R	
	D. canis	*D.c 28S* 110	*D.c 28S 110 F*	~110 bp
			D.c 28S 110 R	
B	*D. brevis*	*Db COI* 350	*Db COI* 63 F	~350 bp
			Db COI 411 R	
	D. folliculorum	*Df 16S* 200	*Df 16S* 78F F	~200 bp
			Df 16S 296 R	
	D. canis	*D.c 28S* 110	*D.c 28S 110 F*	~110 bp
			D.c 28S 110 R	
C	*D. brevis*	*Db COI* 350	*Db COI* 63 F	~350 bp
			Db COI 411 R	
	D. folliculorum	*Df COI* 250	*Df COI* 144 F	~250 bp
			Df COI 396 R	
	D. canis	*D.c 28S* 110	*D.c 28S 110 F*	~110 bp
			D.c 28S 110 R	
D	*D. brevis*	*Dbm COI* 200	Dbm *COI* 1318 F	~200 bp
			Dbm *COI* 1517 R	
	D. folliculorum	*Dfm COI* 320	Dfm *COI* 363 F	~320 bp
			Dfm *COI* 678 R	
	D. canis	*D.c 28S* 110	*D.c 28S 110 F*	~110 bp
			D.c 28S 110 R	

Multiplex PCR assay conditions and quality were tested to identify the combination (A-D, Table 4.4) and protocol that would successfully amplify all three genes in the target DNA and for which species and genes could be reliability differentiated in the PCR product; since this combination would give the broadest utility and potential for real-time multiplex PCRs.

4.2.6 Multiplex PCR amplifications

After ensuring that all primers were species- and gene-specific, and using the optimal annealing temperature for the primer combination, four multiplex PCR assays were conducted using the combinations of primers in Table 4.4.

A QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany, catalogue No 206143) was used and the manufacturer's instructions were followed precisely. For each primer combination, the multiplex PCR mixture was formulated as shown in Table 4.5; a 10× primer mixture was prepared by combining 10 µl each of forward and reverse primer from each of the selected primers for the combination made up with sufficient ddH_2O to reach a final volume of 500 µl and equimolar parity, also as recommended by the manufacturer. A second set of multiplex mixtures were prepared and 5 µl of QIAGEN Q-solution was added and the ddH_2O volume adjusted to achieve a final volume of 50 µl. Q-solution is a QIAGEN® proprietary PCR additive that facilitates amplification by modifying the melting behaviour of target DNA and can, thereby, enable or improve weak amplifications, particularly where the cause is a high degree of secondary structure or GC-richness, resulting in improved quality of the multiplex PCR products.

Table 4.5 Multiplex PCR reaction formulation.

Component	Final volume		Final concentration
2× QIAGEN Multiplex Master Mix		25 µl	1×
10× multiple primer mixture (10 µmo/L)		5 µl	0.2 µM
RNase-free ddH_2O		13 - 18 µl	
DNA template	*D. folliculorum*	1 µl	≤1 µg DNA/50 µl
	D. brevis	1 µl	
	D. canis	1 µl	
Q-Solution (see text)		5 µl	

As recommended by the PCR kit manufacturer, PCR conditions comprised an initial activation hot start at 95 °C for 15 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 90 seconds and extension at 72 °C for 90 seconds, and a final extension for 10 minutes at 72 °C and storage at 4 °C until the sample was required.

4.2.7 Confirmatory investigations

To confirm the utility of the results of this study, further asays were conducted.

1) The selected primers for the multiplex PCR assay were tested to amplify *COI* and *16S* genes in human *Demodex* mites collected from different ethnic groups (see Section 3.3.4).
2) The primers designed for *D. canis* were tested for amplifying the *28S* gene in mite samples collected from *D. canis* infested dogs.
3) The PCR fragments obtained from the human *Demodex* spp. genes were purified by NucleoSpin Gel and PCR purification (Macherey-Nagel kit) and sent to Eurofins Genomics (Germany) for sequencing and compared to equivalent accessions on GenBank.
4) Multiplex PCRs were repeated using low concentractions of DNA (one mite per sample, see Section 4.2.1) from each species, to measure the sensitivity of the test. The DNA concentration of the positive control was between 1.3 ng/µl to 1.7 ng/µl and the negative control was ddH_2O only. The tested DNA concentrations were from 0.01 ng/µl to 1 ng/µl.

4.2.8 Gel electrophoresis

PCR products were further evaluated for purity, concentration and amplicon length by separation using gel electrophoresis. A 2% agarose gel was prepared, as described in 5.2.3, with Ethidium Bromide (5 µl) added as a stain. After loading 5 µl of each PCR product a ladder and the controls, the gel was run at 100V for 60 minutes. The gel was visualised under a transilluminator (Benchtop UV) and images recorded.

4.3 Results

The positive and negative controls in each PCR were checked on the gel separations, and the results as expected in every case, results not shown.

4.3.1 Multiplex PCR discrimination of human *Demodex* spp.

Multiplex PCR assays which included *D. folliculorum* and *D. brevis,* with and without Q-solution, were compared to the results of single genome PCRs. Figure 4.1 shows that the multiplex PCR products produce clear bands of comparable lengths to the single genome assays, following electrophoretic separation. In each of the two-single genome (regular) PCRs there are single, clear bands which discriminate the two species of human follicular mite, and the multiplex PCR produces bands of equivalent sizes, showing that the multiplex PCR conditions and primer combination also clearly discriminate these species. The bands are of lengths: *D. brevis* / COI ~350 bp., *D. folliculorum* / *COI* ~250 bp., *D. brevis* / *16S* ~300 bp., *D. folliculorum* /*16S* ~200 bp, which coincide with the expected lengths for these species / marker combinations (Table 4.1). The effect of adding Q-solution (+Q in Figure 4.1) was to strengthen the bands indicating improved PCR products for *COI* but led to no improvement in 16S amplification.

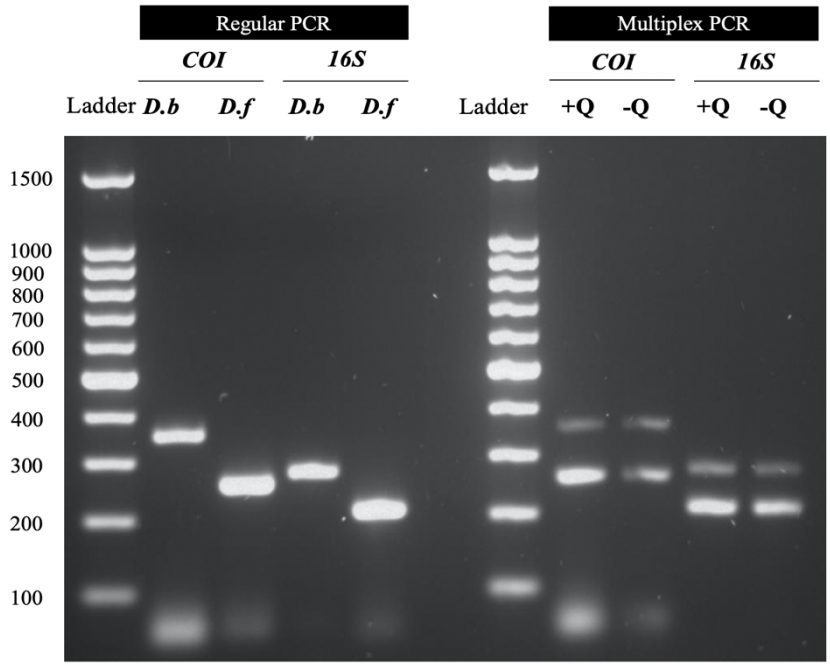

Figure 4.1 Comparison of single species and multiplex PCR products using *COI* and *16S* primer sets (Table 4.1).

Lanes, left to right:
Ladder 100 bp.
Regular PCR reactions: *COI* primers - ***D.b*** band size ~350 bp; ***D.f*** band size ~250 bp. - *16S* primers: ***D. b*** (*16S* primers) band size ~300 bp; ***D f*** band size ~200 bp; -ve control (see Appendix 4.1) – no band.
Multiplex PCR reactions: *COI* primers - +Q and -Q band sizes ~250 bp and ~350 bp. - *16S* primers +Q And -Q band sizes ~300 bp and ~200 bp.

Controls are shown in Appendix 4, figures A4.1 and A4.4. -ve controls showed no band**s.**

4.3.2 Multiplex PCR discrimination of human *Demodex* spp. and *D. canis*

Single genome (regular), primer specific PCR results (Figure 4.2) show strong bands following electrophoretic separation produced by each of the three primer sets and for each of the three *Demodex* species, which all coincide with the expected lengths in Table 4.1 and 4.2. The lanes in Figure 4.2, with their primers and the band lengths are:

- **B1-B3**: *D. brevis* - B1 - *Db 16S* 300 / ~300 bp, B2 - *Db COI* 350 / ~350 bp, B3 - *Dbm COI* 200 / ~200 bp
- **F1-F3:** *D. folliculorum* - F1 - *Df 16S* 200 / ~210 bp, F2 - *Df COI* 250 / ~250 bp, F3 - *Dfm COI* 320 / ~320 bp.
- **C1-C2:** *D. canis* – C1 - D.c 28S 277 / ~270 bp, C2 **-** D.c 28S 110 / ~110 bp.

Only one gel band, for *D. folliculorum* (lane F2) and primer *Df 16S* 200 is potentially longer than expected at ~210 bp. However, in both *D. canis* bands and F1 *D. folliculorum* lanes F1 and F3, the bands are very bright indicating the lanes may have been overloaded and the concentration of the DNA is too high.

It is notable that the strong band produced by *D. canis* using primer D.c *28S* 277 clearly overlaps with bands from other species, notably lanes B1 - *Db 16S* 250, F1 - *Df 16S* 210 and F3 - *Df COI* 300, hence it was not used further.

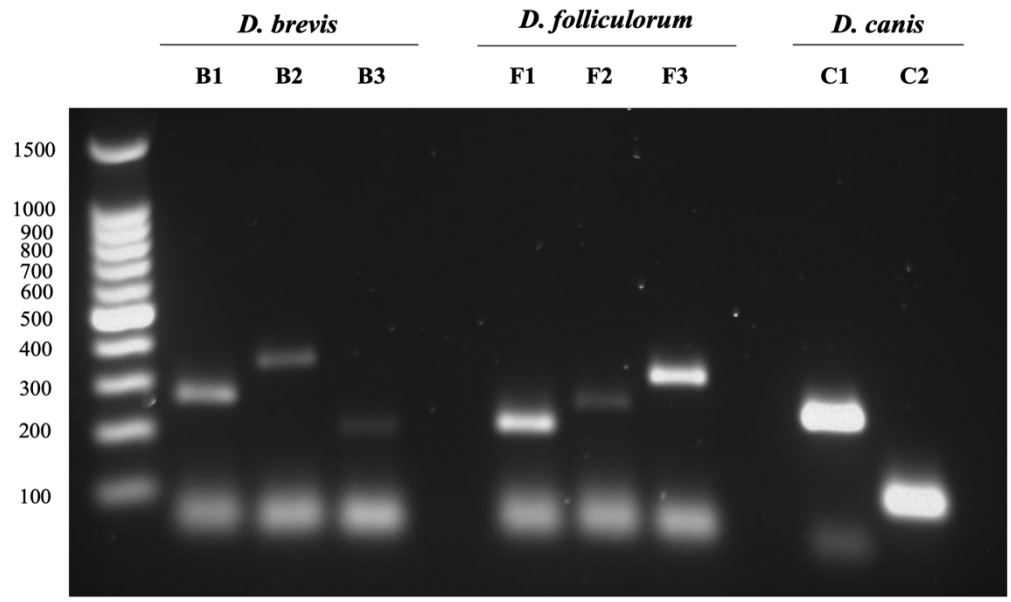

Figure 4.2 Single genome (regular) PCR assay for each selected primer set and all *Demodex* spp.
Lanes, species and primers / fragment lengths:
Ladder 100 bp.
B1-B3: *D. brevis* - B1 - *D.b 16S* 300 / ~300 bp, B2 - *D.b COI* 350 / ~350 bp, B3 - *Dbm COI* 200 / ~200 bp
F1-F3: *D. folliculorum* - F1 - *D.f 16S* 200 / ~210 bp, F2 - *D.f COI* 250 / ~250 bp, F3 - *Dfm COI* 320 / ~320 bp.
C1-C2: *D. canis* – C1 - *D.c 28S 277* / ~270 bp, C2 **-** *D.c 28S 110* / ~110 bp.

Controls are shown in Appendix 4, figures A4.1 and A4.4. -ve controls showed no band**s.**

The four primer mixtures tested (A-D) for discrimination and specificity in multiplex PCR assays each included one primer set for each *Demodex* species. and each gene. The results (Figure 4.3) show that each primer combination has amplified the target DNA fragments and produced three distinguishable bands as expected. The multiplex PCR reactions show clear and distinguishable bands for the primer mixtures A, B and C. However, primer mixture D shows only one definite band, likely representing *D. folliculorum* DNA amplified by the primer *Dfm COI* 300*,* with an approximate size of ~320 bp. and a much fainter band at ~200 bp. likely representing *D. brevis* amplified by

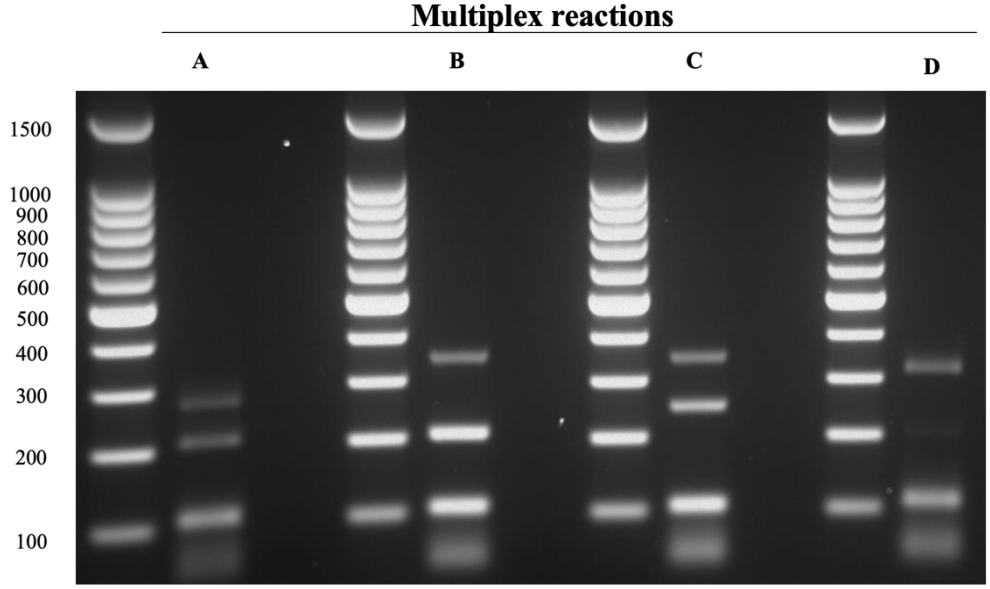

Figure 4.3 Multiplex PCR reaction to distinguish three *D. folliculorum, D. brevis* and *D. canis* using four alternative primer mixtures

Primer mixtures (A-D) are defined in Table 4.4.

Lanes, species and primers / fragment lengths (*D.b = D. brevis*, *Dbm = D. brevis, D.f = D. folliculorum*, *Dfm = D. folliculorum, D.c = D. canis*):
Ladder 100 bp.
A – *D.b 16S* 300 / ~300 bp, *D.f 16S* 200 / 200 bp, *D.c 28S* 110 / ~110 bp.
B - *D.b COI* 350 / ~350 bp, *D.f 16S* 200 / ~200 bp, *D.c 28S* 110 / ~110 bp.
C – *D.b COI* 350 / ~350 bp, *D.f COI* 250 / ~250 bp, *D.c 28S* 110 / ~110 bp.
D - *Dbm COI* 200 / ~200 bp, *Dfm COI* 320 / ~320 bp, *D.c 28S* 110 / ~110 bp.

Controls are shown in Appendix 4, figures A4.1 and A4.4. -ve controls showed no band**s.**

Dbm COI 200. Thus, the multiplex reaction was repeated using the primer mixture D with a higher concentration DNA template (2µl) (Figure 4.4).

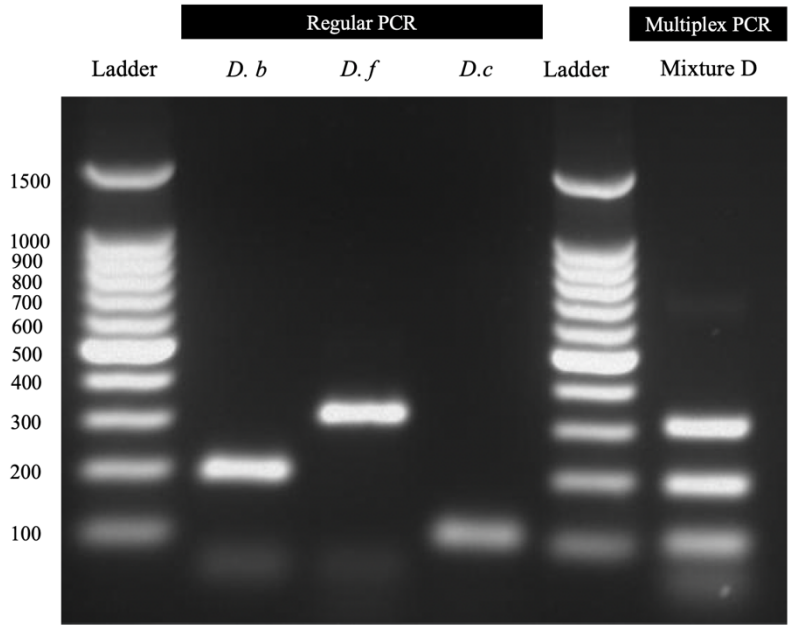

Figure 4.4 Repeated multiplex PCR assay for the primer combination: Dbm COI 200, Dfm COI 320 and Dc 28S 110.

This PCR assay consisted of *Dbm COI* 200 (for *D. brevis)* and produced a **clear** band at ~200bp. and *Dfm COI* 320 (for *D. folliculorum)* band ~320bp and *Dc 28S* 110 band ~110 bp, all as expected.

Controls are shown in Appendix 4, figures A4.1 and A4.4. -ve controls showed no band**s.**

The multiplex PCR repeated for primer combination D showed three definite bands representing the three *Demodex* species. on the gel, which were correctly positioned and clear (Figure 4.4).

4.3.3 Measuring Multiplex PCR sensitivity using low DNA concentrations

The sensitivity of the Multiplex PCR was tested by measuring the minimum concentration of DNA that could be detected in assays of progressively lower DNA concentrations from the three *Demodex* spp. Figure 4.5 shows that the multiplex PCR test

is able to distinguish the species even at low concentrations with finite bands at 0.5 ng/µl. Higher concentrations than this were obtained from a single mite. Below 0.5 ng/µl, bands are too weak for positive attribution.

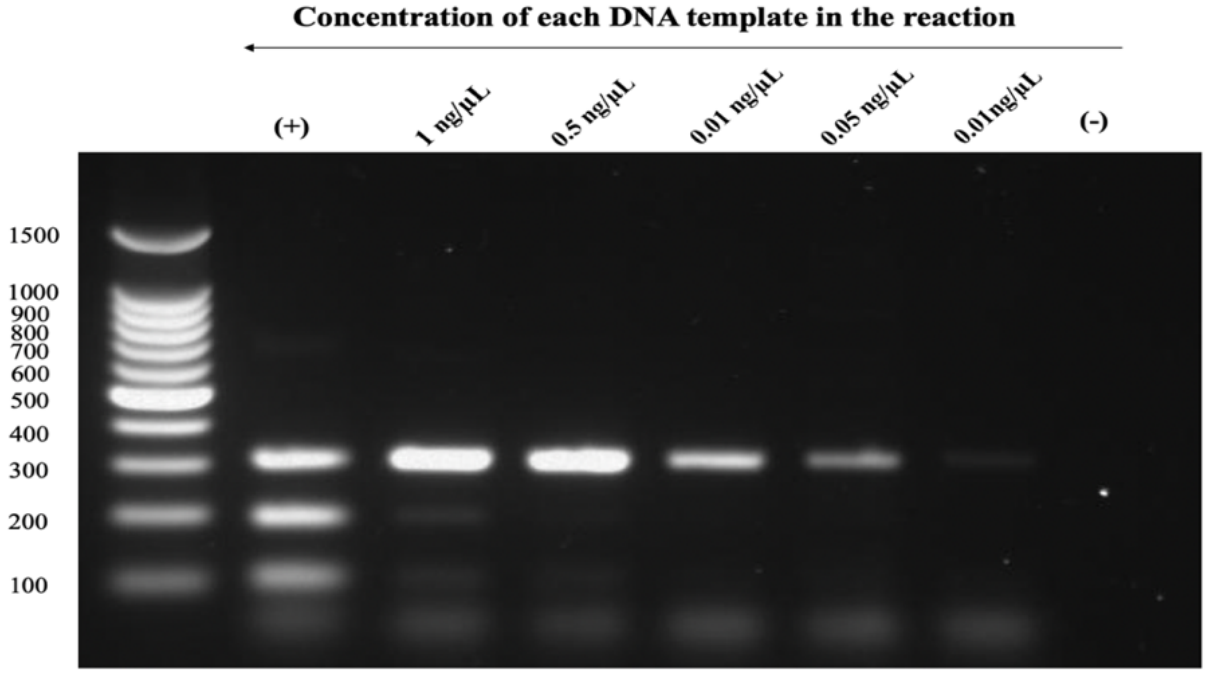

Figure 4.5 Multiplex PCR test using different DNA concentrations of *D. brevis, D. folliculorum* and *D. canis*.

Lanes, species fragment lengths (described top to bottom):

Ladder 100 bp.

(+) **Positive control**: double DNA concentrations of all three *Demodex* species

1 ng/µl: three bands are separated. First strong and bright, size ~300 bp representing *D. folliculorum* amplified by *Dfm COI* primer. Second band strong, size ~200 bp, *D. brevis*. Third band finite, size ~100 bp, *D. canis*.

0.5 ng/µl: has the same bands. First bright, *D. folliculorum.* Second and third finite, *D. brevis* and *D. canis,* respectively.

0.1 ng/µl, 0.05 ng/µl and 0.01 ng/µl: First finite to progressively weak, *D. folliculorum.* Second and third absent.

(-) Negative control: ddH_2O

PCR amplicons obtained from the human *Demodex* spp. assayed using primers for mt*COI* and *S16* rRNA genes were purified and sequenced (Eurofins Genomics, Germany), the results were subjected to a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search to find their closest related sequences in GenBank, the results are sshown in Table 4.6.

Table 4.6 Close matches for PCR sequenced fragments obtained from PCR assays, (BLAST on NCBI / February 2017)

Sequenced fragment	Description of Match	Maximum score	Total score	Query cover (%)	E value	Identical (%)	Accession No.
D. brevis 16S	*Demodex brevis* mitochondrion, complete genome	488	488	100	3e-134	99	KM114225.1
	Demodex brevis isolate Db-3 *16S* rRNA gene, partial sequence; mitochondrial	488	488	100	3e-134	99	JF783999.1
	Demodex brevis isolate Db-2 *16S* rRNA gene, partial sequence; mitochondrial	488	488	100	3e-134	99	HQ844220.1
	Demodex brevis isolate Db-S2 *16S* rRNA gene, partial sequence; mitochondrial	483	483	100	1e-132	99	KC917239.1
D. folliculorum 16S	*Demodex folliculorum* mitochondrion, complete genome	374	374	99	6e-100	99	KM114226.1
	Demodex folliculorum isolate Df-2 *16S* rRNA gene, partial sequence; mitochondrial	385	385	99	3e-103	100	HQ844221.1
	Demodex folliculorum partial mitochondrial *16S* rRNA gene, isolated from human skin	385	385	99	3e-103	100	FN424246.1
	Demodex folliculorum 16S rRNA gene, partial sequence; mitochondria	353	353	99	7e-94	98	KX151162.1
D. brevis COI	*Demodex brevis isolate 2 COI*, partial cds; mitochondrial	315	371	71	5e-82	99	KC917256.1
	Demodex brevis isolate Db-1 COI, partial cds; mitochondrial	311	367	70	7e-81	99	HQ844223.1
	Demodex brevis isolate 2 *COI,* partial cds; mitochondrial	326	382	71	1e-91	99	KC917256.1
D. folliculorum COI	*Demodex folliculorum* partial mitochondrial *COI,* isolated from human skin	468	468	98	3e-128	100	FN424248.1
	Demodex folliculorum isolate Df-4 *COI,* partial cds; mitochondrial	462	462	98	2e-126	99	JF784004.1
	Demodex folliculorum isolate Df-5 *COI*, partial cds; mitochondrial	462	462	97	5e-126	99	JF784005.1
D. canis 28S	*Demodex folliculorum isolate 3-S6D f2 28S* rRNA gene, portal sequence	396	396	94	5e-112	90.5	HQ728004.1
	Demodex canis isolate 3-S6-Dc2 28S rRNA gene, portal sequence	263	263	95	6e-72	82.6	HQ728002.1

For the purpose of minimising the size of this chapter the results for some of the trials described in Section 4.2.7 described above are not shown.

4.4 Discussion

Traditional microscope-based methods to identify *Demodex* species often require specialist morphological knowledge (Izdebska, 2004) and preparation of high-quality microscope slides is time-consuming (Zhao *et al.*, 2011). Molecular identification using *16S* rRNA as the marker was evaluated for *D. canis*, along with the human mites *D. folliculorum* and *D. brevis* but failed to resolve them adequately (Zhao and Wu, 2012). Molecular identification of these species using mtDNA genes has not been attempted but Zhao *et al.*, 2013b recommended mt*COI* as a potential molecular marker at both intra- and inter-species levels and it was investigated in this study.

Multiplex PCR protocols and primer combinations were formulated to distinguishing the two human *Demodex* species and *D. canis.* The two human *Demodex* species were discriminated by a multiplex PCR containing two sets of primers selected to amplify *16S and COI* genes (Figure 4.1). Likewise, discrimination between human *Demodex* species and *D. canis* was achieved with a multiplex PCR containing four primer mixtures (Figure 4.3 and 4.4). Despite the fact that Zhao and Wu (2012) and de Rojas et al. (2012a) found that *16S* is not a suitable molecular marker for distinguishing the two human *Demodex* species, Zhao *et al.* (2013a) later found it was effective at discriminating these species. Both Zhao and Wu (2012) and de Rojas et al. (2012a) agreed on the usefulness of *COI* as a molecular marker. Our results showed that the primers used in the multiplex PCR assay were able to amplify the target fragments (*COI* and *16S*) by using traditional PCR assay in both species with the expected lengths (Appendix 4). *Demodex brevis COI*, for

instance, was amplified using *Db COI* primer and produced a 350 bp band in all *D. brevis* amplifications.

The two human *Demodex* species. were collected from 11 ethnic groups, in this study the results (Appendix 4) showed similar bands from all the ethnic groups for both species of mite and both genes. There is little published evidence in this field, but Zhao *et al*. (2013b) compared the *COI* gene in two *D. folliculorum* populations from Spain and China and their results showed that no lineage differentiations occurred. The sequenced PCR products from this study show close similarities with some isolations of *Demodex* spp. previously sequenced and deposited on GenBank (Table 4.6). Thus, the multiplex PCR test, with the primer combination as used, can be considered a likely universal protocol for discriminating the two *Demodex* spp. from populations worldwide and may therefore be valuable in medical and research applications.

For this work, an average of two *Demodex* mites were used for DNA extractions, which is considerably less than the number that might generally be expected to be recovered in a clinical sample. For instance, *Demodex* species. in rosacea patients were found to be 5.7 times denser than in healthy individuals (Casas et al., 2012) and the mean number of mites collected per patient with a similar condition was 49.4 compared to 10.5 in the control participants (Bonnar et al., 1993). *Demodex canis* in dogs with mange averaged 13.8 mites per sample, which was much higher than in healthy dogs with just 5.3 (Fondati et al., 2010). Previous studies which have conducted multiplex PCRs on other species of mite have not reported the DNA concentrations (Gómez *et al*., 2019, Sinaie *et al*., 2018; Zélé *et al*., 2018), they may, however, have been higher than the DNA concentrations extracted in this study, taking into account the differences in size between the mites species they used and *Demodex* mites. The sensitivity of the multiplex PCR was measured

using progressively lower DNA concentrations from the three *Demodex* species. The utility of the results was limited when using low DNA concentrations, at ≤0.5 ng/µl the multiplex PCR assay was unable to detect *D. brevis* and *D. canis*, this would be equivalent to roughly 1 mite per extraction (Figure 4.5). For *D. folliculorum* the sensitivity was higher, as it was still possible to detect amplification with only 0.01 ng/µl of DNA extract.

Interpretation of the multiplex PCR test was validated against the results of single genome and primer set PCRs undertaken in this study. Therefore, this protocol and all four primer combinations are capable of being developed in the future as a tool for research or clinical studies but are also suitable for expansion. For instance, by designing specific primers for *Demodex* species that infest livestock animals such as sheep, *D. aries* (Desch, 1986), cow, *D. bovis* (Stiles, 1892) and horse, *D. equi* (Owen, 1843) and adding them to the multiplex PCR test. More than one species of mite is known from dogs (including *D. injai*, and *D. cornei*) and they may also be implicated in diseases, such as mange (Izdebska and Fryderyk, 2011), our results, may be capable, with the addition of suitable primers, to be used to better understand the biology of dog mites and its applicability to human disease.

In conclusion, the multiplexed primers selected and combined in these experiments are highly species-specific and efficiently amplified target fragments in the three *Demodex* spp. in this study. Thus, the test can easily be turned into a real-time PCR (automated test) by designing probes for each set of primers. With just a single PCR reaction, clinical practitioners may, therefore, be able to clearly diagnose the mites that may be responsible for dermatologic diseases such as rosacea, blepharitis and gland dysfunctions. They may be able to confirm whether these diseases were caused by *Demodex* mites or not, and if so, to precisely identify which of the *Demodex* species (*D. folliculorum, D. brevis* and *D. canis*) is involved in causing the skin disorder in their patients.

Chapter 5: Genomic DNA extraction from human *Demodex* mites

5.1 Introduction

Isolating a sufficient quantity of genomic DNA (gDNA) is a crucial step for high-quality, whole genome sequencing. The nature of the organism under study may, in some cases, compromise the ability to extract sufficient quantity of gDNA (Gabaldón and Alioto, 2016; Kresse *et al.*, 2018). Difficulties in extracting sufficient gDNA from mites such as *Demodex brevis*, the subject of this study, may have various causes, including: human *Demodex* mites have not been successfully reared *in vitro* (Desch and Nutting, 1972; Carly and Dirk, 2014) and, they are generally only collected from humans opportunistically in very small numbers. Collection rates are often compromised by the invasiveness of the methods and the general unwillingness of trial participants to cooperate, given the subject matter. As *D. brevis* lives deep inside the sebaceous gland duct (Akbulatova, 1963) only small numbers of mites are usually collected when sampling a single host individual (Rufli and Mumcuoglu, 1981), using the widely applied methods. Further, it is not always beneficial to collect and store mites, as obtaining sufficient quantity of high quality, undegraded gDNA yield from preserved samples presents significant challenges, including chemical damage to the DNA, especially with samples fixed for a long time in harsh conditions (for example stored at room temperature for long periods), compared to fresh or frozen samples (Hedegaard *et al.*, 2014). In addition, *Demodex* mites are microscopic organisms, therefore, only small amounts of gDNA (less than a nanogram) are recovered from single mites by typical extraction methods (Richards and Murali, 2015). Building a library of a homozygotic genome for whole genome sequencing requires a great number of *D. brevis* collected from a single

individual host (Ekblom and Wolf, 2014; Richards and Murali, 2015). To achieve this, a resampling process must generally be performed, and the collected mites preserved, usually in ethanol, until sufficient are collected to undertake the gDNA extraction.

For accurate and reliable whole genome sequencing results, most companies who do this task require extracted gDNA of sufficient quantity, the DNA must not be degraded, it must be pure, double-stranded, free of contaminants and must be highly concentrated (Gabaldón and Alioto, 2016). Pure and long-length DNA fragments are also required, which means removing contaminants such as enzymes or other residues. To achieve these requirements, a series of precise and specific molecular protocols are involved in gDNA extraction, amplification, purification and quantification (Ekblom and Wolf, 2014; Biezuner *et al.*, 2017). Each of these steps can be achieved with several commercially available kits combined with widely available quantification techniques (Biezuner *et al.*, 2017).

Only a few drafts of the whole genome of Acari have been sequenced, the most complete being *Tetranychus urticae* (Grbic *et al.*, 2011), *Dermatophagoides farinae* (Chan *et al.*, 2015) and *Metaseiulus occidentalis* (Hoy *et al.*, 2016). These species of mite can all be reared in the laboratory and, therefore, sufficient amount of gDNA easily obtained (Grbic *et al.*, 2011; Chan *et al.*, 2015; Hoy *et al.*, 2016).

Isolating and sequencing eukaryotic mitochondrial DNA (mtDNA) is much easier than nuclear DNA (Robin and Wong, 1988; Smith, 2015). This is because it is shorter, many copies of mtDNA are found in a single cell and, it is also easy to amplify, due to its abundance, by PCR (Sanger sequencing) (Borlado *et al.*, 2010). Modern technologies such as Next Generation Sequencing (NGS) makes whole genome sequencing much easier, cheaper, and quicker while delivering accurate mtDNA sequences (Smith, 2012).

Demodex folliculorum and *D. brevis* mtDNA has been sequenced and is available in GenBank (accession numbers KM114225 and KM114226, respectively) (Palopoli *et al.*, 2014). This explains the great number of recently published molecular studies that have been conducted using *Demodex* mtDNA genes (particularly *16S*, *COI* and *28S*), such as Zhao and Wu (2012), de Rojas *et al.* (2012a), Zhao *et al.* (2013b), Hu *et al.* (2014) and Palopoli *et al.* (2015). This study has taken advantage of the available mtDNA gene sequences which were used to develop a multiplex PCR to discriminate the two human *Demodex* spp. (Chapter 4, and under submission in JAMA Dermatology).

The principal objectives of this Chapter were as follows.

- To compare a variety of protocols to extract of DNA from *D. brevis*.
- To obtain sufficient high-quality genomic DNA from *D. brevis* for whole genome sequencing.
- To determine which DNA extraction methods can be considered the best approach for extraction of gDNA from *Demodex* mites.
- To compare the commercial kits available for DNA purification, as well as the DNA quantification methods, that can yield the required amount of gDNA of *D. brevis*.

5.2 Methods and materials

5.2.1 *Demodex brevis* samples

Demodex brevis was collected from participants (aged 18 to 60) using cellophane tape placed overnight on the nose and forehead (Izdebska, 2009; Ozdemir *et al.*, 2005; Ozdemir *et al.*, 2003). The identity of the mites as *D. brevis* was confirmed by Dr. MA. Perotti, University of Reading, Reading, UK. Only adult mites were recovered from the collections for genomic DNA (gDNA) extraction, they were transferred directly by a

sterile pin into an Eppendorf tube containing either ethanol or a lysing buffer, depending on the experiment. Samples of at least 10 mites of *D. brevis* were used for the experiments and hosts were resampled in some cases to achieve sufficient mites for single-host experiments.

5.2.2 Experimental design and workflow

The following major steps (Figure 5.1) were followed to extract and purify the gDNA from *D. brevis,* in all three experiments, using different proprietary extraction kits and samples. Samples were kept in the final buffer solution in in 1.5 ml Eppendorf tubes between the steps. In some experiments, quantification was performed after both the extraction and amplification steps.

To determine the best genomic DNA preparation technique, different methods and materials were tested in 3 experimental workflows, all following the generalised model in Figure 5.1. For example, the source of samples was either fresh or preserved in fixative for a long period, two gDNA extraction kits were used, three different proprietary kits were used to purify the DNA and different methods were used for quantifying the DNA. Table 5.1 shows the alternative methods and materials compared at each step of the workflow, for each of the 3 experiments. The objective of this Chapter was to compare proprietary methods for extraction and purification; hence amplification was conducted using a tried and previously successful method.

PCR has been used to amplify genomic DNA extracted from both eukaryotic and prokaryotic genomes by using special primers sets. However, in this chapter, TruePrime WGA (SYGNIS, Heidelberg, Germany) was selected for amplification, because it has been used successfully previously, to amplify gDNA from *D. folliculorum* (Dr. M.A. Perotti, University of Reading, Reading, UK. Unpublished study), furthermore, this kit is

relatively cost-effective while producing good quality results. For the other key steps in the workflow, the proprietary kits for the purpose were used so far as possible according to the manufacturer's instructions in order to achieve comparable results (Table 5.1). Where it was necessary for operational reasons, generally relating to the nature of the source material, to vary a protocol, this is also recorded. The non-proprietary steps, such as sampling are described above, with experiment-specific variations shown in the table, and quality visualisation was by gel electrophoresis as described below or the widely used Agilent TapeStation proprietary system which provides a more detailed output.

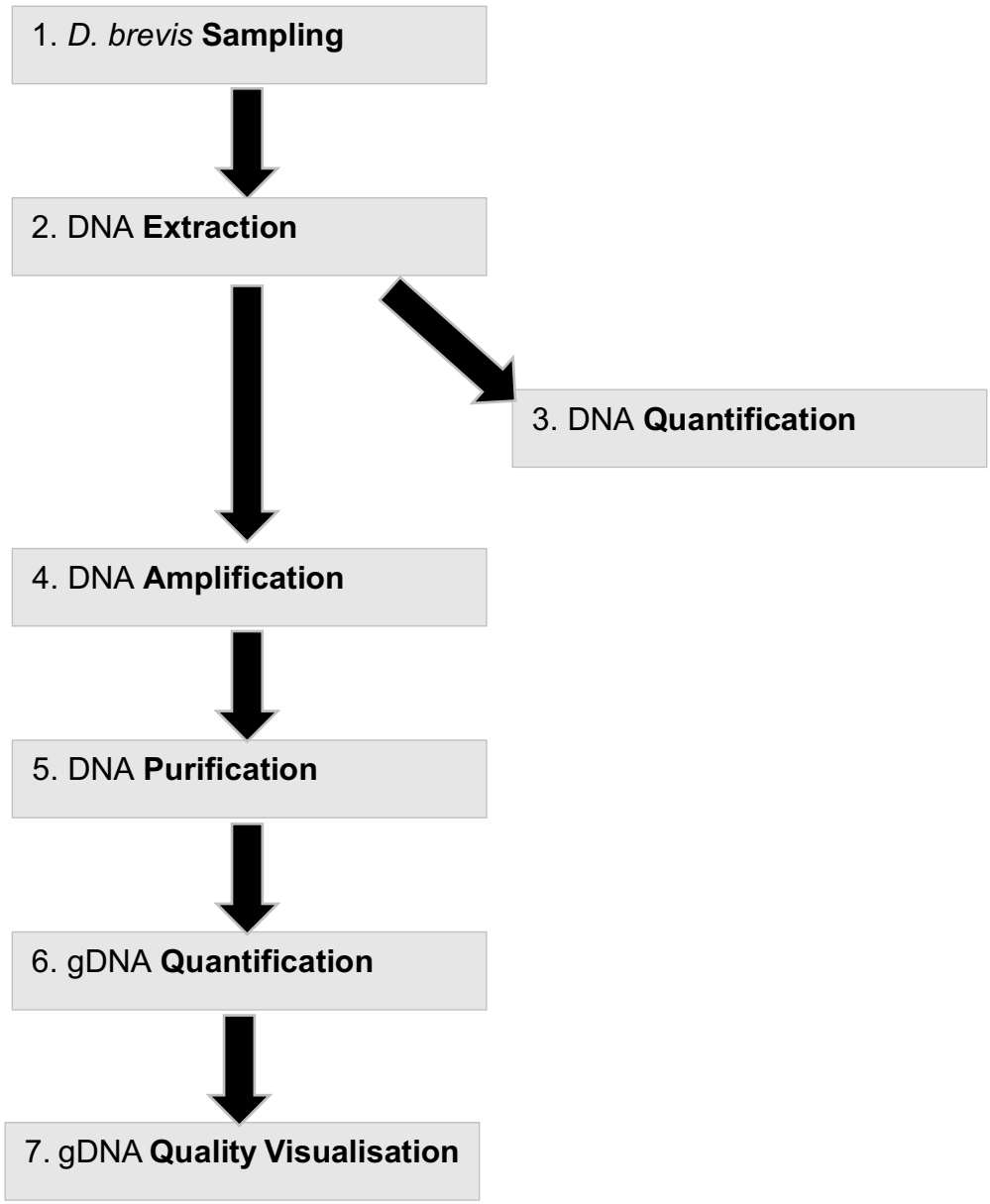

Figure 5.1 Generalised experimental workflow – Genomic DNA extraction steps

5.2.3 DNA Quality visualisations

Gel electrophoresis. The DNA quality was evaluated by separating gDNA samples by gel electrophoresis using 1 % agarose gel + 5µL Ethidium Bromide, run for 30 minutes at 80 volts and a transilluminator (Benchtop UV) was used for the visual inspection. The sample in each lane was 5µL, comprising 2 µL of purified gDNA, 2 µL ddH_2O and 1.8

μL (PROMIGA, M7112). Each set of samples were separated twice on the same gel and 100 bp ladder has been used for comparison.

Spectrophotometry and Fluorometry. Quantification and quality measurement were conducted using a DeNovix (model: DS-11 FX) in standard spectrophotometry and fluorometry modes. DeNovix Fluorescence Broad Range required the addition of a dye to the DNA product. The manufacturer's recommended protocol was followed to achieve this.

Table 5.1 Methodological variations which define Experiments 1-3

The manufacturer's instructions were followed precisely for each kit used, except where indicated in green.

Steps in sequence	Experiment 1	Experiment 2	Experiment 3
Samples (sample no., mites, preservation medium, preservation period) Host details	Preserved 1. 10 mites, 95 % ethanol, 3 mths 2. 23 mites, 95 % ethanol, 10 mths 3. 28 mites, 95 % ethanol, 10 mths All mixed hosts	Fresh 1. 20 mites, 95 % ethanol, 2 weeks Single host	Fresh 1. 13 mites, 95 % ethanol, 1 week (Fresh) 2. 25 mites, lysing buffer (Fresh) Single host (for both samples)
Extraction	DNeasy Blood and tissue kit (QIAGEN 69505) Mite cells disrupted by sonic bath (DELTA model: GT-7810A). Elution step: repeated twice with 30 µl and 20 µl buffer to enhance DNA yield.	QIAamp DNA Micro kit (QIAGEN 56304) Before extraction, alcohol evaporated, 0.5 % bleach added to remove contaminant bacteria, human cells or sebum. Lysing step: sample incubated for 3 days at 56 ˚C, to ensure complete lysing. Elution step: repeated twice, samples incubated at 70 ˚C for 5 min.	QIAamp DNA Micro kit (QIAGEN 56304) Elution step: repeated twice, samples incubated at 70 ˚C for 5 min.
Quantification (interim)	DeNovix (DS-11 FX) Spectrophotometry Agilent 2200 TapeStation (Genomic DNA ScreenTape)	DeNovix (DS-11 FX) Spectrophotometry Agilent 2200 TapeStation (Genomic DNA ScreenTape)	Agilent 2200 TapeStation (Genomic DNA ScreenTape)
Subsampling	Each extracted DNA sample was divided into 2 equal subsamples which were amplified separately	The sample was divided into 2 equal subsamples which were amplified separately	N/A
Amplification	TruePrime WGA kit (SYGIS)	TruePrime WGA kit (SYGIS) ddH_2O was not added in the final step.	TruePrime WGA kit (SYGIS)

Table 5.1 Continued			
Steps in sequence	**Experiment 1**	**Experiment 2**	**Experiment 3**
Subsampling	N/A	N/A	The 2 amplified samples were combined, then 3 equal mixed subsamples created.
Purification	Macherey-Nagel genomic DNA clean-up kit (NucleoSpin, 740609.10)	Macherey-Nagel genomic DNA clean-up kit (NucleoSpin, 740609.10)	*Method 1* Macherey-Nagel genomic DNA clean-up kit (NucleoSpin, 740609.10) Incubated at 70 ˚C for 10 minutes in 75 µl elution buffer. *Method 2* Zymo DNA Clean & Concentrator (D4003T) Elution step: 30 µl of buffer incubated at 70 ˚C for 10 min. *Method 3* AMPure Beads XP (A63380) Elution step: 30 µl of buffer incubated at 70 ˚C for 10 min.
Quantification (final) and quality visualisation	DeNovix (DS-11 FX) 1. Spectrophotometry 2. Fluorometry Agilent 2200 TapeStation (Genomic DNA ScreenTape) Gel electrophoresis	DeNovix (DS-11 FX) Spectrophotometry Agilent 2200 TapeStation (Genomic DNA ScreenTape) Gel electrophoresis	Agilent 2200 TapeStation (Genomic DNA ScreenTape)

5.3 Results

5.3.1 Experiment 1 - DNeasy Blood and tissue kit (QIAGEN 69505)

After the extraction step, spectrophotometry on standard instrument settings indicated higher concentrations of gDNA in samples 1 and 2 compared to sample 3 (Table 5.2).

Before purification, each sample was divided into two subsamples for comparison. The concentration of purified gDNA was measured for each sample using standard instrument settings for spectrophotometry and fluorometry. The results showed that the concentration of the purified gDNA varied but that it was more similar in samples 1 and 2, quantified by both methods, but lower than both 1 and 2 in sample 3 (Table 5.2). Similarly, the concentration of extracted DNA was lower in sample 3. The differences between the subsamples were larger in sample 3.

Purification led to higher gDNA content in all 3 samples. Measured by spectrophotometry, the gDNA concentration appears higher than was found with fluorometry in all 3 samples.

Table 5.2 Experiment 1 - Concentration of DNA after extraction and after purification

Sample	**Genomic DNA concentration**						
	After Extraction (ng/µL)	**After Purification (ng/µL)**					
	Spectrophotometry				**Fluorometry**		
		Subsample 1	*Subsample 2*	*Total*	*Subsample 1*	*Subsample 2*	*Total*
1	2.62	153	132	285	138	104	242
2	2.60	124	116	240	108	104	212
3	1.85	136	66	202	95	28	123

The quality of the purified gDNA was evaluated by gel electrophoresis. Two gel tests were conducted for each sample. The DNA was highly degraded in all 3 samples, in both tests, as

shown by the failure to form a clear fragment band in any column of the gel (Figure 5.2), which instead shows clear smearing.

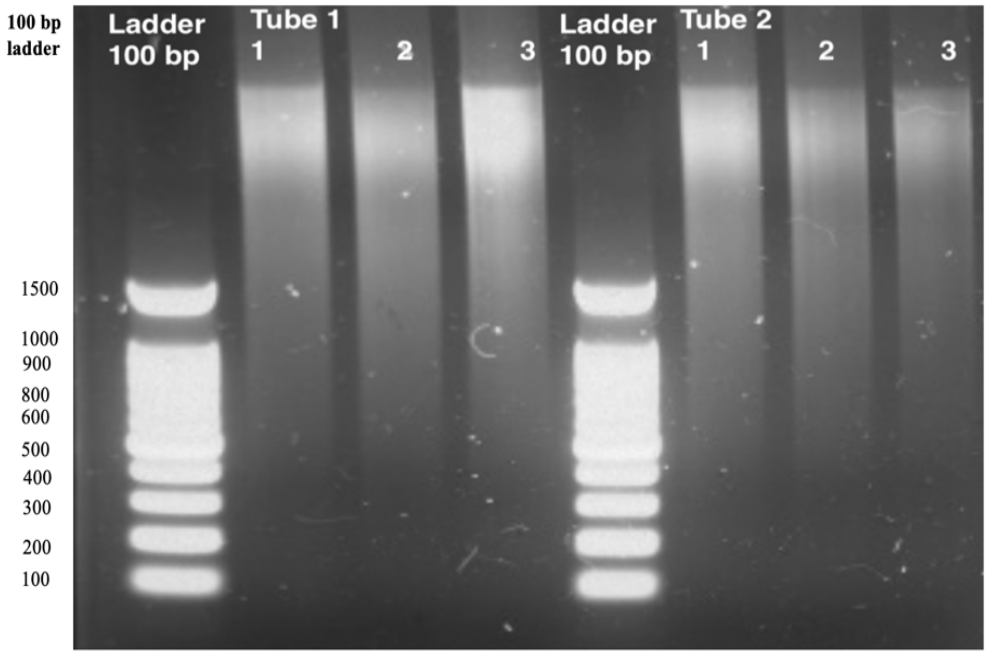

Figure 5.2 Experiment 1 - quality of gDNA after purification in all 3 samples

Tube 1 = subsample 1; Tube 2 = subsample 2. Columns labelled 1-3 = samples 1-3. There is no clear band in any sample or in either subsample separation, so the genetic materials are of different sizes.

5.3.2 Experiment 2 - QIAamp DNA Micro kit (QIAGEN 56304)

The concentration of the gDNA was measured after extraction and after purification. The concentration of the gDNA was 1.62 ng/µL measured by spectrophotometry after extraction only. Two subsamples of the purified gDNA were quantified using the same method, the concentrations were 58 ng/µL and 64 ng/µL. The quality of the purified gDNA from the two subsamples was also evaluated using Agilent TapeStation electropherogram curves, to determine fragment sizes in more detail, and gel electrophoresis. Electropherogram curves from TapeStation showed subsample 1 has various sizes of DNA fragments, ranging from ~15 bp to ~39 bp at the lower scale to ~1400 bp to ~1600 bp at the upper end of the scale. Subsample 2, compared to subsample 1, has more long fragments at ~1100 bp to ~1700 bp and less short fragments at ~23 bp to ~27 bp) (Figure 5.3).

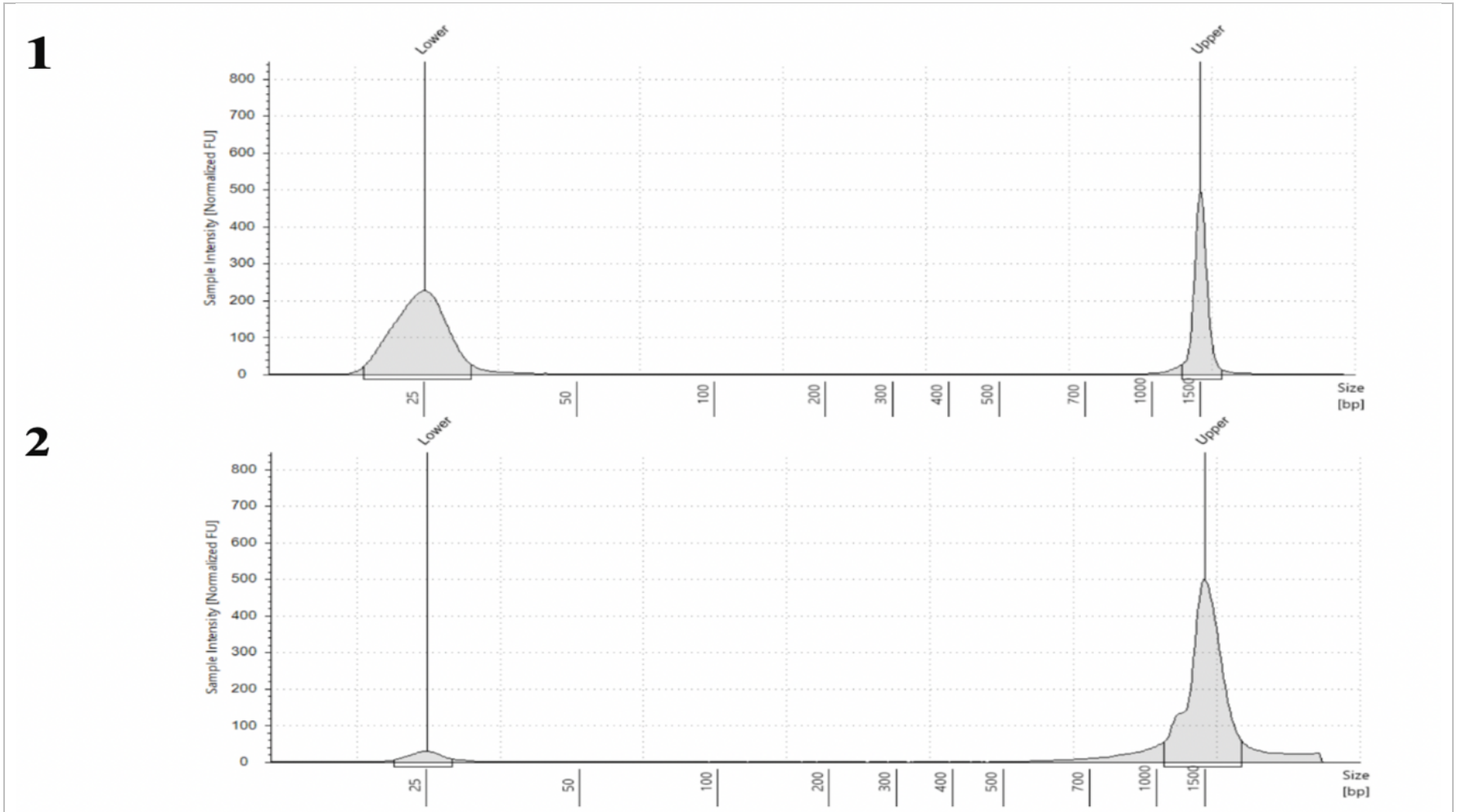

Figure 5.3 Experiment 2 - The quality of purified gDNA shown by Electropherogram curves.

The figure shows the upper and the lower sizes of the fragments in samples 1 and 2. gDNA of both was highly degraded.

The quality of the sample of purified gDNA was evaluated by two gel electrophoresis separations. There was an absence of intact fragments in either column of the gel as shown by high degrees of smearing in both, so the gDNA was highly degraded (Figure 5.4).

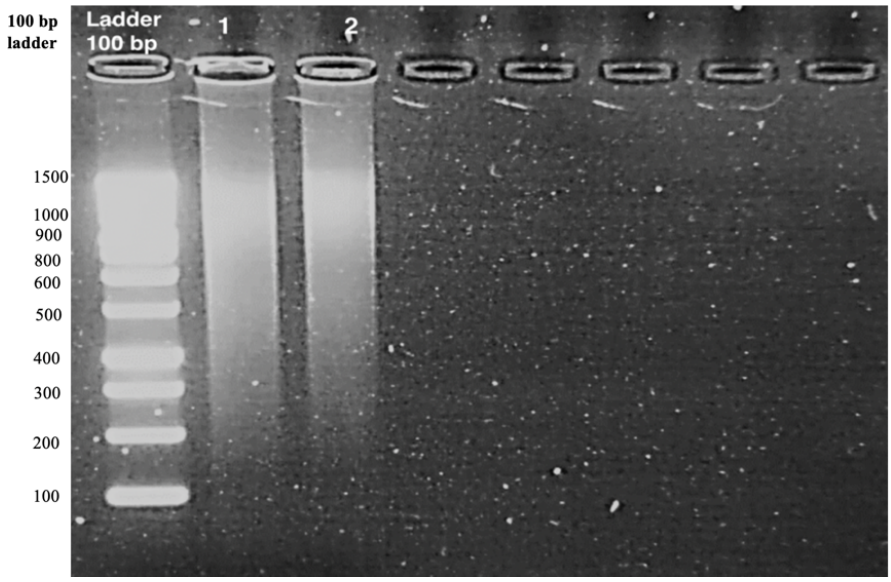

Figure 5.4 Experiment 2 - quality of gDNA after purification.

Column 1 – subsample 1; 2 – subsample 2. There were no clear bands in either subsample separation, therefore, the sample only contains degraded genetic material, of different sizes.

5.3.3 Experiment 3 - Comparison of protocols

One combined sample of differing preservations, containing DNA from 38 mites collected from a single participant, was created by merging two separately extracted samples before amplification. The amplified DNA was divided into three subsamples to test the efficiency of three different purification kits.

Subsamples of the amplified DNA extracted in this and the two previous experiments were quantified using Agilent Genomic DNA ScreenTape assay, to determine and compare DIN (DNA integrity number, as generated by Agilent). DIN represents DNA quality and is measured on a scale of 1-10, 10 being the highest. Quantity ranged from 1.03 ng/µL to 1.68 ng/µL (Figure 5.5). The QIAamp DNA Micro kit extracted more gDNA (1.68 ng/µL) from *D. brevis* (fresh and preserved combined sample, Experiment 3) compared to DNeasy Blood and tissue (preserved samples, Experiments 1 and 2).

However, the concentration of the gDNA extracted by the QIAamp DNA Micro and the DNeasy Blood and tissue kits is approximately the same from all preserved samples.

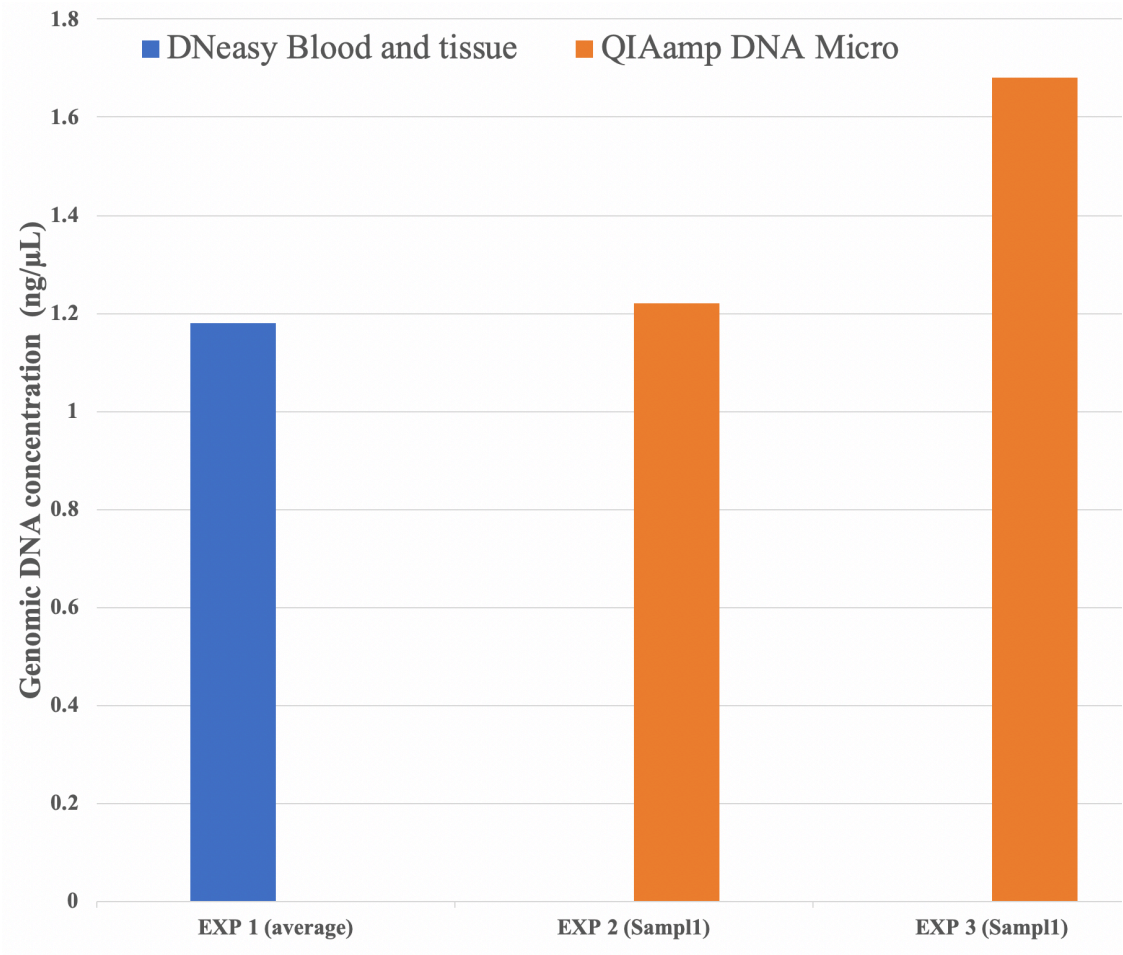

Figure 5.5 Concentrations of gDNA obtained following extraction using two different proprietary kits and study samples preserved for different periods or fresh.

Experiment 1 - combined results - the average gDNA concentration from samples preserved for 3 mths; 10 mths and for 10 mths with an increased DNA template. Experiment 2 - samples preserved 1 - 2 wks. Experiment 3 - combined fresh samples from ethanol and lysing buffers. Exp = Experiment; Sampl = Sample.

One kit only was used for Experiments 1 and 2, but all 3 samples from Experiment 1 were purified (Figure 5.6). The Macherey-Nagel genomic DNA clean-up kit was used to purify samples from all three Experiments. The results ranged from ~71 ng/µL in Experiment 1, Sample (3 mths preservation) down to ~24 ng/µL in Experiment 2 (2 weeks preservation). All three proprietary kits were used to purify the amplified DNA from Experiment 3, The quantity ranged from ~87 ng/µL (Zymo DNA Clean & Concentrator kit) down to ~50

ng/µL (Macherey-Nagel genomic DNA clean-up kit). The former kit was used to purify the amplified fresh DNA sample from Experiment 3 only, and the DNA concentration was also higher than the DNA concentration purified by the AMPure Beads XP kit for the same Experiment and sample.

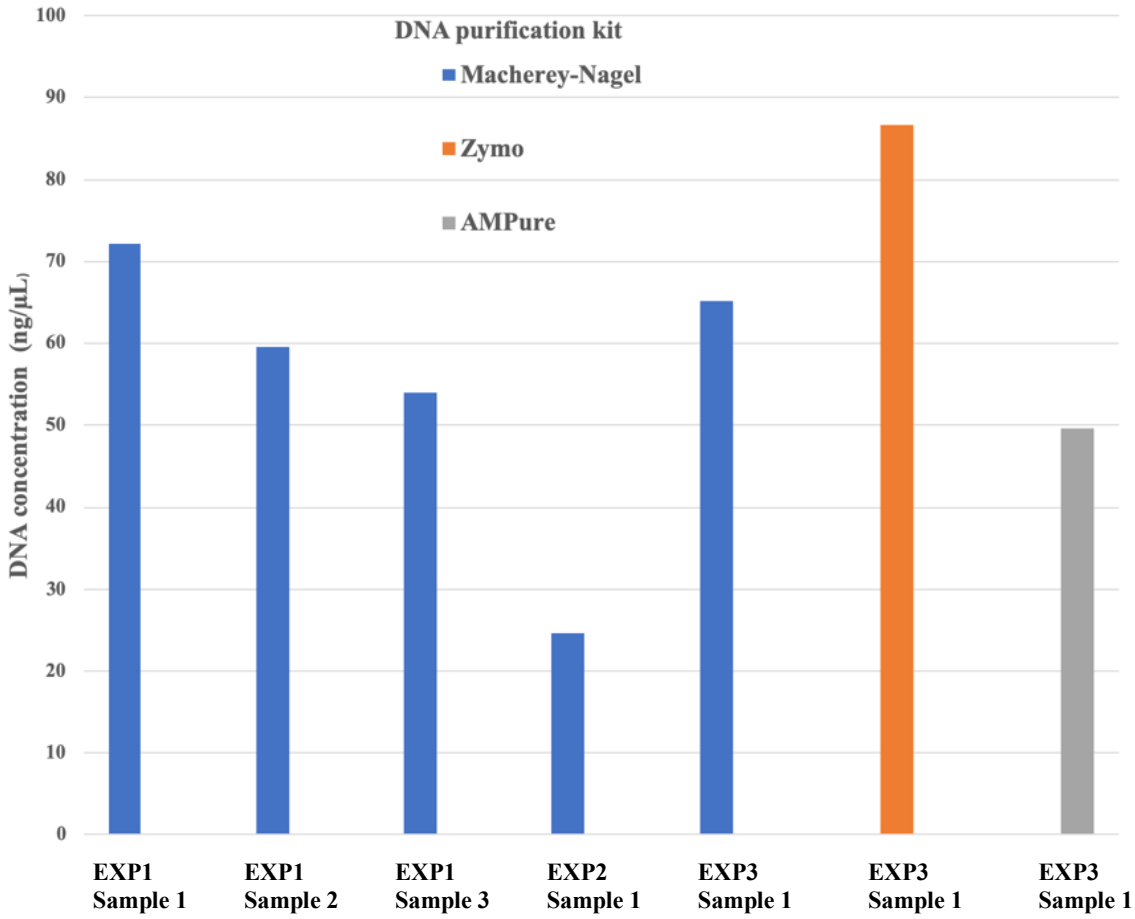

Figure 5.6 The quantity of purified DNA from all Experiments and samples purified by different kits.

Experiment 1 (EXP1) preserved samples: 1 - 3 mths; 2 10 mths; 3 – 10 mths (higher DNA); Experiment 2 (EXP2) preserved: 1 - 2 wks; Experiment 3 (EXP3) fresh: 1 - ethanol and lysate combined.

The quality of the extracted gDNA was evaluated with the Agilent Genomic DNA ScreenTape system (Figure 5.7). DIN from all extracted gDNA samples (lanes A, B, C, G, I) was below the measurable range for the special gel separation, and the system was unable to calculate DIN in any. While the purified DNA quality value for all purified

samples was detectable, ranging from DIN 5.5 to 6.2 (lanes D, E, F, H, G, K, M) with lower DIN representing more highly degraded DNA. The quality of the DNA was lower than expected, as shown on the ScreenTape gel image as a smear in all samples, except M.

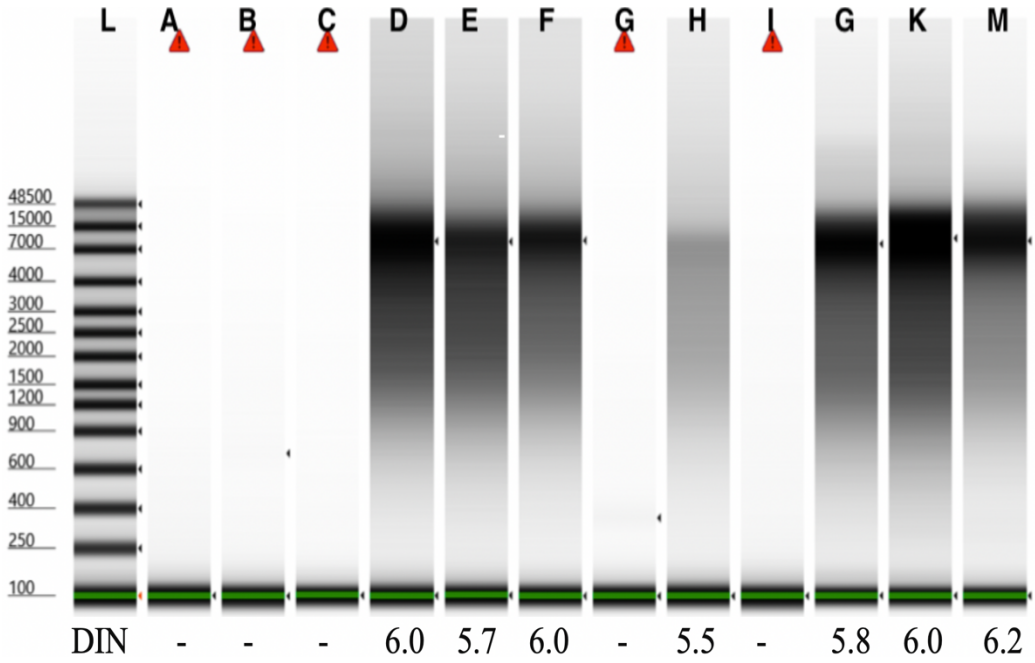

Figure 5.7 gDNA quality evaluation from extracted and purified samples using Agilent Genomic DNA ScreenTape visualisation.

The gel separation image shows DIN values below each column. gDNA is highly degraded in all extracted samples.

Experiment 1 samples: lanes A, B, C extracted; D, E, F purified gDNA from 3 samples.

Experiment 2 samples: lane G extracted; H purified gDNA.

Experiment 3 samples: lane I extracted; G, K, M purified gDNA using 3 kits.

5.4 Discussion

Adequate quality of extracted genomic DNA (gDNA) is essential for successful analysis by high-throughput DNA sequencing (Kresse *et al.*, 2018). Obtaining high quality gDNA yield from preserved samples presents significant challenges, such as chemical damage to the DNA, especially with samples fixed for a long time in harsh conditions (for example stored at room temperature for long periods), compared to fresh or frozen samples (Hedegaard *et al.*, 2014). In this study, comparisons were made using different

nucleic acid extraction kits, purification kits and quantification methods. DNA quantity and quality were the benchmarks for the performance of the methods and materials.

Two different commercial extraction kits were compared to isolate *D. brevis* gDNA from preserved and fresh samples. QIAamp DNA Mico kit (QIAamp) was used in two experiments and the concentration of gDNA obtained by this kit was 1.22 ng/µL from a preserved sample (Experiment 2, 20 mites) and 1.63 ng/µL from a fresh sample (Experiment 3, 38 mites). Less than 1 ng/µL of DNA is typically recovered from single mites (Richards and Murali, 2015), so our results are considerably below some published results and DNeasy Blood and tissue (DNeasy) produced similar results, from preserved mites, maximum gDNA was 1.3 ng/µL (Experiment 1, 10-28 mites per sample).

As the gDNA quantity was considerably below the expected limits for an organism such as *D. brevis*, it was not enough to build a solid DNA library. Building a library of a homozygotic genome for whole genome sequencing requires a great number of *D. brevis* collected from a single individual host (Ekblom and Wolf, 2014; Richards and Murali, 2015) and high-quality DNA (for example, Keats *et al.*, 2018). Very few studies have published the results of mite DNA purity following extractions, as this is usually only a step towards a final goal, or if they have, they do not use concentration as a measure of quality/quantity, often the A260/A280 ratio is preferred (for example Per and Ercan, 2015). Desloire *et al*. (2006) measured 20.5 ng/µL DNA measured with a biophotometer (Eppendorf, Hamburg, Germany) (spectrophotometry), following extraction using QIAamp from a single mite of *Dermanyssus gallinae,* an ectoparasite of poultry, which had previously been frozen. In a recent study comparing DNA extraction techniques using orabitid mites Lienhard and Schäffer (2019) were also unable to recover gDNA of sufficient concentration for sequencing from single mite samples, and also, therefore, found that no bands were displayed by a TapeStation analysis. Their mites were not fresh,

having been preserved for "several weeks" (Lienhard and Schäffer, 2019, p. 3) in either ethanol or propylene glycol, and so were comparable to ours. They used QIAamp and, measured by Nanodrop spectrophotometry and Quantifluor fluoroscopy, achieved recovery of 1.83 to 3.5 ng/μL and 0.053, to 0.969 ng/μL, respectively, their smallest mite species, *Tectocepheus* sp. produced the lowest DNA concentrations, and is similar in size to human *Demodex* mites at 300-350 μm. They further note that their photometers were operating at the lower limits of detection in making these readings. Overall, compared to the other six extraction kits and protocols tested in their study, QIAamp was outperformed by one, a modified Chelex resin protocol, however, it produced most DNA for the smallest mite, *Tectocepheus* sp. While, therefore, Desloire *et al*. (2006) exceeded our levels of DNA concentration considerably and they used single mite samples, Lienhard and Schäffer (2019) only matched our results, albeit also from single mites. Given our samples contained 20-38 mites, our extraction was very low, ~10× lower than Lienhard and Schäffer (2019) (based on multiplying their result by 30) and similar for DNeasy. Other authors, such as Zhao *et al*. (2012), have extracted similar amounts of DNA to us, they achieved equivalent to149.2 ng/μl from 1500 *D. folliculorum* mites, or 0.01 μg per mite, however, they're objective was single gene PCR sequencing, not gDNA preparation, therefore, this result was adequate. The quality of gDNA can also be checked using a PCR with well-tried primers selected to produce amplicons of known size, such as ITS2, followed by gel electrophoresis, this is a common approach.

Tectocepheus sp. are probably larger in volume than *Demodex*, since they are generally subrotund, which may mean they contain more DNA, Zhao *et al*. (2012) compared similar shaped mites of *Sarcoptes*, an ectoparasite of horses, and concluded, that although they were similar in length at 300–500 μm (females) to *D. folliculorum, Demodex* were "remarkably smaller" (Zhao *et al*., 2012, p. 48). However, size alone is unlikely to account for the entire differences in DNA recovery.

Corriveau *et al*. (2010) noted that although temperature and storage period had little effect on DNA recovery, a lysis buffer, such as we used for storage of one sample in Experiment 3, and for all samples during both the QIAamp and DNeasy kit extractions, resulted in poor recovery. However, although this effect may have been experienced in other research which used these kits, it is not necessarily apparent from their results, for example Leinhard and Schäffer (2019).

There was no great difference between DNA quality as measured by Agilent TapeStation (TapeStation) DIN of ethanol-preserved samples and fresh samples (Figure 5.6). Corriveau *et al*. (2010) found that even longer storage periods, up to 8 months, had little effect on DNA quality of *Balaustium* sp. mites, and several authors report using mites after periods of preservation without attributing any loss in DNA quantities to this method (for example, Leinhard and Schäffer, 2019), therefore, it is unlikely that storage adversely affected our DNA quality and it is reasonable, in future, to use this method where necessary.

Although the Zymo DNA Clean & Concentrator kit (Zymo) purified DNA with the highest concentration of the three purification kits (86.6 ng/µL), DIN was 6.0, lower than DIN 6.2 reported by the TapeStation system for the DNA amplified by AMPure Beads XP kit, 49.6 ng/µL (AMPure). Macherey-Nagel genomic DNA clean-up kit (M-N) amplified DNA from the same fresh sample in Experiment 3, the DNA concentration was 65.2 ng/µL but the DIN was 5.8, lower than the other two purification kits (Figure 5.6). All of these values are <7, the threshold for DNA quality recommended to progress to whole genome sequencing on common platforms such as Illumina (Keats *et al*., 2018).

The comparison between quantification methods in this study showed that spectrophotometric methods measured high gDNA concentration in all three samples in Experiment 1 (Table 5.2) compared to the concentrations of the same samples by the

TapeStation system (Figure 5.7 lanes A, B, C). DNA integrity was evaluated by both the ubiquitous gel electrophoresis method and TapeStation system. Many authors solely rely on gel electrophoresis, to evaluate the results of DNA extractions, for example Desloire *et al*. (2006) and many others, however an end goal such as PCR amplification, as used in their case, and sequencing, constitute more robust validation of the DNA qualities. Both methods showed low intactness of DNA and no clear fragments from the samples in this study, which means they are both likely to be reliable methods for evaluating DNA quality. However, the TapeStation system is more convenient, less laborious and produces DIN values for decisive evaluation of the results. An automated system such as TapeStation system has a very low error rate, thus, its results compare favourably to gel electrophoresis, since it produces quantified results rather than relying on visual comparisons with a ladder, which can be inaccurate particularly if bands are not crisply defined. A comparison of spectrophotometry and fluoroscopy was carried out by Leinhard and Schäffer (2019) to analyse mite DNA, who found higher concentrations from equivalent samples were measured by the former, using a Nanodrop instrument but questioned the accuracy of all their readings, given the limitations of this instrument at such low concentrations of DNA as they found. As we also found such low concentrations of DNA, we are unable to draw conclusions about the differences in these two quantification methods, for the reasons given by Leinhard and Schäffer (2019).

The weight of a single nucleotide base pair, on average, is 650 Daltons (1 Dalton (Da) = weight of one Hydrogen atom = 1.67×10^{-24} g. Given the human genome is 3.3×10^{9} bp in length, the molecular weight of a DS-DNA molecule is the number of bases pairs $\times$ 650 Da. Thus, the weight of the human genome is $\approx 3.3 \times 10^{9}$ bp $\times$ 650 Da $\approx 2.15 \times 10^{12}$ Da. Therefore, human genome weighs $2.15 \times 10^{12} \times 1.67 \times 10^{-24} \approx 3.59 \times 10^{-12}$ grams. If the human body has 100 trillion cells that means each cell has $3.59 \times 10^{-12} \times$

$2 \times 10^{12} = 7.2$ pg of Genomic DNA. Integrated and Technology (2011). If this estimation is correct, and one mite has $\pm 16 \times 10^3$ cells then ~128 pg genomic DNA might be extracted from a single *D. brevis*, which is equal to 0.128 µg, a very small amount of gDNA. Therefore, a large number of *D. brevis* must be processed to produce enough DNA to sequence the whole genome, Eurofins, for example, require a minimum of least 10 µg of double-stranded, purified DNA of concentration ~80 ng/µl (Eurofins Genomics, https://www.eurofinsgenomics.eu/en/eurofins-genomics/product -faqs/next-generation-sequencing/, accessed 15-09-2019) which is 100× the generalised calculation for a *Demodex* mite. The collection methods employed for this study, which could not include re-sampling of participants because of the participant's sensibilities, are likely, therefore, to have been a significant factor in our inability to collect sufficient DNA.

In conclusion, the quantity of the gDNA obtained from *D. brevis* in this study was in the expected range with different extraction kits, and comparable to some published studies. However, the quantity and quality of the DNA was below the requirements for most gDNA sequencing approaches, such as commercial sequencing by companies including Eurofins, Novogen and GeneDx; there are several potential reasons that may account for this, however, clearly it is an issue that is commonly encountered with mite DNA extraction and purification. This is evidenced by the low DIN for the obtained DNA in all the samples, because the DNA was degraded in small fragments and the low concentrations of DNA throughout. However, many studies clearly use DNA of similar concentrations and quality to achieve other goals, notably, successful PCRs for identification and phylogenetic studies. Genomic DNA extracted from a sufficient number of *D. brevis* preserved in alcohol at -20 ˚C or using liquid nitrogen to improve the rupturing of the mites' chitin exoskeletons should be considered as either might result in higher yields of gDNA with higher quality.

Chapter 6: Novel Growth Media for rearing *D. folliculorum* mites *in vitro*

6.1 Introduction

To study human *Demodex* mites, to date the only source of samples has been a human host (Zhao *et al.*, 2011). In practice, sampling human mites has some disadvantages, such as unstandardised collection methods, the frequent collection of dead mites and it being a time-consuming and laborious process (Zhao *et al.*, 2011). It is widely accepted that *Demodex* species depend on their natural hosts for survival, consuming hair follicle epithelial cells, sebum from the sebaceous gland and interstitial fluid secretion (Nutting, 1963; Zhao *et al.*, 2011) which precludes rearing them in vitro

Rearing human *Demodex* mites *in vitro* has been highly recommended, to get a better understanding of their life cycle and their relationship with humans (Carly and Dirk, 2014; Zhao *et al.*, 2011). There are a few reports of attempts to rear human follicular mites on an artificial medium, yet reproduction has never been successfully achieved *in vitro*. For example, Spickett (1961) studied the mites' habitat, behaviours and their responses to light under several sets of environmental conditions, including various temperatures and humidities. He cultured *D. folliculorum* on a medium that contained human sebaceous material with antimicrobials such as Penicillin and Streptomycin. He found that adult mites are able to remain alive for up to 6 hours at 20 to 35 °C. He also found that humidity and darkness are significant factors for most human *Demodex* life stages. Shufang *et al.* (2003) reported the survival time for *D. folliculorum* in several media, such as paraffin oil, a cell culture medium called Iscove's Modified Dulbecco's Medium (IMDM) and glycerine, under varying temperature and humidity conditions. They concluded that the mites could live for 17 days in paraffin oil under high humidity, 70% at 15 °C. In a similar study, Zhao *et al.* (2011) attempted to culture human *Demodex*

mites using five types of liquid media: human serum, paraffin oil, pig fat, a 1640-seroculture solution and normal saline. They compared the survival time of the two human *Demodex* spp. in each and found that mites can live in temperatures below normal for the human body (16 to 22 ˚C), especially in human serum at high humidity. Also, they reported that human serum is suitable for preserving mites for five days however, this cannot be repeated, as the mites drown in the liquid. Recently, Santana *et al.* (2015) reported a medium under development for culturing *Demodex* sp., their results indicated the best survival rate after 8 days is 60 % at 15 °C in Roswell Park Memorial Institute (RPMI) cell culture medium and human serum media.

Human serum and cell culture media (RPMI) have been claimed to preserve *D. folliculorum,* mites alive *in vitro* for more than ten days (Shufang *et al.*, 2003; Zhao *et al.*, 2011; Santana *et al.*, 2015), although there was no evidence that the mites reproduced in these conditions. This was nevertheless an important step towards producing new media. Pig fats were used by Zhao *et al.* (2011), also for *D. folliculorum*, with paraffin oil as the control medium, an approach also used by Shufang *et al.* (2003).

The ecological niche of human *Demodex* mites is the pilosebaceous unit in which the mites are surrounded by sebaceous gland secretions and epithelial cells on the hair follicle wall. Seemingly, the mites are consuming these materials (Desch and Nutting, 1972; Desch and Nutting, 1978; Carly and Dirk, 2014). Sebaceous glands at the skin surface produce sebum, which mainly consists of a complex mixture of fatty acids (Boughton and Wheatley, 1959; Picardo, 2009 and Smith and Thiboutot, 2008), principally, free and ester cholesterol, squalene, wax ester and free fatty acids (Picardo, 2009). There is no substantial difference in sebum from the sebaceous glands of men's and women's faces, (Boughton and Wheatley, 1959; Ramasastry *et al.*, 1970). Extracting natural sebum from humans is a difficult task and involves using special medical instruments such as

"Sebutapes" (Lu *et al.*, 2009). Thus, a number of artificial human sebum-like media have been devised as an alternative to human sebum for use in a variety of research requirements (Spangler *et al.*, 1967; Friberg and Osborne, 1986 and Nordstrom *et al.*, 1986). Four such artificial sebums were compared to natural human sebum and a formulation of their own by Lu *et al.* (2009) who concluded that their own artificial sebum, likely because of its physical and chemical similarity to natural human sebum, outperformed the others.

To formulate artificial media and growth conditions suitable for rearing *D. folliculorum in vitro* an understanding of the mite's natural habitat and behaviour and the environmental and microenvironmental conditions surrounding the human hair follicle is important. Human skin pH, ambient humidity and temperature may have a great influence on mites' life cycle and behaviour. The skin surface pH of healthy individuals ranges between 4.0 to 7.0, based on location (Parra and Paye, 2003; Lambers *et al.*, 2006). A large number of studies have shown that the skin surface is acidic, (pH 4.7 to 5.0) (Fluhr and Elias, 2002), the importance of which is to control the numbers of the microfauna and to maintain significant physiological processes, such as stratum corneum homeostasis and to main an optimal structure for the lipid barrier (Lambers *et al.*, 2006). Variable facial skin temperatures are reported in the literature, which fall between 29 and 33 ˚C (Ring *et al.*, 2000; Niu *et al.*, 2001; Or and Duffy, 2007). Although Zhao *et al.* (2009b) concluded that 15 ˚C is the ideal temperature for human *Demodex* to thrive, which is less than normal human body temperature, the optimal temperature for rearing the mites remains unknown. In addition, low-humidity environmental conditions result in loss of moisture content from the skin (Egawa *et al.*, 2002), so it is assumed that high humidity is an important *in vitro* requirement. Biochemical properties may also be important for mites to thrive *in vitro*. The hair follicle is covered by layers of different types of epithelial cells with protein connections (McGrath *et al.*, 2004). The most abundant protein in the

dermis, and therefore worthy of consideration, is Type I Collagen (Nakatsuji *et al.*, 2013). Collagen is primarily an animal protein and has been widely used in a variety of medical applications because it can be easily polymerized *in vitro* to many fabricated forms (Silver *et al.*, 1989; Badylak, 2004; Koide, 2007). Due to its non-toxic nature, biodegradability and flexibility, it has been used for various clinical purposes such as wound dressings, dermatological disorders, pharmaceutical capsules and tissue engineering (Aszodi *et al.*, 2006; Chattopadhyay and Raines, 2014).

Rearing *D. folliculorum in vitro* on an artificial medium is much needed, to better understand the mites' life cycle stages and their behaviour. Our understanding of these aspects of *Demodex* mite's biology is very incomplete (see Section 2.2) It could also have significant benefits for the development of medications for treating human skin disorders related to *Demodex* mites.

It has been hypothesized that *D. folliculorum* mites will survive for several days under simulated (artificial) growing conditions similar to ones in the follicle micro-environment if appropriately formulated taking into consideration the mite's natural environmental preferences.

The principal objectives of this Chapter were as follows.

- To analyse and improve the survival time of *Demodex folliculorum* mites in *de novo* artificial media.
- To analysis how long mites survive in replicated formulations of media and environmental conditions previously reported in the literature.
- To analyse the mites' reproductive rate (if any) in *de novo* artificial media.

6.2 Materials and Methods

6.2.1 *Demodex folliculorum* samples

Skin scraping samples were obtained from study participants aged between 18 and 60. Two facial areas were sampled, the forehead and the nose, by a using beauty, metal instrument called a black head remove. The skin samples were spread on microscopic slides and searched for *Demodex* mites. *Demodex folliculorum* were identified, according to Desch and Nutting (1972) and Izdebska (2009), and selected for rearing on the artificial media. Different sizes of metal pins used for transferring the mites from the slide onto the media. The number of *D. folliculorum* mites and their life stages, transferred to each medium, were recorded and later used for the statistical analyses.

The information sheet for participants of this human follicular mites' project (Appendix 2) was approved by the Ethics Committee of the University and it contained a brief explanation of the processes involved in the project, which had previously been reviewed and approved by the School of Biological Sciences Ethics Research Committee in 2013, before the project started.

6.2.2 Observing mites *in vitro*

The mites were kept on the surface of the medium inside sterilised, transparent disposable Petri dishes 4.5 cm in diameter (Fisher Scientific, Germany), with the lid open throughout. A stereo microscope (Leica M125) was used to observe the development of the mites, and movements were observed every 8 hours after the incubation period. A mite was considered alive when found moving on the medium or in the holes (see Figure 6.2, Materials and Methods) provided, or if their legs or chelicera were moving. If the mites remained immobile for two minutes of observation, another observation was conducted after 15 minutes. The second observation was performed for 1 minute with a video recording of the mite movement, taken with a camera (Moticam 10.0 MP)

connected to the microscope. Because the camera is able to record very small details and subtle movements, the video was watched carefully after recording. Only if the mite was found not moving after this process was it considered dead.

Thus, the purpose of this experiment was:

1) to trial human serum, Roswell Park Memorial Institute (RPMI), and an oil mixture medium for rearing *Demodex folliculorum* mites;

2) to improve these media by adding supplements to develop a novel growth medium for rearing *D. folliculorum*; and

3) find the optimal temperature for rearing *D. folliculorum.*

Some media previously reported in the literature were repeated in this study and a number of *de novo* media were produced and tested. Hence the experimental workflow was divided into two main approaches.

6.2.3 Previously trialled media

In this study, three different, previously published media and a control were used, as follows, to test their effectiveness as media for *Demodex folliculorum* mite rearing *in vitro*.

- Human natural serum (H4522, Sigma);
- RPMI 1640 Medium;
- an oil mixture (consisting of coconut oil, argan oil, jojoba oil and olive oil); and
- immersion oil (VWR collection), experimental control

These media were tested for their efficacy in supporting mite survival for the trial period. Three replicates of each medium were incubated. The optimal temperature for rearing the

mites remains unknown, therefore, three different incubation temperatures were used for each medium.

Procedure

1) The oil mixture was prepared by mixing 200 µL of each oil (coconut oil, argan oil, jojoba oil and olive oil) in one Eppendorf tube.
2) Human serum and RPMI media were kept at room temperature.
3) Immersion oil was kept at room temperature.
4) Twelve petri dishes were prepared, 500 µl of each medium and the control were pipetted into a petri dish (four plates for each medium), and it was then labelled with the medium name.
5) Fresh skin scrape samples were spread on a microscopic slide and searched for *Demodex* mites
6) The number of *D. folliculorum* mites transferred into each dish and the control was recorded.
7) Each medium was incubated at three different temperatures (20-23 ˚C, 28-32 ˚C and 35-37 ˚C)
8) The humidity was maintained at a constant 70- 90 % for all the trial treatments.
9) The pH for RPMI medium was kept at 6.9 ± 1.2, human serum at 7.2 ± 2.5 and the oil mixture and immersion oils at 5.3 to 5.6.
10) The mites were observed every 8 hours after the incubation and mortality was recorded.

This experiment was repeated three times (three replicates).

6.2.4 *De novo* media

The human body has approximately 5 million hair follicles at birth (Paus and Cotsarelis, 1999). No more hair follicles are formed; however, their sizes and shapes change throughout the human lifespan (Botchkarev and Paus, 2003). The shapes and sizes of the follicles depend on their location, but they all have the same basic structure (Zhou *et al.*,

1995) of papillae, matrix cells, bulbs, hair shafts, inner-root sheaths, outer-root sheaths, *arrector pili* muscles and sebaceous glands (Paus and Cotsarelis, 1999). The diameter of the hair canal is 85.93 ± 10.07 µm and the hair follicle diameter is 268.41 ±- 24.88 µm, in normal terminal hairs (Lee, 1995), however, follicles affected by androgenetic alopecia are significantly smaller, with the hair canal and follicle diameters 236.34 ± 17.23 µm and 68.83 ± 13.60 µm, respectively.

The assumed prevalent microclimatic conditions of *Demodex folliculorum* habitat (Figure 6.1) in the human skin were mimicked so far as practically achievable in this study. A generalised illustration of the typical media used in this study is shown in Figure 6.2.

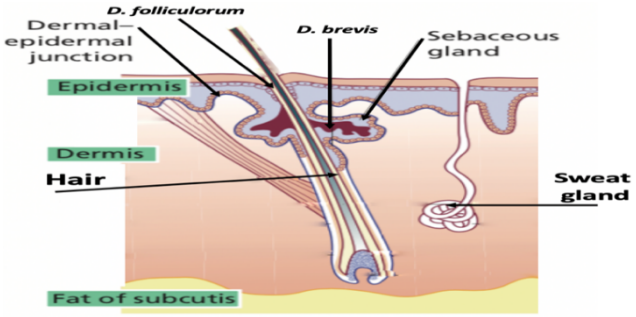

Figure 6.1 Cross section of the human skin, showing the microhabitat of *Demodex* mites *D. folliculorum* and *D. brevis*.

Figure adapted from (McGrath *et al.*, 2004).

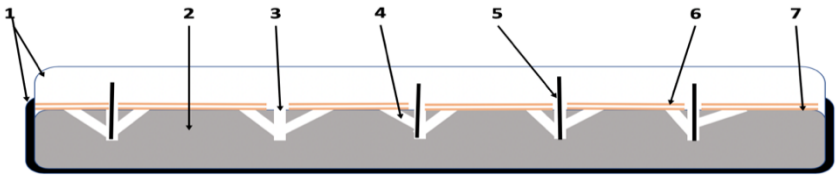

Figure 6.2 Generalised illustration of typical media configuration tested in this study.

1: Petri dish. 2: solid base of the medium. 3: vertical hole. 4: slide hole. 5: human hair. 6: first layer of additive materials. 7: second layer of additive materials. Detailed explanation of key features is provided below.

Base medium and artificial follicle procedure

1) **Petri dishes**: in most experiments disposable Petri dashes (Fisher Scientific, Germany) were used as made from transparent plastic allowing easy views of the mites on the surface of the medium or inside the holes provided.

2) **Solid base**: was usually 2 % agarose gel (Thermo Fisher Scientific, Germany). Gel preparation: 2 g of agarose gel was weighed and added to 100 ml of distilled water in a 300 ml flask, the mixture was heated until the gel completely dissolved then cooled to 40 ˚C and 1 to 2 ml of the mixture poured into a petri dish. In some media, oils were added to the gel or RPIM medium was used instead of agarose gel. The solid base of the media was an important to carry these added materials, and also to provide extra humidity inside the petri dish.

3) & 4) **Holes** in the solid base: *D. folliculorum* live inside the hair follicle, to imitate that, holes were made in the agarose gel using a sterile pin. Two types of hole were made when the gel was set, vertical holes were approximately 2 mm in depth and about 300 µm in diameter, slide holes were about 1 to 1.5 cm long and about 300 µm in diameter, the petri dish was then incubated at 37 ˚C for 30 minutes to allow water to evaporate. The slide or diagonal holes were connected to the vertical holes to allow mites to escape vertical holes in the case of becoming affixed to the gel, they were dug so that they had two openings, one leading to the surface of the medium and the other opening in the main hole, the dish was incubated again at 37 ˚C for 2 hours to allow water to evaporate from the slide holes.

5) Human hair: human hair was cut into approx. 1 cm lengths and cleaned with distilled water, then dried by towel paper and autoclaved at 121 ˚C for 15 minutes at 1.5 Atmospheres. The sterile hair was kept in a sealed bottle, forceps were used to place a hair in a vertical hole, to prevent contamination, resulting in an imitated human follicle arrangement (Figure 6.3).

6) **Additional materials**: the materials selected for rearing in this study are shown in Table 6.1 and their preparation is described below. These materials were variously applied to the base gel to create the trial conditions.

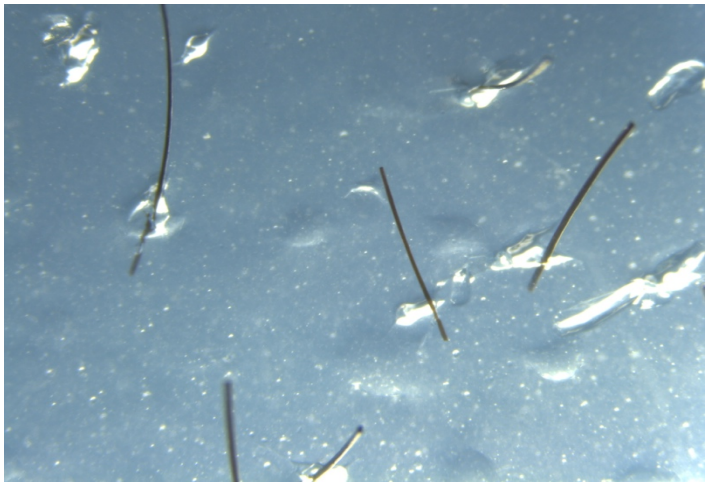

Figure 6.3 General view of the typical agarose gel base before addition of further materials, showing the imitated hair follicles.

Table 6.1 List of all the materials used to trial rearing of *D. folliculorum*

Where no source reference is given, the material was sourced and prepared from our own design UOR – University of Reading, Reading, UK.

Materials	Description	Reference/Source
Human serum	Human serum (H4522, Sigma); contains all the vitamins in suitable portions as found in natural human serum.	(Zhao et al., 2011) (Shufang et al., 2003)
RPMI	Roswell Park Memorial Institute (RPMI) or 1640 Medium is mainly used for culturing mammal cells. Does not contain proteins.	(Zhao et al., 2011) (Shufang et al., 2003)
Multivitamin	Wellman (brand) pharmacological multivitamin contains 12 different vitamins.	
Organic sponge	Honeycomb natural sponge, used as scaffold	
Argan oil	Extra virgin organic argan oil	
Jojoba oil	Extra virgin organic jojoba oil	
Coconut oil	Extra virgin organic coconut oil	
Olive oil	Extra virgin organic olive oil	
Cottonseed oil	Extra virgin organic cottonseed oil	
Melatonin	Human hormone: regulates the sleeping cycle, it peaks during night-time (15 pg/ml) in the plasma. In this study a concentration of 30 pg/ml was used (Cerilliant).	
Antimicrobial	A solution containing Penicillin, Streptomycin and Amphotericin used to prevent growth of bacteria and fungi (100×)(Fisher Scientifics).	(Su et al., 2009)
Collagen Type 1	liquid collagen Type 1 (3.3 mg/mL) produced from Rat tail (Temecula).	Prof. Dash, UOR
Gelatine	Gelatine produced from bovine skin (Sigma)	Prof. Dash, UoR
Human Artificial sebum	Comprising: squalene 15 ml, paraffin wax 10 ml, jojoba oil 15 ml, olive oil 10 ml, coconut oil 10 ml, cottonseed oil 25, oleic acid 1.4 ml, palmitoleic acid 5 ml, palmitic acid 5 ml, cholesterol 1.2 ml and cholesterol oleate 2.4 ml	(Lu et al., 2009)
Melanoma cell Pellets	Melanoma cells cultured at the University of Reading	Prof. Dash, UoR

Selection of main trial media and approaches

A total of thirty different media components were tested for rearing *D. folliculorum*. The process was iterative, during each set of experimental conditions knowledge of both, the mites' behaviour on the artificial media and their physiological needs were gained as well as drawbacks in the medium under consideration, this knowledge was fed into subsequent treatments. Three main *de novo* artificial media and experimental approaches emerged as candidates for long term rearing of *D. folliculorum*, Agarose / Gelatine Based Medium (AGBM), Agarose / RPMI Based Medium (ARBM) and Melanoma Cells with Human Artificial Sebum medium

Preparation of Agarose / Gelatine Based Medium (AGBM):

1) Base medium was a 1:1 mixture of 50 ml of 2 % agarose gel and 50 ml of 0.1 % bovine gelatine (0.1 g bovine gelatine dissolved at 60 ˚C in 100 ml distilled water).
2) To 100 ml of heated base mixture, 25 µl each of: argan, jojoba, coconut and olive oils were added.
3) Then 15 µL of melatonin, 200 µL of multivitamin, 5 µl of the antimicrobial cocktail was added the mixture when it had cooled to 38 ˚C.
4) The mixture was vortexed for 1 minute.
5) Prepared mixture, 1 ml, was poured into a petri dish and placed in a safety cabinet with the lid off to prevent steam condensing on its surface. When solidified, vertical and slide holes were created and dried as described above.
6) Additive layer: 100 µl of human serum mixed with 100 µl bovine gelatine (0.1g Bovine gelatine dissolve in 100 ml distilled water) was added to entirely cover the surface of the medium, including the holes, and left to be absorbed for 5 minutes.
7) Holes and imitated follicles were created, as above, and a number of mites were inoculated near them. The prepared treatment was incubated at 30 ˚C, 45 % humidity.

8) The overall pH of the medium was measured and was 6.6 ± 0.3.

Table 6.2 summarises the final rearing medium for AGBM.

Table 6.2 Components of the Agarose / Gelatine Based Medium (AGBM)

Medium layer	Components	Volume / weight	≈ Final concentration
Base (100 ml)	Argon oil	25 µl	0.025 %
	Jojoba oil	25 µl	0.025 %
	Olive oil	25 µl	0.025 %
	Coconut oil	25 µl	0.025 %
	Multivitamin	200 µl	0.2 %
	Melatonin (100 pg/ml)	300 µl	0.3 %
	Antibimicrobial (100×)	5 µl	0.005 %
	Agarose gel	0.2 g	0.2 %
	Bovine gelatine	0.01 g	0.01 %
	Distilled water	100 ml	99.2 %
Additive (100 µL)	Human serum	100 µl	0.01 %
	Bovine gelatine	100 µl	0.01 %

Preparation of Agarose / RPMI Based Medium (ARBM)

1) Base medium was 100 ml RPMI cell culture mixed with 2 % agarose gel and autoclaved at 121 ˚C, at 1.5 atmospheres for 15 minutes.
2) After cooling to 40 ˚C, 5 µl of antibiotic and 300 µl of melatonin were added.
3) Prepared mixture, 1 ml, was poured in a petri dish, dried, holes introduced, and re-dried as described above.
4) An additive was created from two pre-mixed components which were combined 1:1 and vortexed for 1 minute. Pre-mixes:
5) 1**:** 800 µL of human serum and 0.001 g Bovine gelatine, mixed in an Eppendorf tube and incubated for 2 hours at 60 ˚C in a water bath;
6) 2: 25 µL each of coconut, argan, jojoba and olive oils and 100 µl of RPMI, thoroughly mixed.
7) Additive, 100 µl, was spread on the surface of the base medium.
8) Holes and imitated follicles were created, mites introduced, and the treatment incubated as described for AGBM.
9) The overall pH of the medium was 6.1 ± 0.3.

Table 6.3 summarises the final rearing medium for AGBM.

Table 6.3 Components of the Agarose / RPMI Based Medium (ARBM)

Medium layer	Components	Volume / weight	≈ Final concentration
Base (100 ml)	RPMI	99 mL	98.7 %
	Agarose gel	2 g	2 %
	Melatonin (100 pg/ml)	300 µl	0. 3 %
	Antimicrobial (100×)	5 µl	0.005 %
Additive (1000 µL)	Coconut oil	25 µl	0.025 %
	Olive oil	25 µl	0.025 %
	Jojoba oil	25 µl	0.025 %
	Argon oil	25 µl	0.025 %
	RPMI	100 µl	0.01 %
	Human serum	800 µl	0.7 %
	Bovine gelatine	0.001 g	1 %

Preparation of Melanoma Cells with Human Artificial Sebum medium (MASM)

1) Base medium was 98 ml of RPMI medium mixed in a 150 ml beaker, with 2 ml of collagen Type 1, 0.5 g of bovine gelatine and 2 g of agarose gel. This mixture was heated in a microwave 7 times for 20 seconds each time until the gel had dissolved completely.
2) To 100 ml of base mixture cooled to 40 ˚C: 5 µl of antibiotic and 300 µl of melatonin were added and the mixture vortexed for 1 minute.
3) Prepared mixture, 1 ml, was poured in a petri dish, dried, holes introduced, and re-dried as described above.
4) An additive was created, 250 µl melanoma cell pellet was added to 250 µl human serum, then mixed for 1 minute.
5) Additive, 100 µl, was spread on the surface of the base medium and the treatment dried as described above.
6) Holes were created and the treatment incubated as described for AGBM.
7) Human serum, 100 µl, was added on the surface of the medium particularly around the holes and incubated for 30 min to allow absorption by the medium.
8) Human serum, a further 100 µl, was added and a sterilised hair was used to force the serum inside the holes (one hair for each hole). The prepared

medium was kept for 10 minutes at room temperature to allow the serum to enter the holes.

9) Human artificial sebum, 200 µl, was added on the medium surface, particularly around the holes.

10) A number of mites were inoculated around the holes and the treatment incubated at 30 ˚C and humidity 70 %.

11) This medium was used for rearing *D. folliculorum* inside a capillary tube with very minor differences in the method, however, the survival time was similar in both experiments.

12) The overall pH of the medium was 6.8 ± 0.3.

Table 6.4 summarises the MASM.

Table 6.4 Components of the Melanoma Cells and Human Artificial Sebum medium

Medium layer	Components	Volume / weight	≈ Final concentration
The base (100 ml)	RPMI	98 mL	97 %
	Agarose gel	2 g	2 %
	Bovine gelatine	0.5 g	0.5 %
	Collagen type 1	2 mL	2 %
	Melatonin (100 pg/ml)	300 µl	0.29 %
	Antimicrobial (100 X)	5 µl	0.005 %
The additive (layer 1) (100 µL)	Melanoma cell pellet	50 µl	0.05 %
	Human serum	50 µl	0.05 %
The additive (layer 2) (100 µL)	Human serum	200 µl	0.19 %
The additive (layer 1) (100 µL)	Human Artificial Sebum	200 µl	0.19 %

6.2.5 Statistical analyses

While observing the mites on the prepared media, the number of dead mites and the time of death was recorded. Based on this data collected, survival probability curves of *D. folliculorum,* on each medium and in each different incubation temperature, were compared using Kaplan-Meier estimate curves (Kleinbaum and Klein, 2012). A Log-rank test was used to investigate the differences in overall survival of mites between media

and incubation temperatures (Iachine *et al.*, 2010; Kleinbaum and Klein, 2012). If the Log-rank test was significant, a pairwise comparison test was performed to find which medium or incubation temperature was significantly different from the others (Appendix 5). The Cox proportional model was used to test the influence of different incubation temperatures and media with different components on the survival time of *D. folliculorum.* The hazard ratio covariates of the model were plotted using forest plots for visual comparison of the media and incubation temperatures (Iachine *et al.*, 2010). Survival analyses tests and figures were produced using ggsurvplot in the survminer package (Kassambara *et al.*, 2017) of R in RStudio version 1.0.135 (RStudio Team. *RStudio: Integrated Development for R*. Boston, MA: RStudio Inc; 2016. http://www.rstudio.com/) for Mac.

6.3 Results

6.3.1 Comparing previously trialled media

Three media that have been used in the literature, an oil mixture, RPMI and human serum were compared for rearing *D. folliculorum* in this study. The estimated survival time for the mites on these three media is illustrated by Kaplan- Meier estimated curves (Figure 6.4). The results show that there is significant difference between the mites' survival time reared in these media ($p < 0.001$). For the oil mixture, the maximum survival time was 56 hours compared to 24 hours on the control medium. The survival period for both the human serum and the RPMI cell culture media was 48 hours (Table 6.5).

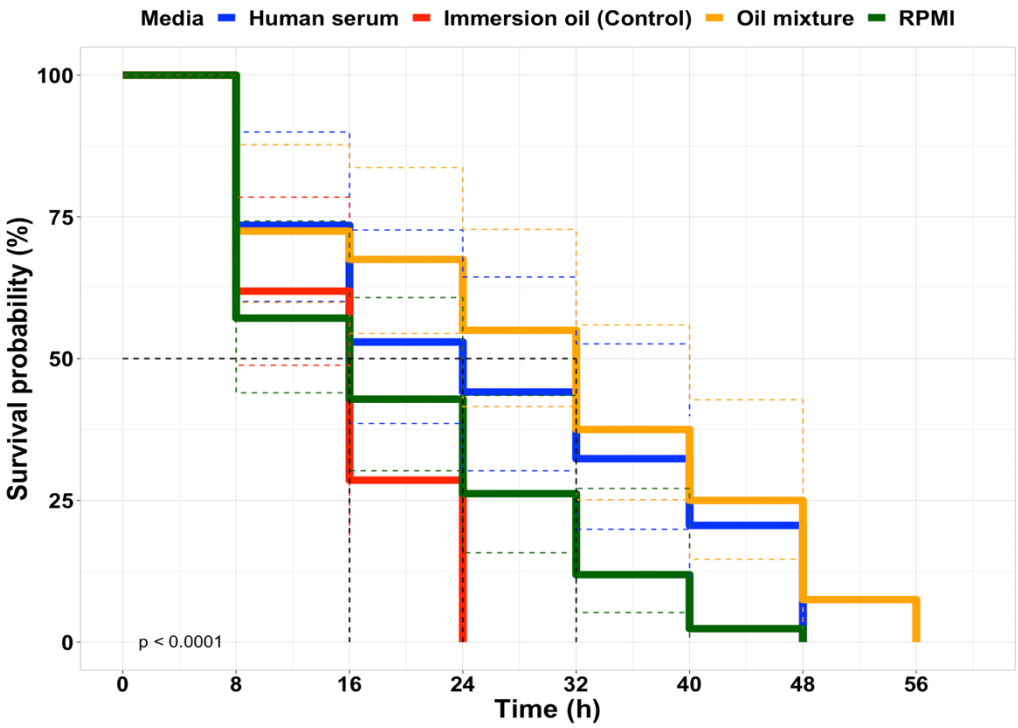

Figure 6.4 Kaplan-Meier estimated survival curves for *D. folliculorum* reared on three media used in the literature

Dotted lines represent low and high 95 % CI

Log rank test results show significant differences (Chi^2 = 31.2, df = 3, p < 0.001)) (Appendix 5, Table A5.2) between the survival times of *D. folliculorum* reared on the control and the three media. The survival time for the mites was 16 hours on the immersion oil (Control) and RPMI media, whereas it was 24 hours and 32 hours on the human serum and the oil mixture media, respectively (Table 6.5).

Table 6.5 Summary statistics for the survival time of *D. folliculorum* on the media and the incubation temperatures used in the literature.

	recor	n.ma	n.sta	event	rmean	se(rmea	median	0.95LC	0.95UC
Temperatur									
20-23 ˚C	55	55	55	55	25.01	1.63	24	24	24
28-32 ˚C	52	52	52	52	31.07	1.76	32	24	40
35-37 ˚C	51	51	51	51	9.41	0.42	8	8	8
Media									
Human ser	34	34	34	34	25.88	2.62	24	16	40
Immersion	42	42	42	42	15.23	1.0	16	8	16
Oil mixture	40	40	40	40	28.60	2.44	32	24	40
RPMI	42	42	42	42	19.20	1.84	16	8	24

D. folliculorum was also reared on each medium at three different incubation temperature (20-23 ˚C, 28-32 ˚C and 35-37 ˚C) reflecting previous studies. The estimated survival time for mites at these three temperatures is illustrated by Kaplan-Meier estimated curves (Figure 6.5). The results show that there is significant difference between the mites' survival time reared in these incubation temperatures ($p < 0.001$). The maximum survival time is 56 hours for *D. folliculorum* incubated at 28-32 ˚C, while the survival probability curve of the mites incubated at 20-23 ˚C is 48 hours. Whereas the minimum survival time for the mites was at 35-37 ˚C incubation temperature (Table 6.6).

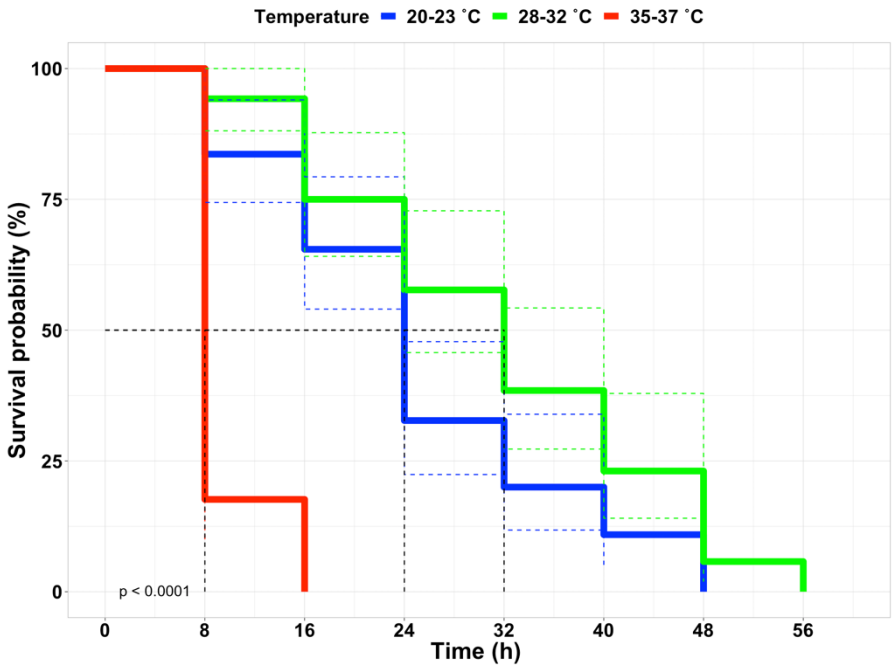

Figure 6.5 Kaplan-Meier estimated survival curves for *D. folliculorum* reared in three different incubation temperatures used in the literature

Dotted lines represent low and high 95 % CI.

Log rank test shows there is a significant difference between the three-incubation temperatures (Chi^2 = 170, df = 2, $p < 0.001$) (Appendix 5, Table A5.4). The median survival time for *D. folliculorum* is 32 hours incubated at 28-32 ˚C, whereas it is 16 hours and 24 hours for the incubation temperatures 35-37 ˚C and 20-23 ˚C, respectively (Table 6.6).

Table 6.6 Summary statistics for the survival time of *D. folliculorum* on the media used in this research.

Mediums	**records**	**n.max**	**n.start**	**events**	**rmean**	**se(rmean)**	**median**	**0.95 LCL**	**0.95 UCL**
Immersion oil (Control)	15	15	15	15	18.1	1.4	16	8	16
Human Cells with Sebum	15	15	15	15	52.8	2.3	56	48	96
MMAG based medium	15	15	15	15	34.1	3.0	32	24	48
RPMI based medium	15	15	15	15	43.4	4.2	48	32	72

The Cox proportional model shows significant differences in the survival time of *D. folliculorum* reared on the three media. By comparing the human serum (as a reference medium) to the two other media and the control in the experiment, the model results showed there was no significant difference in the survival time of *D. folliculorum* reared on human serum and oil mixture media, the hazard ratio decreased by only -0.12, which was not significant. However, when rearing the mites on the control and the RPMI media, the hazard ratio increased to 1.23 and 0.79, respectively indicating a significant decrease in the survival time, compared to the human serum medium. The model results showed that there was a significant difference in the survival time when rearing the mites at three different incubation temperatures. The hazard ratio decreased by -0.4 when rearing the mites at 28-32 ˚C compared to the incubation temperature 20-23 ˚C. while it increased by 2.4 when incubating the mites at 35-37 ˚C, so that survival time was low compared with the lower incubation temperature 20-23 ˚C (Figure 6.6), (summary statistics and the model output tables can be found in Appendix 5).

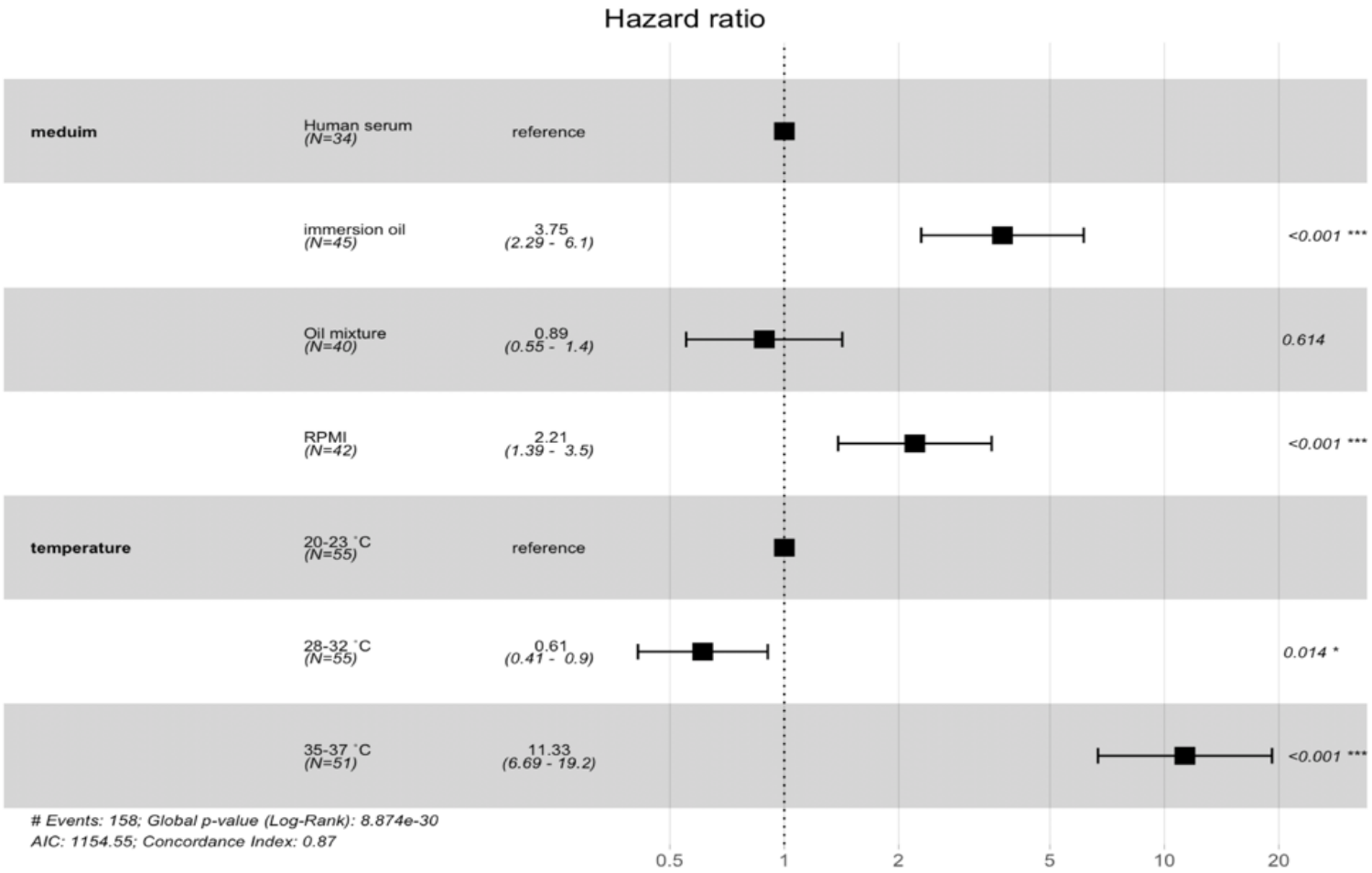

Figure 6.6 Forest plot showing hazard ratios a of the survival analysis of mites in previously trialled media and conditions

The data are derived from Cox's model hazard ratio covariates. Horizontal bars are 95 % CI.

6.3.2 Comparing the *de novo* media used in this study

Three different artificial media were mixed *de novo* and trialled in this study, from constituents previously recommended in the literature, for rearing *D. folliculorum*, Agarose / Gelatine Based Medium (AGBM), Agarose / RPMI Based Medium including human sebum (ARBM) and Melanoma Cells with Human Artificial Sebum medium (MASM). Some visual results are shown in Appendix 5.

The estimated survival time for the mites on these three media is illustrated by Kaplan-Meier estimation curves (Figure 6.7). The results show that there is significant difference between the mites' survival times reared in these media ($p < 0.001$). The maximum survival time for *D. folliculorum* is 96 hours in MASM and the human artificial sebum, whereas the minimum time for surviving on the control was 24 hours. While the survival probability for AGBM and ARBM was 48 hours and 72 hours, respectively.

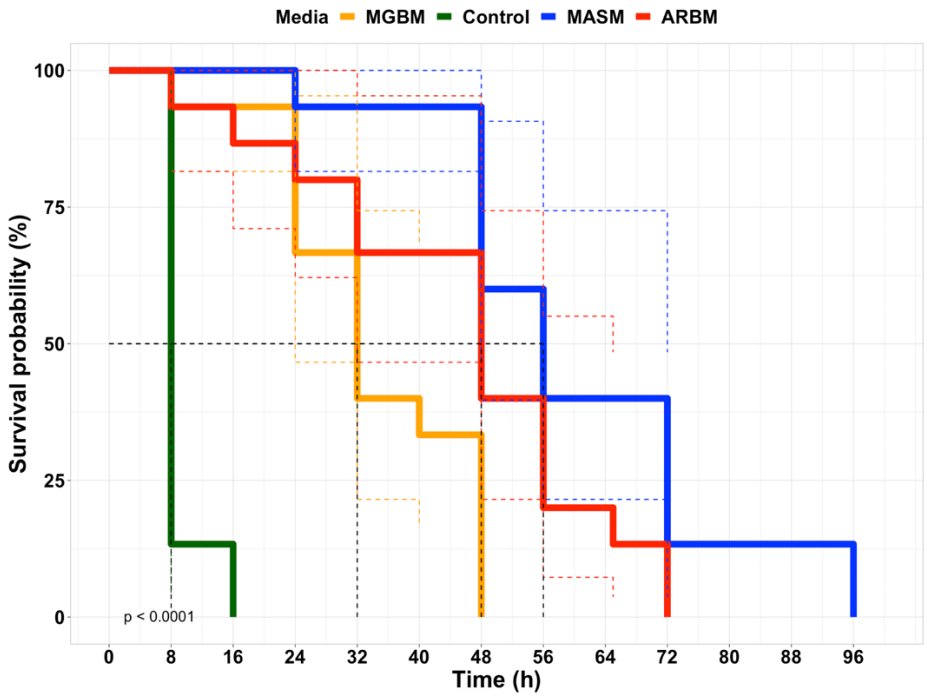

Figure 6.7 Kaplan- Meier estimated survival curves for *D. folliculorum* reared on three media developed *de novo* in this study.

Dotted lines represent low and high 95 % CI. MMGA = AGBM and RPMI = ARBM.

Log rank test showed that there is a significant difference between the three media and the control ($Chi^2 = 51.2$, df = 3, $p < 0.001$). The median survival time for *D. folliculorum* reared on the human melanoma cell medium and the human artificial sebum medium was 56 hours, whereas it was 16 hours in the control. The median survival time for the mites reared on RPMI-based medium and the agarose/gelatine-based medium was close to 48 hours and 32 hours, respectively (Table 6.7).

Table 6.7 Log rank test for the survival curves of *D. folliculorum* reared on four different media in this study

Mediums	Number	Observed	Expected	(O-E)^2/E	(O-E)^2/V
Immersion oil (Control)	15	15	4.36	25.9	38.2
Human Cells with Sebum	15	15	27.2	5.48	16.8
MMAG based medium	15	15	10.7	1.65	2.88
RPMI based medium	15	15	34.4	0.39	0.83
$Chi^2 = 51.2$, df = 3, $p < 0.001$					

The Cox proportional model shows significant differences in the survival time of *D. folliculorum* reared on the four media. By comparing agarose / gelatine-based medium (as a reference) to the two other media and the control in this experiment, the model results showed that there were significant differences of the survival time of *D. folliculorum* reared on these media (Appendix 5). The hazard ratio was decreased by -0.189 when rearing the mites on the Melanoma cells and Artificial Sebum medium ($p < 0.001$), which means an increase in the survival time of the mites. In addition, rearing the mites on ARBM medium decreases the hazard ratio to -1.15 ($p < 0.005$), which also indicates a significant increase in survival time compared to the agarose / gelatine-based medium. However, rearing the mites on the control increases the hazard ratio to 1.83, indicating a significant decrease in the survival time (Figure 6.8) (summary statistics and the model output tables can be found in the Appendix 5).

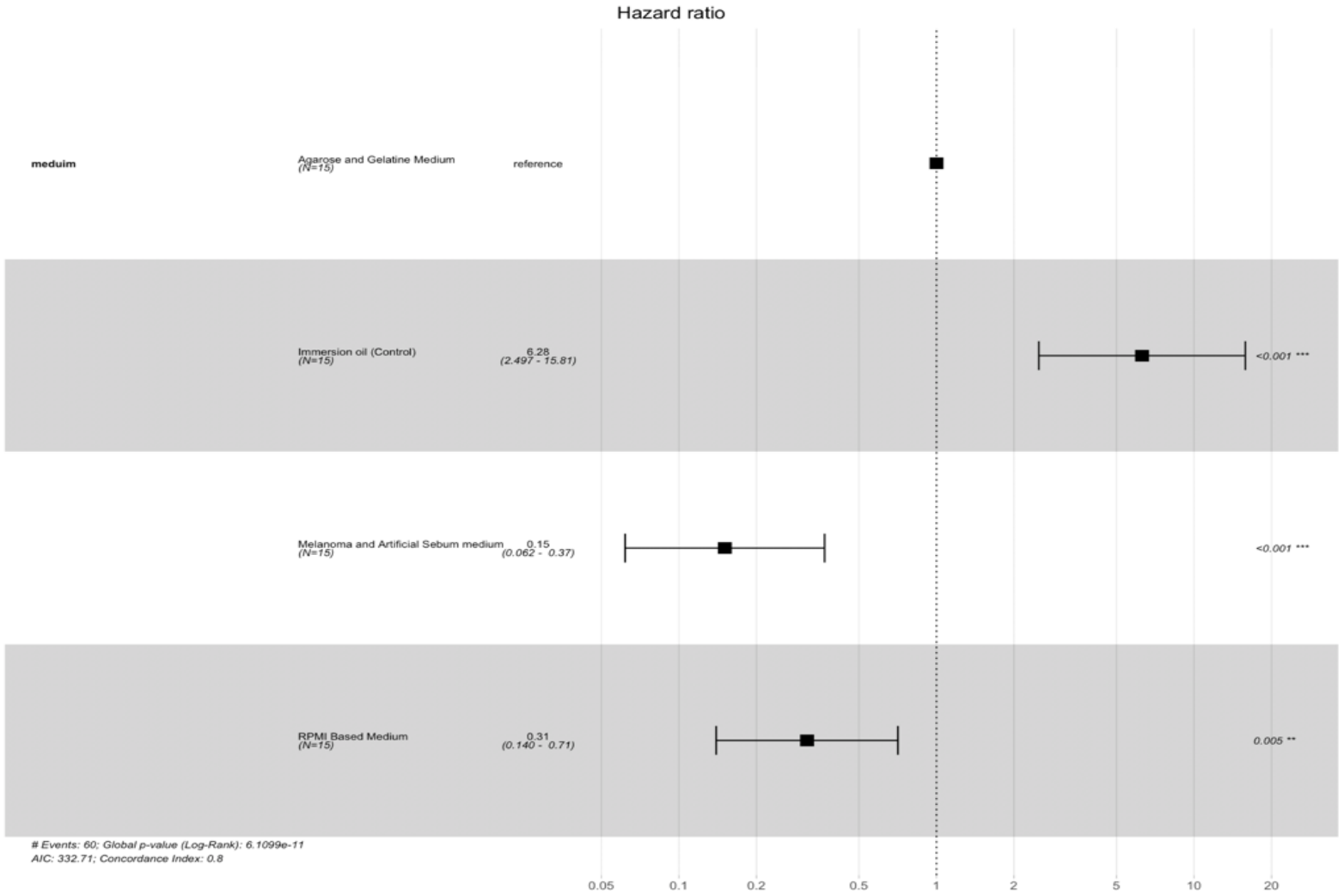

Figure 6.8 Forest plot showing hazard ratios a of the survival analysis of mites reared on three different *de novo* media
The data are derived from Cox's model hazard ratio covariates. Horizontal bars are 95 % CI.

6.4 Discussion

Rearing *Demodex* mites of humans on artificial media has always been a highly sought objective due to its potential utility in both biological and medical studies (Zhao *et al.*, 2011; Carly and Dirk, 2014). These mites have been linked to the development of various skin lesions (Coston, 1967; Liu *et al.*, 2010), nevertheless there is a lack of knowledge of how these mites feed or live naturally on the skin (Zhao *et al.*, 2009b). Some attempts have been made to growing the mites in the laboratory (Spickett, 1961; Shufang *et al.*, 2003; Zhao *et al.*, 2009b; Zhao *et al.*, 2011; Santana *et al.*, 2015), but none was successful in breeding a new generation of mites. Oil mixture, PRMI and human serum medium are artificial media that have been used tried previously (Shufang *et al.*, 2003; Zhao *et al.*, 2011; Santana *et al.*, 2015) for preserving the human *Demodex* spp. and these media were repeated in this study with similar methods and incubation conditions for humidity and temperature to those described in the literature. Shufang *et al.* (2003) reported that *D. folliculorum* was preserved in an oily medium for 17 days at 15 ˚C, our results differed, and it is concluded that their methods are not reproducible. Mites reared on oily media in our experiments showed a maximum survival time for *D. folliculorum* of just 24 hours in immersion oil (control) and 56 hours in the oil mixture (Figure 5.6). A human serum medium was preferred by Zhao *et al.* (2011) and achieved survival rates of more >5 days, but our results showed that the mite can only survive for 48 hours in the same medium (Figure 5.4). Santana *et al.* (2015) reared the mites on RPIM medium for 8 days, however, using as near identical conditions as was practical, the maximum survival time for *D. folliculorum* was 48 hours in the same medium (Table 6.6).

Demodex folliculorum incubated at 35–37 ˚C have a shorter survival time compared to lower incubation temperatures used in this experiment (Figure 5.5). This result is very

similar to the results reported by Zhao *et al.* (2009b), Zhao *et al.* (2011) and Shufang *et al.* (2003). However, our maximum survival time for mites was 56 hours at 25–28 ˚C, whereas in these published studies it was longer at an incubation temperature between 5–20 ˚C (Table 6.6 and Figure 6.6).

The experiments in this chapter initially aimed to develop a solid based media for rearing *D. folliculorum in vitro* or at least to preserve them alive for as long as possible. To achieve this objective, the microhabitat of the *Demodex* mites was simulated in shape and the surrounding natural materials and other environmental conditions such as humidity and pH, so far as was practical and easily reproducible. As there were few reliable, published references to the exact components of the materials surrounding these mites in their natural habitat, thirty media with different nutrition elements and levels with various environmental conditions were tested. Only three of the thirty media tested are discussed in this chapter, due to lower survival times in most, compared to results achieved in previously trialled and published media. Some natural factors reported to influence survival could not be trialled, for example, the skin has a bacterial flora including *Staphylococcus* and *Corynebacterium* (Fredricks, 2001), however, the health and safety standards of the laboratory were incompatible with culturing bacteria for these trials.

Melatonin is a human hormone that regulates the sleeping cycle (Benloucif *et al.*, 2008) and peaks between 2–5 am (Nogueira *et al.*, 2013). It is likely that melatonin is present in the environment surrounding the mites and a likely correlation has been postulated between high levels of melatonin (15 pg/ml of plasma) during sleeping (Benloucif *et al.*, 2008) and the activity of human *Demodex* mites at night (Rufli and Mumcuoglu, 1981).

This research was based on improving the experimental methods by trial and error and learning from the unsuccessful trials how to improve the survival rates for the mites *in*

vitro. These media are, therefore, the final versions of a series developed from combinations of similar components.

The results showed that *D. folliculorum* has the longest survival time on the medium containing the human melanoma cell and the human artificial sebum followed by RPMI-based medium and agarose / gelatine-based medium, respectively (Figure 6.7). They all have higher survival time than the control medium ($p < 0.001$) and also the survival time of the mites between them is significant ($p < 0.001$) (Table 6.7).

In conclusion, it is likely, from observations of our own reared colonies, that *D. folliculorum* was unable to live in the liquid media used in the literature for two main reasons, firstly, they were unable to move naturally and secondly because of a lack of oxygen which caused them to suffocate. In this study, *D. folliculorum* mites may also have consumed the media they were reared in for their normal physiological functions, but, not for a long time. Perhaps these media allow the mites to move freely and to moult their exoskeleton but do not supply all of their essential nutrition or requisite environmental conditions for living and proliferating. For instance, there are other factors that have not been identified that may need to be included in the artificial medium and mite's environment, to sustain proliferation. Adding human skin commensal bacteria, and using tight spaces to allow mites mating are, for example, worthy of further consideration. The human melanoma cell and the human artificial sebum increases the viability of *D. folliculorum* for four days but, after that the melanoma cells dried out, and the mites may subsequently have been unable to consume them, so methods to replace them in the media, for example. to replenish the available nourishment in the rearing medium, is also worthy of further trials.

Chapter 7: Conclusions

Demodex folliculorum and *D. brevis* are ubiquitous inhabitants of our hair follicles and sebaceous glands (Desch and Nutting, 1972). Although the pathogenesis of human *Demodex* mites is far from being understood, they have been linked to severe skin lesions in humans and diseases such as rosacea, pityriasis and gland dysfunction (Gao *et al.*, 2005; Lacey *et al.*, 2007). The close association between humans and *Demodex* mites seems to be an ancient one (Thoemmes *et al.*, 2014). It has been suggested that studying this ancient association may reveal significant details of human evolution (Palopoli *et al.*, 2015). The medical and ecological importance of these mites may explain the great interest of scientists in this branch of evolutionary biology (Sastre *et al.*, 2016). This project is an attempt to fill some of the important gaps in our knowledge, many of which have regularly been noted in published research and to contribute to a better understanding of the biology of the two human *Demodex* spp. and of their association with human beings.

The introduction to this study provided an overview of the background to various aspects of the current lines of research regarding human *Demodex* mites. The literature that specifically links to the work in this project was cited in the context of its relevance. It was also an attempt to show various points of view and areas of contention and even disagreement between dermatologists and acarologists in some aspects of this field. For example, the nature of the role of *Demodex* mites in human disease is contentious and the morphological characteristics of the two species used in microscopic identifications are recognised to be sometimes inadequate for reliable diagnoses.

Chapter two aimed to emphasise the importance of the identification of the human *Demodex* mites and how to distinguish between the two mites using two of the most

common microscopy methods. Recruiting a large cohort to undertake this study was challenging, given the nature of the research and human sensibilities, nevertheless, 256 participants agreed to take a part and to be sampled. Because of the limitations on the time available for the participants, who were all academic staff and students, the collection of the samples was mostly an opportunistic process.

Although microscopic identification of the two human *Demodex* spp. is a very useful, easy task, collecting the samples from humans is a time-consuming process. In addition, creating permanent slides of mounted *Demodex* mites using the traditional techniques failed, because, the lactic acid used in most of these methods dissolved the thin exoskeleton of the *Demodex* mites, making it impossible to recover intact from the solution. Thus, temporary slides were relied upon, obtained by mounting the mites directly from the sample into a Hoyer's solution or Phosphate-Buffer Saline (PBS), this technique allows preservation of the samples for approximately a week without any apparent change in the external features of the mites. The mites can be identified based on key morphological characteristics using either of the two types of microscopes tested in this Chapter, and which are generally available in scientific laboratories. The described methods, and in particular the indications of their limitations and potential pitfalls, will serve as useful guidelines for anyone studying human *Demodex* mites for the first time by these methods.

In Chapter 3, the prevalence of human follicular mites was assessed using a small, highly educated population of academic staff and students at the University of Reading. However, most of the participants had not heard of human *Demodex* mites before or had only vague notions about them, so it was a great opportunity to educate the participants, by explaining some background facts about *Demodex* mites of human hosts. The

transmission mechanism of human *Demodex* mites from one individual to another is still poorly understood, but this study showed that that breastfeeding (as an indicator of very close skin to skin contact) increased the possibility that a person will have mites, a result which has not previously been found in most of the studies which have considered it.

The multiplex PCR test that has been designed, in Chapter 4, for distinguishing three *Demodex* spp. (*D. folliculorum*, *D. brevis* and *D. canis* which occur on dogs) will allow medical practitioners in the field to identify which mite species is present when investigating any particular skin disorder in their patients. *Demodex canis* was included as an example because of its widely reported pathogenesis, which has regularly been confirmed, and because dogs are the favoured pet amongst pet owners in the UK. Our findings, in particular the multiplex PCR protocol with a selected combination of tested primers, may open the door widely to allow more species of *Demodex* mites to be included in the test, species such as *D. aries* (sheep), *D. bovis* (cattle), *D. cati* (cats) and *D. equi* (horses). These mites infest livestock animals and so humans might also be affected by them. It is also possible that the multiplex PCR assay will allow more *Demodex* spp. to be identified and named. The multiplex PCR can be upgraded to a Realtime PCR test by designing probes for each primer set in the test, such an approach would potentially be convenient and time saving for those needing to study these mites in medical and scientific laboratories.

Rearing the human *Demodex* mites on artificial media as we investigated in Chapter 5, is still an unfulfilled biological need. It is needed because of the time-consuming nature and unreliability of collecting mites from human hosts. Thus, an investigation of sustainable rearing of *D. folliculorum* on artificial media was conducted. Media containing the natural-habitat substances of the mites, such as human cells and human sebum were

considered in this trial, as they were considered likely to be effective at preserving the mites alive outside of the human body for long periods. However, none of the media tested kept mites alive beyond the first generation. However, the limitations of these and other substances tried out may be useful for future research into media. For example, rearing the mites in short and narrow capillary tubes to mimic their human habitat, with a medium consisting of these two substances could prove effective. The narrowed spaces may allow the mites to come closer together for mating and this environment allows easy visualising of the mites during the study while consuming smaller amounts of medium during experiments, hence may also prove more cost-effective.

All of the molecular approaches for studying the human *Demodex* mites were carried out using mitochondrial genes, but *D. brevis* whole genome mitochondrial DNA has not yet been sequenced to our knowledge, making this a valuable area for study. In Chapter 6, a comparison was made of different methods to extract, amplify, purify and evaluate genomic DNA from *D. brevis* for potential next generation sequencing. Several sample types and proprietary kit-based protocols were compared to achieve this aim. The amount of extracted gDNA from *D. brevis* was in the expected quantity range for single gene or multiplex PCRs, such as we conducted successfully in Chapter 4, but, the quality and quantity of the obtained gDNA was not adequate for reliable gDNA sequencing. For future work, gDNA should be extracted from larger samples of mites, at least 100 recommended, however, this is a very considerable number, given the difficulties inherent in collection, especially if sampling a single individual. Therefore, tests are needed to see if samples preserved in alcohol at -20 until DNA is extracted may also suffice. Having genomic DNA sequenced will allow more molecular studies to be carried out and comparisons with other species, notably *D. folliculorum* and *D. canis*. Such an

achievement may reveal important information about the nature and evolution of the close association between humans and mites of humans.

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Appendixes

Appendix 1 (Chapter 1)

Table A1.1 List of some common *Demodex* species and their mammalian hosts

***Demodex* species**	**Mammalian host**	**Publishing Author(s)**
D. acutipes	Red deer	Bukva and Preisler, 1988
D. aries	Sheep	Desch, 1986
D. aurati	Golden hamster	Nutting, 1961
D. bovis	Cow	Stiles, 1892
D. brevis	Human	Akbulatova, 1963
D. caballi	Horse	Nutting *et al.*, 1975
D. cafferi	African buffalo	Nutting and Guilfoy, 1979
D. canis	Dog	Leydig, 1859
D. caprae	Goat	Nutting *et al.*, 1975
D. cati	Cat	Mégnin, 1877
D. cervi	Indian deer	Bukva, 1987
D. equi	Horse	Owen, 1844
D. folliculorum	Human	(Simon, 1842)
D. gapperi	Red-backed vole	Nutting *et al.*, 1971
D. gracilentus	Mouse	Izdebska and Rolbiecki, 2013
D. kutzeri	Moose	Bukva, 1987
D. nycticeii	Bat	Desch, 1996
D. odocoilei	White-tailed deer	Nutting, 1979
D. ovis	Sheep	Hirst, 1919
D. phylloides	Pig	Csokor, 1879
D. pseudaxis	Japanese deer	Bukva, 1987
D. saimiri	Squirrel monkey	Lebel and Nutting, 1973

Appendix 2 (Chapter 2)

1. Flier designed to recruit human experimental subjects for the study.

University of Reading

School of Biological Science

Volunteers Needed

Human follicular mites are group of mites that live on our skin all life. They can be found on healthy individual's skin (face, nick, forehead and eyelashes). Dermatologists think these mites are responsible for some skin lesions such as acne, alopecia, blepharitis and dermatitis. We do not know for sure who these small creatures transmit to human. In this project we try to study more in this aspect, and we need to collect mites as we can from variety of ethnic group, because of that we are looking for**:**

- Male or female.
- Aged 18 to 80 years old.
- Willing to attend to the school of Biological science and fill out a very short questioner and sign a participate consent form.
- The process is safe and painless, and the instructions should not take more than 5 minutes.
- A photo of your own follicular mites will be sent to your email, if we found them.

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2. Information Sheet

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Information Sheet for Participants of Experimental Project

Reading, 2013

Dear participant,

The following text will give you information about the project in which you are participating.
Project title: *"Human follicular mites"*.
Summary: The main purpose of this project is to collect and study the biology of human follicular mites. Follicular mites live inside the follicles of the skin. They are very common, occurring in almost all humans, rarely causing any problems. They are easy to spot on the face. There are two species of mites in humans, *Demodex brevis* and *Demodex folliculorum*.

Volunteers will be asked to provide their follicular mites for further identification and studies. The studies will be on mites, Acari, Demodex. The mites focus of this study are localised in the pores of the skin of the face: forehead, nose, cheeks, chin and ears (external ear, and in the wax). Only volunteers in good health and aged 18 and above will be selected for this project. The technique to collect mites is very common, using a blackhead remover, tape or similar. The technique is used in beauty shops. The sample is collected in a tube provided by the researcher. The following questions will also be asked to the volunteers (and this forms the basis of the questionnaire):

You will need to match in the following ranges:
Age: 18-30, 31-40, 41-60, 61-100 years old.
Sex: Male or Female
Childbirth: How were you born? Vaginal delivery or Caesarean section
Breastfeeding: Were you breastfed? Yes or No
Ethnicity (origin): Asian Indian, Pakistani, Bangladeshi, Chinese, Japanese, any other Asian background; Black African, Black Caribbean, or any other Black background; Central Europe-White, East-Europe-White, British White, Irish White, Scandinavian White, Mediterranean White, any other White background (these categories are based on the reports of the Office of National Statistics).

By matching these data (either age, sex, childbirth, breastfeeding or ethnic origin) with the diversity of mites found in each volunteer (either one of the two species, the two species or a new species) we will be able to understand the nature and history of this association of mites and humans.

The project does not involve the study of humans or human subjects/tissues and no human material will be collected and or stored. All participants will remain anonymous and they will not be identified by name in the final project report, nor in scientific publications; their information, in the answered questionnaires will be stored and kept safe as 'confidential' in Dr. Perotti's office until the end of the project, when these documents will be destroyed. The collected mites will be stored and further preserved in the Acarology collection of the acarology lab, School of Biological Sciences, University of Reading. The participants are asked to return the signed consent form to the researcher, Dr. Perotti. The Researcher's contact details are found in the signature and the headline; a copy of the signed consent form will be kept by the participants.

This project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

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Sub

3. Participant Consent Form

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Participant Consent Form

1. I have read and had explained to me by Researcher, Dr. *M. Alejandra Perotti* the accompanying Information Sheet relating to the project on: *"Human follicular mites"*

2. I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation.

3. I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time, and that this will be without detriment.

4. This project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

5. I have received a copy of this Consent Form and of the accompanying Information Sheet.

Name: .....

Signed: .....

Date: .....

4. Participant Questionnaire

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Questionnaire for the participants of the project: *Human follicular mites*

Please fill in the following fields (if the answer is 'Unknown', please do not underline any of the options in the field):

Name of participant:

Age (please underline as required): 18-30, 31-40, 41-60, 61-100 years old.

Sex (please underline as required): Male or Female

Childbirth: (please underline as required): How were you born? Vaginal delivery or Caesarean section

Breastfeeding: (please underline as required): Were you breastfed? Yes or No

Ethnicity (origin) (please underline as required): Asian Indian, Pakistani, Bangladeshi, Chinese, Japanese, any other Asian background; Black African, Black Caribbean, or any other Black background; Central Europe-White, East-Europe-White, British White, Irish White, Scandinavian White, Mediterranean White, any other White background.

Appendix 3 (Chapter 3)

1. Facial Area

Demodex mites were collected from two facial areas (nose and forehead) and compared, Figure 3.6 A. Facial areas combined with host's sex were compared (Figure 3.6 B) based on the median number of mites collected per sample from each site. The median of *Demodex* spp. collected from both sites for each sex is 0. A Mann Whitney test showed no significant differences in the numbers of mites collected from the nose ($U = 6131$, $p > 0.05$) and from the forehead ($U = 6584$, $p > 0.05$) between the two host's sex (Figure 3.6).

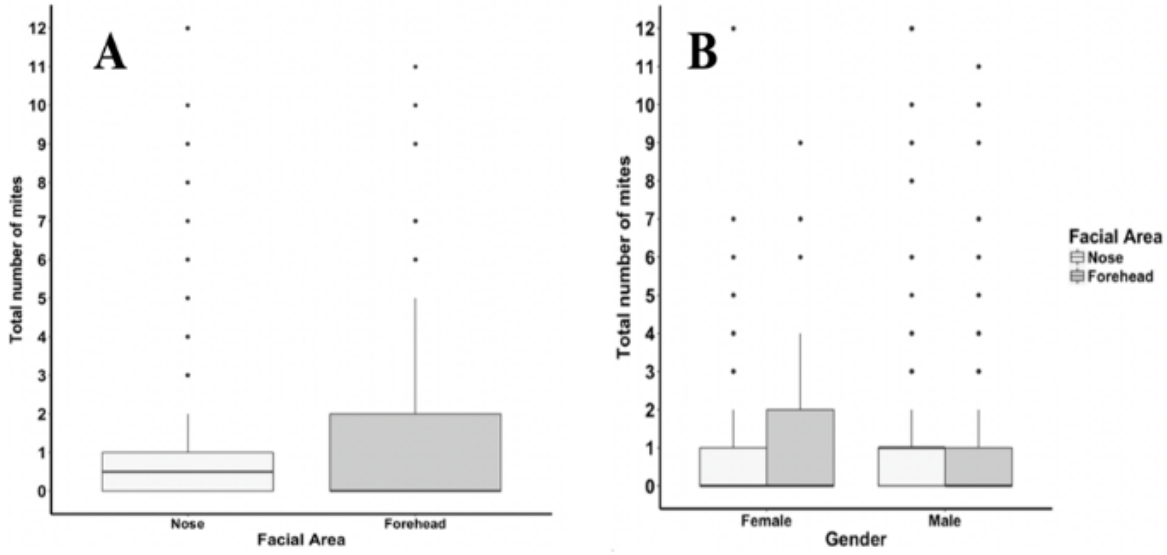

Figure A3.1 Boxplot diagram comparison of the nose and forehead collection of both sexes showing the total number of human follicular mites per sample.

A: B: In females (women) the number of mites collected from the forehead is 254 (median = 0.0, range 0-21) whereas, from the nose it is 160; (median 0.0, range 0-62). In males(men) the total number of mites collected from the forehead is 309 (median = 0.0, range 0-32) and from the nose it is 189 (median = 0.0, range 0-16). Only proximal outliers are shown

2. Zero-inflated Negative Binomial model output

Prevalence of the human follicular mites, *Demodex folliculorum* and *D. brevis* (Acari: Demodicidae) on a population of students and academics at the University of Reading.

Table A3.1a Zero-inflated Negative Binomial model output

Count model coefficients (negbin with log link):				
	Estimate	**Std. Error**	**z value**	**Pr (>|z|)**
Host's sex - Male	-0.0525	0.2354	-0.223	0.8234
Childbirth- Vaginal delivery	0.2990	0.4173	0.717	0.4736
Breastfeeding Yes	-0.8455	0.3511	-2.408	0.0005*
Age31-40	0.7638	0.3446	2.216	0.0266*
Age41-60	1.0928	0.3143	3.477	0.0005*
Age61-100	0.9022	0.4870	1.853	0.063943
Log (theta)	-0.6708	0.1685	-3.981	0.0005*
Zero-inflation model coefficients (binomial with logit link):				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
Host's sex - Male	-0.952	1.685	-0.565	0.572
Childbirth- Vaginal delivery	0.5488	3.882	0.141	0.888
Breastfeeding Yes	0.2259	2.1283	0.106	0.915
Age31-40	1.1219	2.2964	0.489	0.625
Age41-60	-12.5485	483.0241	-0.026	0.979
Age61-100	-10.1999	201.6795	-0.051	0.960
Theta = 0.5113 **Number of iterations in BFGS optimization:** 80 **Log-likelihood:** -506.5 on 11 Df				

Zero-inflated Negative Binomial model output Continued - Descriptive results:

Table A3.1b Zero-inflated Negative Binomial model output

		Exposure to *Demodex* spp.						
		Negative (no mites found)			Positive (mites found)			Total
		Female (n = 45)	Male (n = 51)	Sub-total (n = 96)	Female (n = 69)	Male (n = 91)	Sub-total (n = 160)	Total (n = 256)
Age group								
18 – 30		22	31	53	24	32	56	109
31 – 40		13	15	28	11	22	33	61
41 – 60		9	4	13	29	28	57	70
60 +		1	1	2	5	9	14	16
Early-life conditions								
Vaginal	**Breastfed**	38	42	80	52	76	128	208
	Bottle	5	3	8	11	7	18	26
Caesarean	**Breastfed**	2	4	6	2	4	6	10
	Bottle	0	2	2	4	4	8	10
Ethnic group								
Asian Indian		4	2	6	5	8	13	19
Black African		2	8	10	3	7	10	20
British White		15	10	25	26	25	51	76
Central Europe		0	3	3	4	3	7	10
Chinese		6	3	9	7	13	20	29
East-Black Caribbean		1	0	1	1	2	3	4
Japanese		1	0	1	0	3	3	4
Mediterranean White		2	4	6	9	5	14	20
Other Asian		12	13	25	7	19	26	51
Pakistani		0	5	5	1	4	5	10
South American		2	3	5	6	2	8	13

Appendix 4 (Chapter 4)

1. Multiplex PCR identification for the two human *Demodex* mites and *D. canis* (Dog mites).

Amplifying the two human *Demodex* species collected from different human ethnicities and *D. canis* with primers used in the multiplex PCR test, with varying DNA templates to provide positive and negative controls.

Checking the specificity of the selected *COI* primers

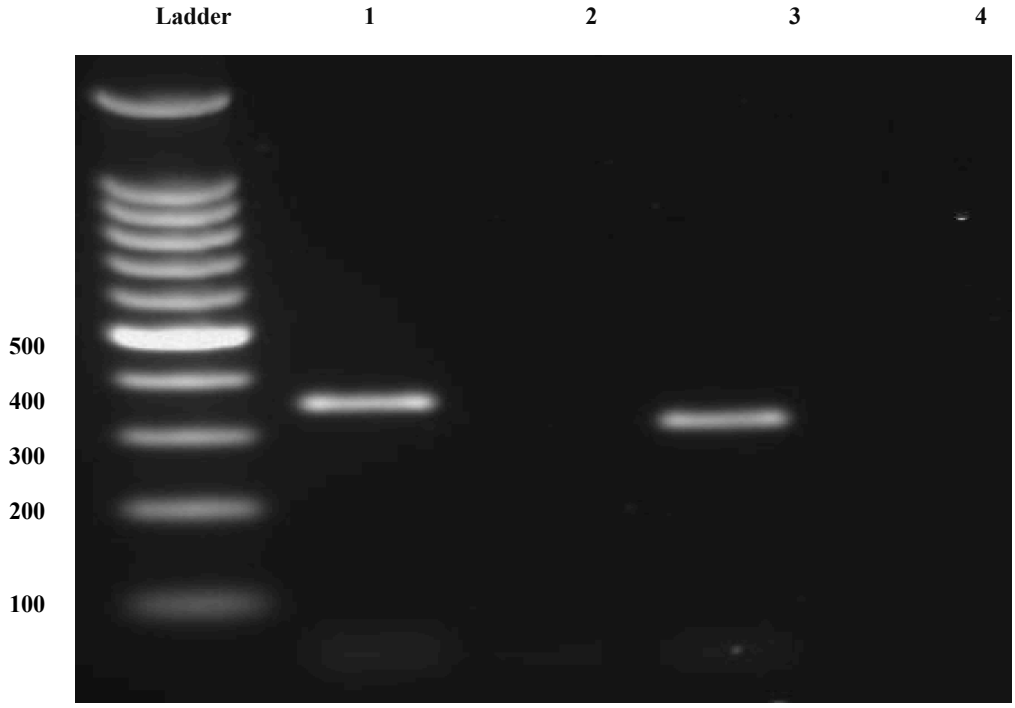

Figure A4.1 Checking the specificity of *COI* primers in both human *Demodex* species (Controls).

Ladder 100bp

Primer pair Db COI 63F and Db COI 411R (*D. brevis* primers)

Lane **1**: positive control, with *D. brevis* DNA - one band of 350 bp. **2**: negative control - with *D. folliculorum* DNA - no band.

Primer pair Df COI 144F and Df COI 396R (*D. folliculorum* primers)

Lane **3**: positive control, with *D. folliculorum* DNA - one band of 250 bp. **4**: negative control - with *D. brevis* DNA - no band.

1 % agarose gel.

Checking the specificity of selected *16S* primers

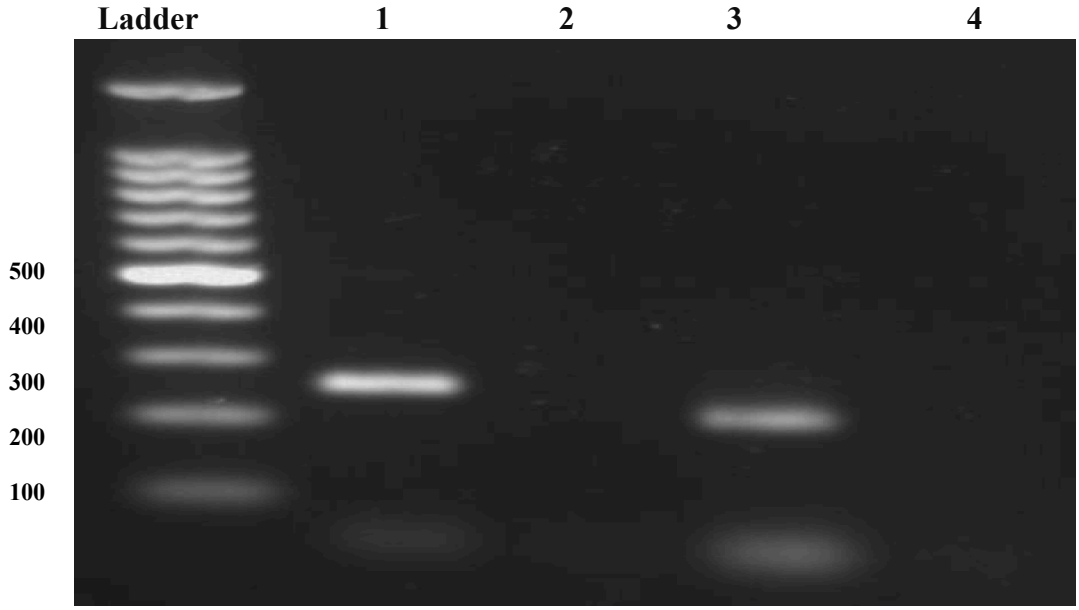

Figure A4.2 Checking the specificity of *16S* primers in both human *Demodex* species (Controls).

Ladder 100bp

Primer pair Db 16S 80F and Db 16S 357R (*D. brevis* primers)

Lane **1**: positive control, with *D. brevis* DNA - one band of 300 bp. **2**: negative control - with *D. folliculorum* DNA - no band.

Primer pair Db 16S 80F and Db 16S 357R (*D. folliculorum* primers)

Lane **3**: positive control, with *D. folliculorum* DNA - one band of 200 bp. **4**: negative control - with *D. brevis* DNA - no band.

1 % agarose gel.

Checking the specificity of *D. canis* primers

The figure shows the spscificity of *D. canis* primer sets *D. c 28S 277* and Dc 28S 237.

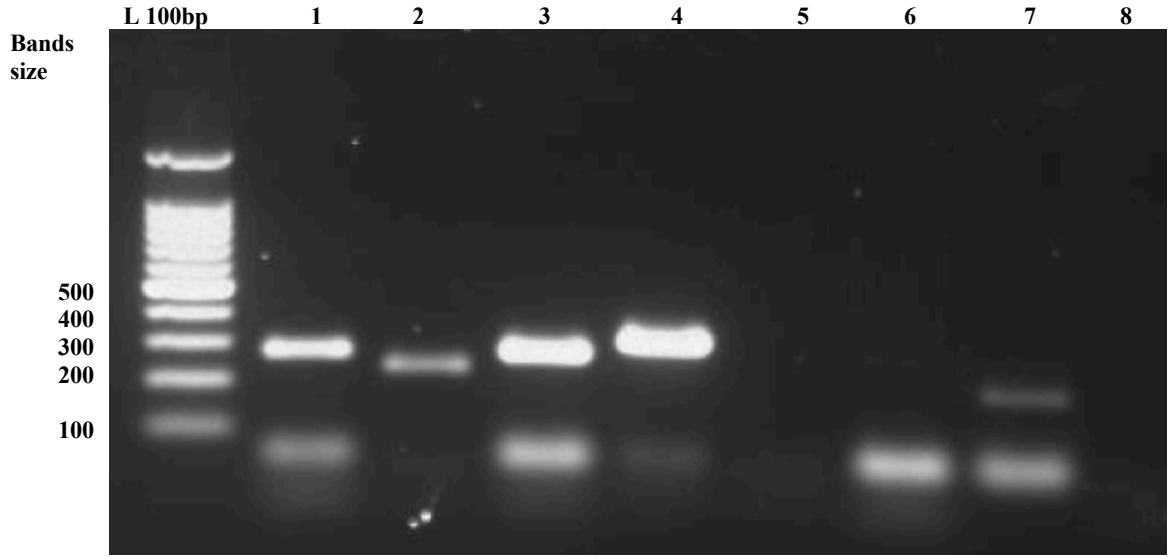

Figure A4.3 Checking the specificity of the new primers for *D. canis* (Controls)

Ladder 100bp

Primer pair Dc 28S 277F and Db 28S 277R

Lane **1**: positive control, with *D. canis* DNA - one band of 300 bp. **3:** negative control, with *D.folliculorum* DNA - one band ~300 bp. **6**: negative control, with *D. brevis* DNA – no band.

Primer pair Dc 28S 237F and Db 28S 237R

Lane **2**: positive control, with *D. canis* DNA - one band of 250 bp. **5**: negative control, with *D. folliculorum* DNA – no band. **8**: negative control, with *D. brevis* DNA – no band.

Primer pair Dfm COI

Lane **4**: positive control, with *D. folliculorum* DNA - one band ~300 bp.

Primer pair Dbm COI

Lane **7**: positive control with *D. brevis* DNA - one band of ~200 bp

1 % agarose gel.

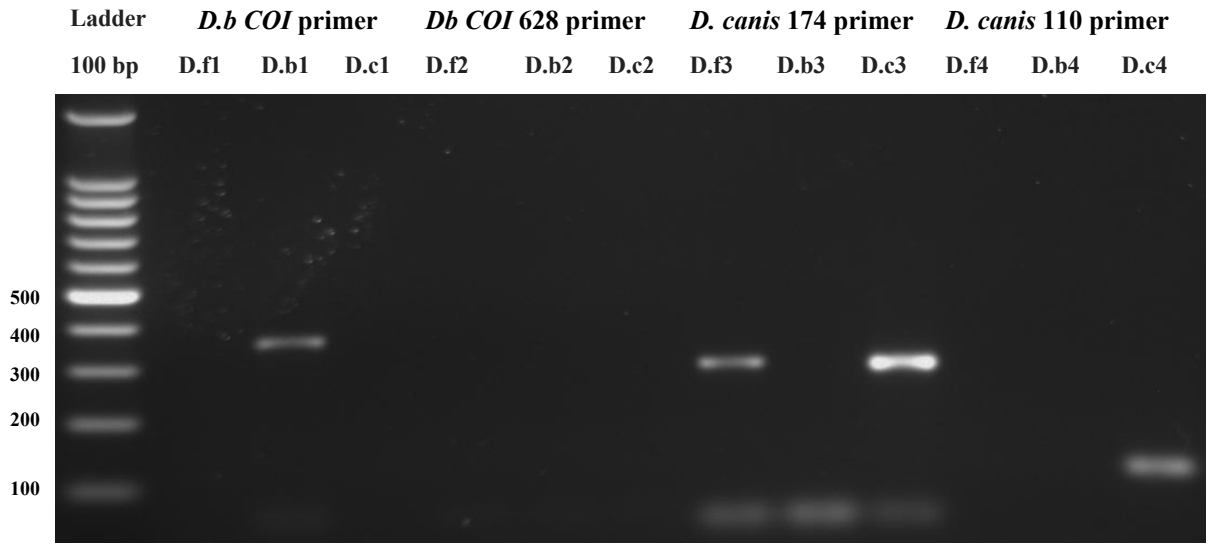

Figure A4.4 PCR amplification products for the new primers with the three *Demodex* species (Controls).

Ladder 100bp

Primer pair Db COI

Lane **D.f1**: negative control, with *D. folliculorum* DNA - no band. **D.b1:** positive control, with *D. brevis* DNA - one band ~350 bp. **D.c1**: negative control, with *D. canis* DNA - no band

Primer pair Db COI 628

Lane **D.f2**: negative control, with *D. folliculorum* DNA - no band. **D.b2**: positive control, with *D. brevis* DNA – no band. **D.c1**: negative control, with *D. canis* DNA - no band.

Primer pair Dc 174

Lane **D.f3**: negative control, with *D. folliculorum* DNA - one band ~310 bp. **D.b3**: negative control, with *D. brevis* DNA – no band. **D.c3**: positive control, with *D. canis* DNA - one band ~300 bp.

Primer pair Dc 110

Lane **D.f4**: negative control, with *D. folliculorum* DNA - no band. **D.b4**: negative control, with *D. brevis* DNA – no band. **D.c4**: positive control, with *D. canis* DNA - one band ~100 bp.

1 % agarose gel.

Amplifying the two human *Demodex* species collected from different human ethnic groups using the primers that have been used in the multiplex PCR test.

D. brevis collected from different ethnic groups amplified by different primer sets used in previous experiments

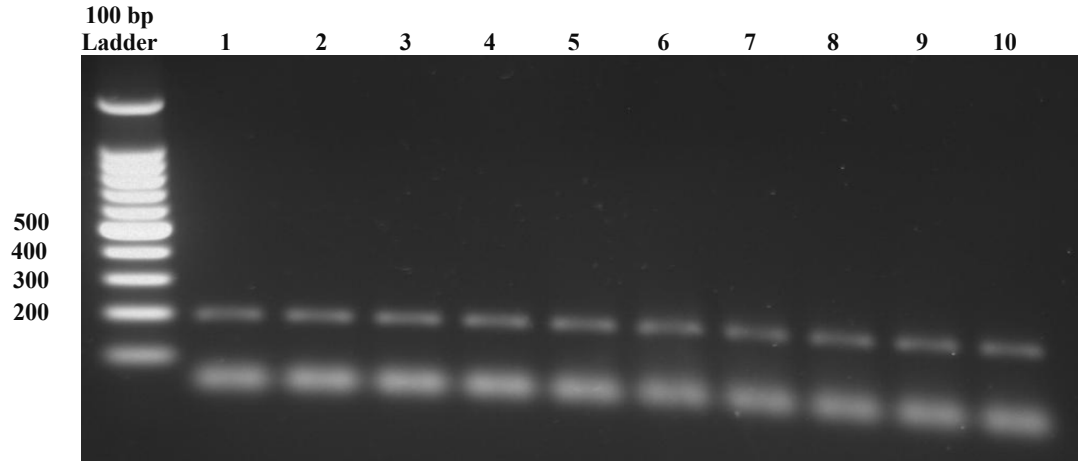

Figure A4.5 PCR amplification of *D. brevis* collected from different 11 different human ethnic groups using Dbm COI primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American. **11**: Pakistani.

In each reaction there is one band at ~200 bp.

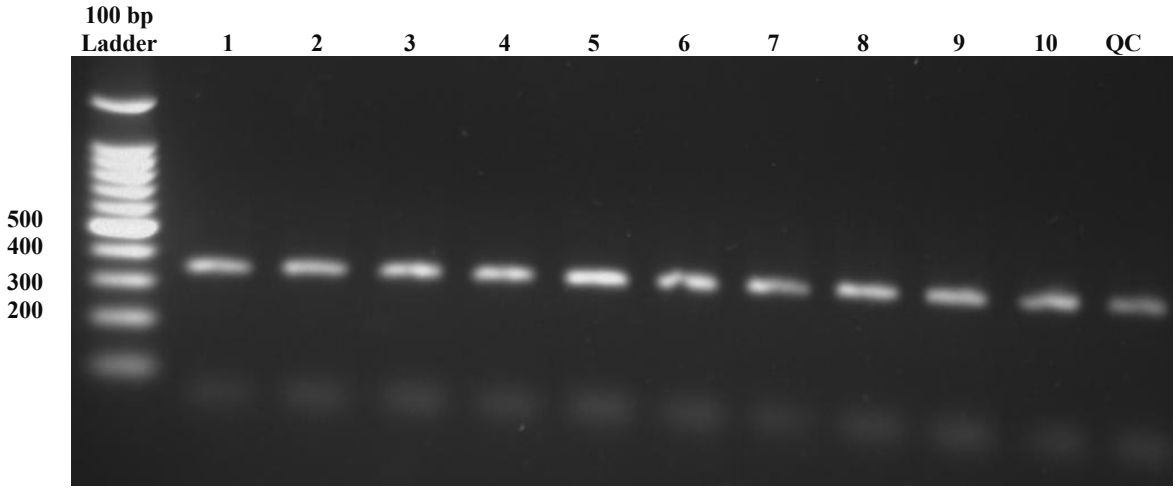

Figure A4.6 PCR amplification of *D. brevis* collected from 10 different human ethnic groups using Db COI primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American.

QC: Positive Control.

In each reaction there is one band at ~350 bp.

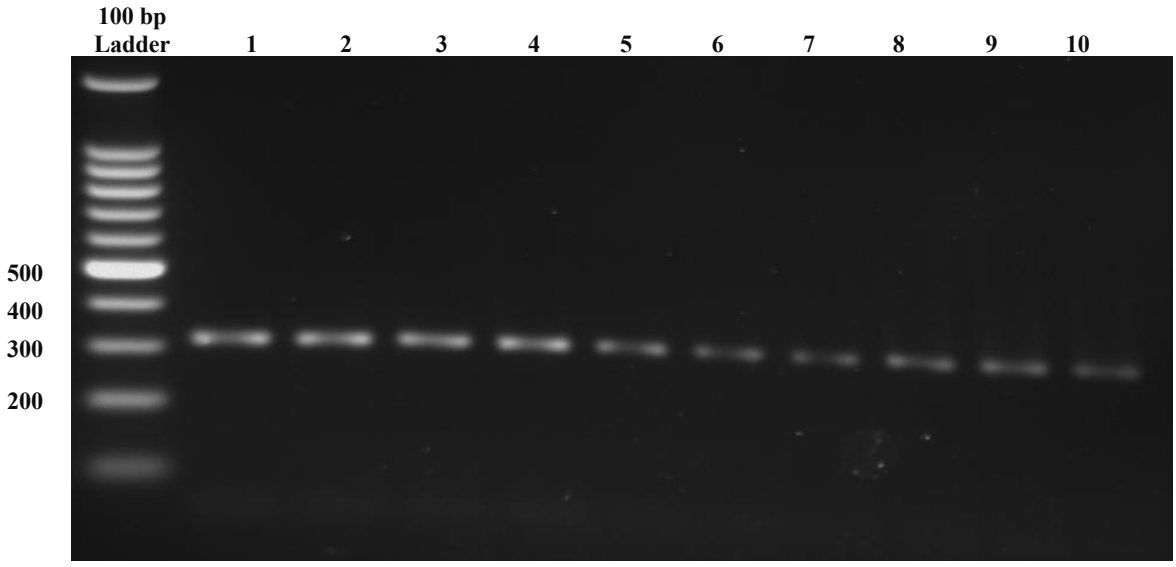

Figure A4.7 PCR amplification of *D. brevis* collected from 10 different human ethnic groups using Db 16S primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American.

In each reaction there is one band at ~300 bp.

D. folliculorum collected from different ethnic groups amplified by different primer sets used in the experiments

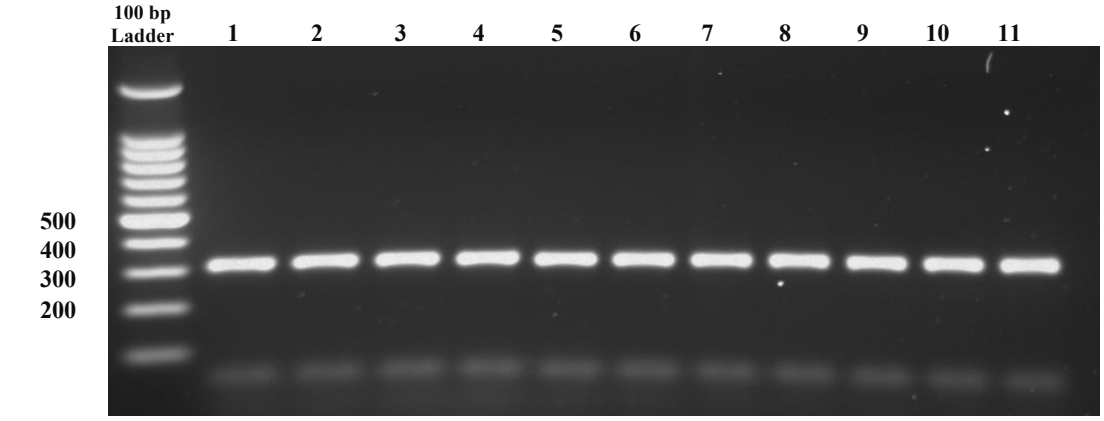

Figure A4.8 PCR amplification of *D. folliculorum* collected from 11 different human ethnic groups using Dfm COI primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American. **11**: Pakistani.

In each reaction there is one band at ~300 bp.

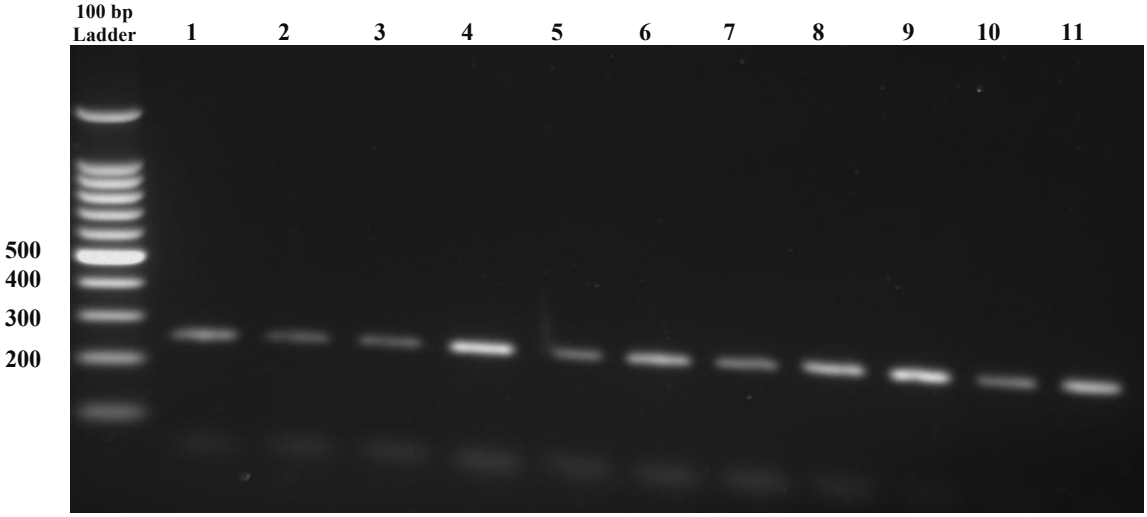

Figure A4.9 PCR amplification of *D. folliculorum* collected from 10 different human ethnic groups using Df COI primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American. **11**: Pakistani.

In each reaction there is one band at ~250 bp.

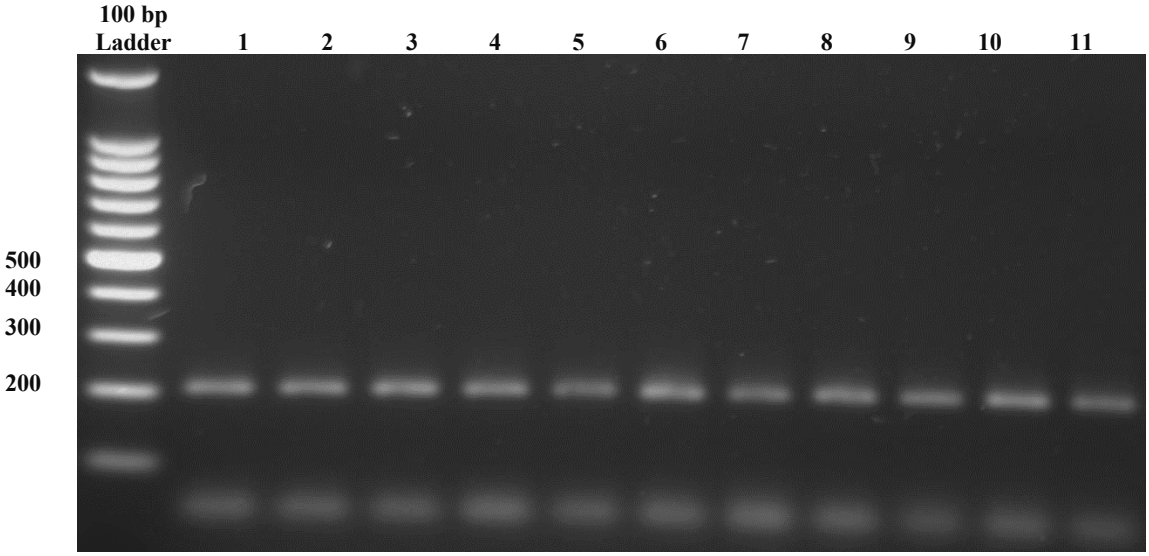

Figure A4.10 PCR amplification of *D. folliculorum* DNA collected from 11 different human ethnic groups using Df 16S primer.

Ladder 100 bp.

Lane **1**: Black African. **2**: British white. **3**: Indian. **4**: Mediterranean White. **5**: Central Europe. **6**: Chinese. **7**: Other Asian. **8**: East- Black Caribbean. **9**: Japanese. **10**: South American. **11**: Pakistani.

In each reaction there is one band at ~200 bp.

Examining dog samples for *D. canis* using Dc 28S 327 primers.

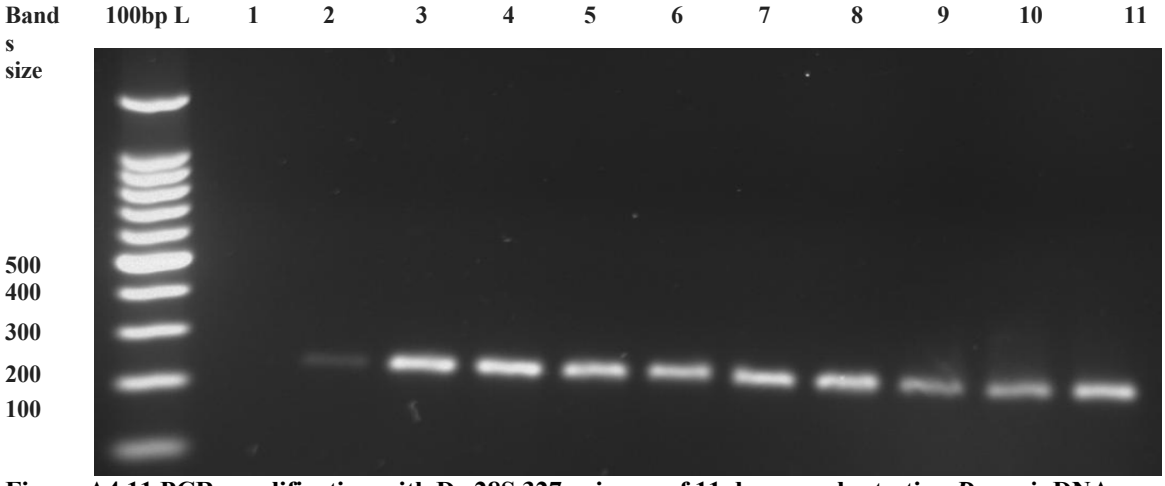

Figure A4.11 PCR amplification with Dc 28S 327 primers of 11 dog samples testing *D. canis* DNA.

Ladder 100 bp.

Lane **1**: negative control, sample number 1 (dog) - no band. **2- 11**: positive controls for *D. canis*, show one band at ~230 bp.

Appendix 5 (Chapter 6)

1. Novel growth medium for rearing *D. folliculorum* in vitro

Table A5.1 Cox proportional hazard model results comparing survival time of *D. folliculorum* reared on four different media and incubated at three different temperatures.

	Coef	exp (Coef)	se (Coef)	z-scare	p-vale
Immersion oil	1.32	3.7	0.25	5.2	0.00001
Oil mixture	-0.12	0.8	0.24	0.5	0.6
RPMI	0.79	2.2	0.23	3.3	0.00001
28-32 ˚C	-0.4	0.6	0.20	-2.4	0.01
35-37 ˚C	2.4	11.3	0.26	9.0	0.00001
	exp(Coef)	exp(-Coef)	lower CI	upper CI	
Immersion oil	3.7	0.2	2.2	6.1	
Oil mixture	0.88	1.1	0.5	1.4	
RPMI	2.2	0.4	1.3	3.5	
28-32 ˚C	0.6	1.6	0.4	0.9	
35-37 ˚C	11.3	0.08	6.6	19.1	
Concordance = 0.868 (se = 0.014)		Likelihood ratio test = 146.1, df = 5, p <0.005			
Wald test = 138.2 on 5 df, p <0.005		Score (log-rank) test = 180.9 df = 5, p <0.005			

Table A5.2 Log rank test for the survival curves of *D. folliculorum* reared on three different media in the literature

Mediums	**Number**	**Observed**	**Expected**	**(O-E)^2/E**	**(O-E)^2/V**
Immersion oil (Control)	42	42	25.9	9.97	91.44
Human Serum	34	34	40.8	1.14	2.60
Oil mixture	40	40	56.9	5.02	13.97
RPMI	42	42	34.4	1.70	3.42
Chi^2 = 31.2, df = 3, pc< 0.001					

Table A5.3 Cox proportional hazard model results for comparing the survival time of *D. folliculorum* reared on three different media used in this research.

	Coef	exp (Coef)	se (Coef)	z-scare	p-vale
immersion oil (Control)	1.83	6.2	0.47	3.90	0.0001
Melanoma cells with Artificial Sebum medium	-0.189	0.15	0.45	-4.1	0.0001
RPMI Based Medium	-1.15	0.31	0.41	-2.7	0.0001
	exp(Coef)	**exp(-Coef)**	**lower CI**	**upper CI**	
Immersion oil	6.2	0.15	2.4	15.81	
Melanoma cells with Artificial Sebum medium	0.15	6.6	0.06	0.3	
RPMI Based Medium	0.3	3.1	0.13	0.70	
Concordance = 0.799 (se = 0.028) Likelihood ratio test = 50.55, df - 3, p <0.0005					
Wald test = 43.18 on 3 df, p <0.0005 Score (log-rank) test = 62.91, df = 3, p < 0.0005					

Table A5.4 Log rank test for the survival curves of *D. folliculorum* reared at three different incubation temperatures

Temperature	Number	Observed	Expected	(O-E) ^2/E	(O-E) ^2/V
20-23 ˚C	55	55	60.6	0.51	1.37
28-32 ˚C	52	52	77.5	8.38	28.83
35-37 ˚C	51	51	19.9	1.70	101.6
$Chi^2 = 170$, df = 2, p < 0.001					

Figures A5.1-A5.3 - some visual results showing the media, construction such as artificial follicles and mites surviving on the media. Photographs were all taken during the rearing experiments and are not to scale.

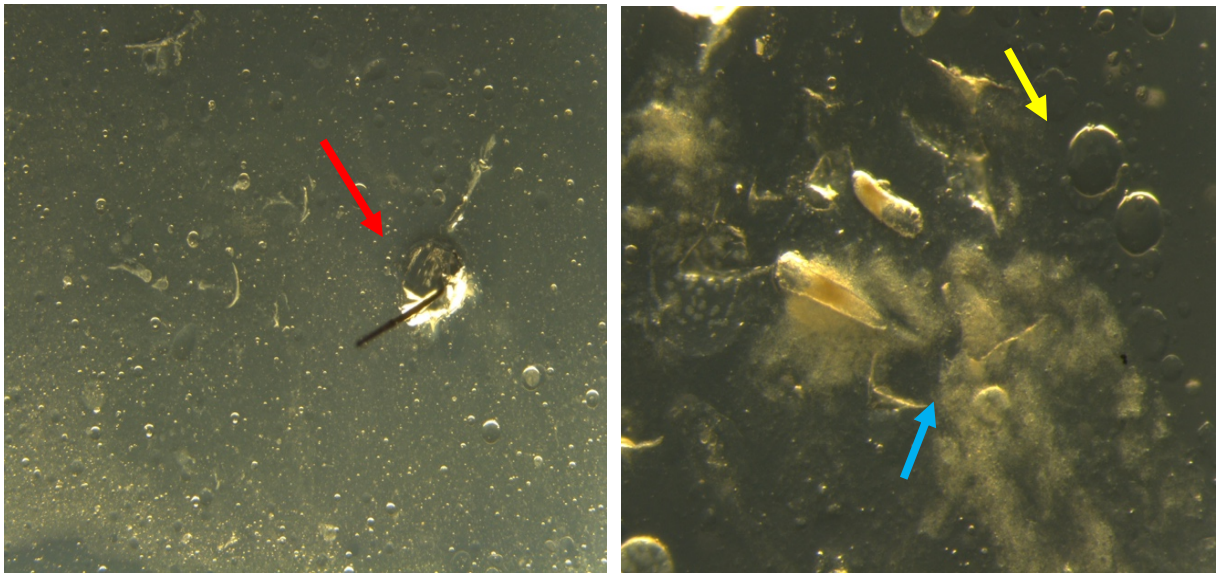

Figure A5.1 An overview of the Agarose / Gelatine Based Medium

Left: medium before incubation with mites: the red arrow is pointing to the artificial hair follicle. Right: mites moving freely on the surface of the medium 24 hours after incubation; the yellow arrow is pointing to the oil drops in the medium base and the blue arrow is pointing to the additive layer

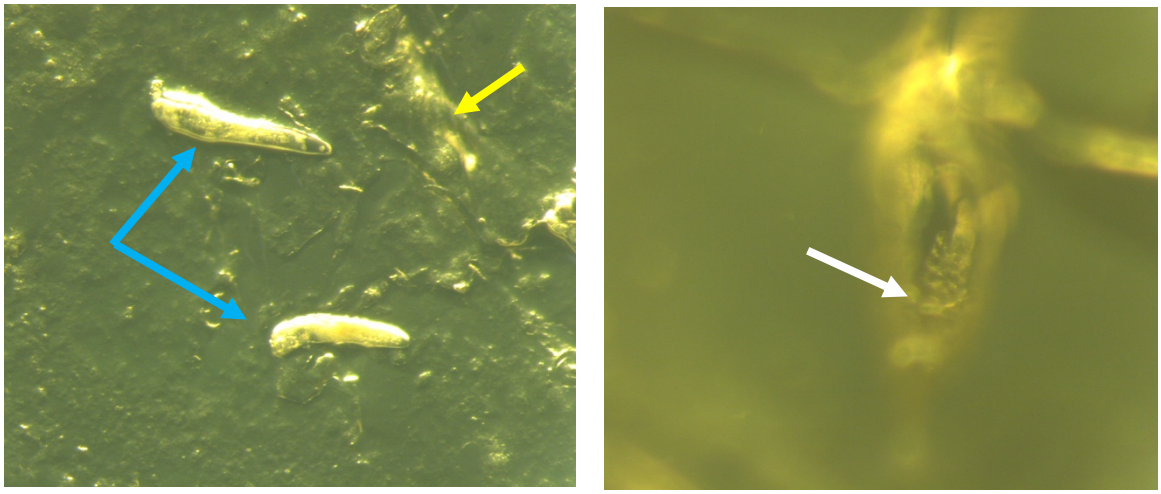

Figure A5.2 An overview of the Agarose / RPMI Based Medium.

Left blue arrows are pointing to two *D. folliculorum* moving freely on the surface of the medium shortly after being transferred to the medium and the yellow arrow is pointing to the additive layer on the medium. Right, the white arrow is pointing to a mite which has made its way to one of the holes and is still alive after 24 hours of incubation.

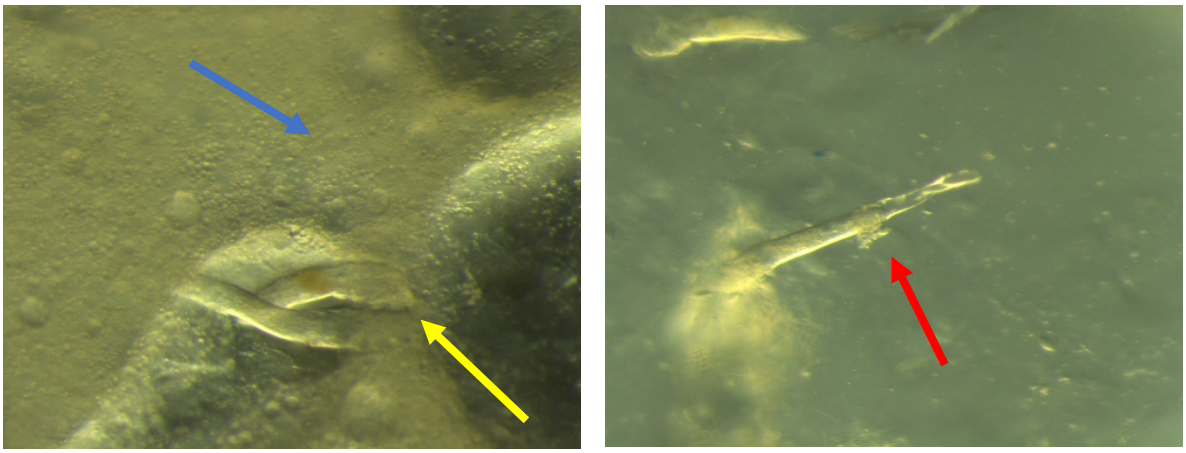

Figure A5.3 Melanoma Cells and Human Artificial Sebum medium for rearing *D. folliculorum*.

Left, yellow arrow is pointing to two *D. folliculorum* moving close to each other on the surface of the medium shortly after the incubation, and the blue arrow is pointing to the additive layers of the medium. Right, red arrow is pointing to a mite moulting its exoskeleton after 48 hours of incubation..

Table A5.5 List of media tested in this chapter with brief component descriptions and limitations

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
1	Agar based medium: contains 2 % agarose agar, 500 µL Argan oil and 0.2 g solid Multivitamins. Hairs were placed horizontally on the surface. The mites were placated on the surface of the medium and then incubated inside in a controlled environment (22 ℃ with 95 % humidity).	Mites were floating in the oil surface and could not swim.	32 - 48 hours
2	Agar based medium: contains of 2 % agarose agar, 240 µL Argan oil, 240 µL Jojoba oil and 20 µL solid Multivitamins (mixed together and place on the surface of the agar). Hair were placed horizontally on the surface. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	Mites were floating in the oil surface and could not swim.	32 - 48 hours
3	Agar based medium: contains of 2 % agarose agar melted by microwave and poured in a petri dish. Then a 1.5 mm-thick sponge scaffold was placed in the agar when still liquid. The components were 240 µL Argan oil, 240 µL Jojoba oil and 20 µL solid Multivitamins. This components mixture dropped directly on the sponge layer. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	The sponge was very thick, and it was difficult to visualise the mites	unknown
4	Agar based medium: contains of 2 % agarose agar melted by microwave and poured in a petri dish. Then a 1.5 mm-thick sponge scaffold place in the agar when still liquid. The components were 240 µL Argan oil, 240 µL Jojoba oil and 20 µL solid Multivitamins. This mixture of components was dropped directly on the sponge layer. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	The sponge was thick, and it was difficult to visualise the mites	unknown
5	Agar based medium: contains of 2 % agarose agar melted by microwave and poured in a petri dish. Then a 1.5 mm-thick sponge scaffold place in the agar when still liquid. The components were 240 µL Argan oil, 240 µL Jojoba oil and 20 µL solid Multivitamins. This components mixture dropped directly on the sponge layer. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	The sponge was still thick, and it was difficult to visualise the mites	unknown
6	Agar based medium: consist of 10 ml (2 % agarose agar) mixed with 800 µl oil mixture contains of four oils Argon, Jojoba, Coconut, Olive (200 µl from each oil) and heated by microwave and poured in a petri dish. When the mixture got to 37 ˚C 20 µL of multivitamins were added. Then 3 ml of medium was poured in the petri dish and a 400 µm- thick sponge scaffold was placed in the medium before it became solid. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	The sponge was still thick, and it was difficult to visualise the mites	unknown
7	Agar based medium: consist of 10 ml (2 % agarose agar) mixed with 800 µl oil mixture contains of four oils Argon, Jojoba, Coconut, Olive (200 µl from each oil) and heated by microwave and poured in a petri dish. When	The sponge was still thick, and it was hard	24 hours

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
	the mixture become to 37 ˚C 20 µL of multivitamin were added. Then 3 ml of medium poured in the petri dish and a 200 µm- thick sponge scaffold was divided into five pieces and then placed in the medium before it became solid. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	to visualise the mites	
8	Agar based medium: consist of 100 ml (2 % agarose agar) mixed with 800 µl oil mixture containing of four oils Argon, Jojoba, Coconut, Olive (200 µl from each oil) and heated by microwave and poured in a petri dish. When the mixture become to 37 ˚C 100 µL of multivitamin were added. A pin was used to dug vertical holes (2 mm in depth) in the medium and a hair was plated each hole. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	The mites moved inside in the holes and drowned. They probably died because of drowning and were unable to use the provided nutrition of the medium.	16 hours
9	Bovine gelatine-based medium consists of 10 ml of distilled water, 9 g gelatine, and 800 µl oil mixture contains of four oils Argon, Jojoba, Coconut, Olive (200 µl from each oil) and heated by water bath at 60 ˚C and mixed for 10 minutes. Then it poured in a petri dish. When the mixture become to 37 ˚C 100 µL of multivitamin were added. A pin was used to dug vertical holes (2 mm in depth) in the medium and a hair was plated each hole. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	This medium was sticky, and the mites couldn't move because of the gelatine concentration was high. fungi grow on this medium after 4 days.	32 hours
10	Bovine gelatine-based medium consists 8 ml of distilled water, 2 g gelatine, and 2 mL of oil mixture contains of four oils Argon, Jojoba, Coconut, Olive (500 µl from each oil) and heated by water bath at 60 ˚C and mixed for 10 minutes. Then it poured in a petri dish. When the mixture become to 37 ˚C 100 µL of multivitamin were added. A pin was used to dug vertical holes (2 mm in depth) in the medium and a hair was plated each hole. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ℃ with 95 % humidity).	It was difficult to visualise the mites because the media was cloudy due to high oil concentration.	Unknown
11	Bovine gelatine-based medium consists of 9 ml of distilled water, 1.2 g of gelatine, and 100 µl of oil mixture contains of four oils Argon, Jojoba, Coconut, Olive (25 µl from each oil) and heated by water bath at 60 ˚C and mixed for 10 minutes. Then it was poured in a petri dish. When the mixture become to 37 ˚C; 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added. A pin was used to dug vertical holes (2 mm in depth) in the medium and a hair was plated each hole. The mites were placated on the surface of the medium and then incubated inside a controlled environment (22 ˚C with 95 % humidity).	Although the mites seem to move freely on the surface, they were unable to use the provided materials as a source of nutrition.	56 hours
12	Filter paper medium with oil mixture contains 200 µl of four oils Argon, Jojoba, Coconut, Olive. A petri dish with	Due to the filter paper	Unknown

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
	a pad (47 mm filter paper) was used for rearing the mites. The mites were placated on the surface of the filter paper and then incubated inside a controlled environment (22 ˚C with 95 % humidity).	being opaque it was difficult to visualise the mites	
13	Agarose / Gelatine Based Medium (AGBM): it was consisted 50 ml of the 2 % agarose gel and 50 ml of the 1 % gelatine mixed together to get a ratio of 1:1. In 10 ml of this mixture 100 µl of four oils Argon, Jojoba, Coconut, Olive 25 µl from each oil was added. Then 3 ml of this medium was poured in a petri dish. Then it was incubated inside a controlled environment in three different temperature 28 ˚C, 30 ˚C and 37 ˚C and 95 % humidity	At the incubation temperature 37 ˚C the media tended to be liquefied and the oil drops formed on the surface of the medium. the mites were unable to use the nutrition in the media	48 hours at temperatures 28 ˚C and 30 ˚C
14	Agarose / Gelatine Based Medium (AGBM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 1 % gelatine mixed together to get a ratio of 1:1. In 10 ml of this mixture 100 µl (of four oils Argon, Jojoba, Coconut and Olive, 25 µl from each oil) and Then it poured in a petri dish. When the mixture become to 37 ˚C; 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added. Then 3 ml of this medium was poured in a petri dish. Then incubated inside a controlled environment in three different temperature 28 ˚C, 30 ˚C and 37 ˚C and 95 % humidity	the incubation temperature 37 ˚C the media tended to be liquefied and the oil drops formed on the surface of the medium. the mites were unable to use the nutrition in the media	48 in hours temperatures 28 ˚C and 30 ˚C
15	Agarose / Gelatine Based Medium (AGBM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 1 % gelatine mixed together to get a ratio of 1:1. In 10 ml of this mixture 100 µl (of four oils Argon, Jojoba, Coconut and Olive, 25 µl from each oil), 200 µl of multivitamin and 500 µl melatonin (30 pg/ml) were added. Then 3 ml of this medium was poured in a petri dish. Then incubated inside a controlled environment in three different temperature 28 ˚C, 30 ˚C and 37 ˚C and 95 % humidity	the incubation temperature 37 ˚C the media tended to be liquefied and the oil drops formed on the surface of the medium. the mites were unable to use the nutrition in the media	48 in hours temperatures 28 ˚C and 30 ˚C
16	Agarose / Gelatine Based Medium (AGBM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture 100 µl (of four oils: Argon, Jojoba, Coconut and Olive, 25 µl from each oil), Then it poured in a petri dish. When the mixture become to 37 ˚C; 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added. Then 3 ml of this medium was poured in a petri dish. After the medium cooled down 500 µl human serum was added to the surface of the medium. Then incubated inside a controlled environment with temperature 37 ˚C and 95 % humidity.	High humidity inside the medium	48 in hours

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
17	Agarose / Gelatine Based Medium (AGM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture Then it poured in a petri dish. When the mixture become to 37 ˚C; 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added (when the media is cooled down at 38 ˚ C). Then 3 ml of this medium was poured in a petri dish. After the medium cooled down 500 µl human serum was added to the surface of the medium. Then incubated inside a controlled environment with temperature 37 ˚C and 95 % humidity.	High humidity inside the medium	48 in hours
18	Agarose / Gelatine Based Medium (AGBM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture 100 µl (of four oils: Argon, Jojoba, Coconut and Olive, 25 µl from each oil), 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added (when the media is cooled down at 38 ˚ C).Then 3 ml of this medium was poured in a petri dish with a filter paper stacked in the lid. After the medium become solid a pin was used to dug vertical holes (2 mm in depth) in the medium and kept for 2 hours to allow water to evaporate. Then hair covered by yeast extract (0.1 %) was plated each hole. Then 150 µl human serum was added to the surface of the medium. Then incubated inside a controlled environment with temperature 37 ˚C and 70 % humidity.	Filter paper to prevent high humidity but, seemed to be infected by fungi very easily. Oils can help moisturising the medium this cannot be used by the mites as nutrition.	48 in hours
19	Agarose / Gelatine Based Medium (AGM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture 100 µL of multivitamin and 500 µL (30 pg/ml) of melatonin were added (when the media is cooled down at 38 ˚ C). Then 3 ml of this medium was poured in a petri dish with a filter paper stacked in the lid. After the medium become solid, a pin was used to dug vertical holes (2 mm in depth) in the medium and kept for 2 hours to allow water to evaporate Then the hair was covered by yeast extract (0.1 %) was plated each hole. After the medium cooled down 150 µl human serum was added to the surface of the medium. Then incubated inside a controlled environment with temperature 37 ˚C and 70 % humidity.	filter paper to prevent high humidity but, it seems to be infected by fungi very easily. Oils can help moisturising the medium this cannot be used by the mites as nutrition.	48 in hours
20	Agarose / Gelatine Based Medium (AGM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture 100 µl (of four oils: Argon, Jojoba, Coconut and Olive, 25 µl from each oil), 200 µL of multivitamin, 3 µl the antibiotic and 3 µL (30 pg/ml) of melatonin were added (when the media is cooled down at 38 ˚ C). Then 3 ml of this medium was poured in a petri dish plates and kept in the safety cabin with the led open to allow steam to get out and not to stick on the lid surface. After the media is solid, a pin was used to dug vertical holes (2 mm in depth) “main hole”. The plates then incubated in 37 ˚C for 2 hours to evaporate the water	There were signs of fungal infection in the medium	48 in hours

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
	from the holes. Then slide holes were dug from the surface lead to the main holes (one opening on the surface of the media and the other opening in the main hole).Then 100 µl of human serum mixed with (0.1 g) gelatine was added to cover all the surface of the media including the holes and lift to absorb by the gel for 5 minutes. A Hair was placed in each hole. The media incubated at 30 ˚C, 45 % humidity.		
21	Agarose / Gelatine Based Medium (AGM): it was consisting of 50 ml of the 2 % agarose gel and 50 ml of the 0.1 % gelatine mixed together to get a ratio of 1:1. In 100 ml of this mixture 100 µL of multivitamin, 3 µl the antibiotic and 3 µL (30 pg/ml) of melatonin were added (when the media is cooled down at 38 ˚ C). Then 3 ml of this medium was poured in a petri dish plates and kept in the safety cabin with the led open to allow steam to get out and not to stick on the lid surface. After the media is solid, a pin was used to dug vertical holes (2 mm in depth) "main hole". The plates then incubated in 37 ˚C for 2 hours to evaporate the water from the holes. Then slide holes were dug from the surface lead to the main holes (one opening on the surface of the media and the other opening in the main hole).Then 100 µl of human serum mixed with (0.1 g) gelatine was added to cover all the surface of the media including the holes and lift to absorbed by the gel for 5 minutes. A Hair was placed in each hole. The media incubated at 30 ˚C, 45 % humidity.	There were signs of fungal infection in the medium	48 in hours
22	Agarose / RPMI Based Medium: it was consisting of 100 ml RPMI cell culture medium mixed with 4 g agarose gel and autoclaved at 121 ˚C, 1.5 atmosphere for 15 minutes. After the media cooled at 40 ˚C add 3 µL of the antibiotic and 3µL of the melatonin. Then 3 ml of this medium was poured in a petri dish. After the media is solid, a pin was used to dug vertical holes (2 mm in depth) "main hole". The plates then incubated in 37 ˚C for 2 hours to evaporate the water from the holes. Then slide holes were dug from the surface lead to the main holes (one opening on the surface of the media and the other opening in the main hole). A 100 µL of mixture (Solution 1) consists of 500 µL of human serum and 0.01 g gelatine (mixed together in an Eppendorf tube and kept for 2 hours in 60 ˚C water bath) was spread on the surface of the medium. A hair was used to fill the holes with the mixture and then placed in the holes. Then the plates were incubated in three different temperature (37 ˚C, 30 ˚C, and 25 ˚C, with 45 % humidity).	The mites cannot use the provided nutrition, namely oils and vitamins.	48 hours At 30 ˚C only
23	Agarose / RPMI Based Medium: it was consisting of 100 ml RPMI cell culture medium mixed with 4 g agarose gel and autoclaved at 121 ˚C, 1.5 atmosphere for 15 minutes. After the media cooled at 40 ˚C add 3 µL of the antibiotic and 3µL of the melatonin. Then 3 ml of this medium was poured in a petri dish. After the media is solid, a pin was used to dug vertical holes (2 mm in depth) "main hole". The plates then incubated in 37 ˚C for 2 hours to evaporate the water from the holes. Then slide holes were dug from the surface lead to the main holes (one opening on the surface of the media and the other opening in the	The mites cannot use the provided nutrition, namely oils vitamins.	56 hours At 30 ˚C only

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
	main hole). A 100 µL of mixture (Solution 2) contains (25 µL of 4 oils (Coconuts, Argan, Jojoba and Olive) and 92 µL of RPMI media mixed thoroughly until they combined) was spread on the surface of the medium. A hair was used to fill the holes with the mixture and then placed in the holes. Then the plates were incubated in three different temperature (37 ˚C, 30 ˚C, and 25 ˚C, with 45 % humidity).		
24	Agarose / RPMI Based Medium: it was consisting of 100 ml RPMI cell culture medium mixed with 4 g agarose gel and autoclaved at 121 ˚C, 1.5 atmosphere for 15 minutes. After the media cooled at 40 ˚C add 3 µL of the antibiotic and 3µL of the melatonin. Then 3 ml of this medium was poured in a petri dish. After the media is solid, a pin was used to dug vertical holes (2 mm in depth) "main hole". The plates then incubated in 37 ˚C for 2 hours to evaporate the water from the holes. Then slide holes were dug from the surface lead to the main holes (one opening on the surface of the media and the other opening in the main hole). A 100 µL of Solution 1 and solution 2 (this mixture is containing 50 µL of solution 1 and 50 µL solution 2 mixed thoroughly with vortex for 30 seconds) was spread on the surface of the medium. A hair was used to fill the holes by the mixture and then placed in the holes. Then the plates were incubated in three different temperature (37 ˚C, 30 ˚C, and 25 ˚C, with 45 % humidity).	The mites cannot use the provided nutrition, namely oils vitamins.	72 hours At 30 ˚C only
25	Human artificial sebum medium: consist of (100 ml) Squalene 15 ml, Paraffin wax 10 ml, Jojoba oil 15 ml, olive oil 10 ml, coconut oil 10 ml, cottonseed oil 25, oleic acid 1.4 ml, Palmitoleic acid 5 ml, Palmitic acid 5 ml, Cholesterol 1.2 ml and Cholesterol oleate 2.4 ml. the Human artificial sebum was heated in a water bath at 60 ˚C then 500 µL was poured in the Petri dish and then incubated at 28 ˚C with 45 % humidity.	Water concentration in the mites' body is higher that it is in the medium (osmotic action), therefore they shrunk and died.	24 hours
26	Human artificial sebum with collagen type 1 medium: consist of 250 µL human artificial sebum (heated in a water bath at 60 ˚C) mixed thoroughly with 250 µL collagen type 1 (ratio 1: 1) and poured in the Petri dish. Then incubated at 28 ˚C with 45 % humidity.	The mites were found shrunk because of the differences of osmotic pressure between medium and the mites' body, which causes lethal damage to the mites' cells and promotes their death	24 hours
27	Human carcinoma cell medium: consist of 100 µL of human carcinoma cell in a sterile tube (obtained from Dr. Dash's laboratory) mixed thoroughly with 30 µL of RPMI, 50 µL collagen type 1, 5.0 µl Antibiotic, 15.0 µl	The human artificial sebum could not mix with	ours

A5.5 Medium	Summary description of the methods and materials	Limitations	Survival time
	Melatonin, 50.0 µl Human serum. The human artificial sebum is heated for 2 minutes at 60 ˚C, when it became liquid, the cooled down until about 37 ˚C. Then 250 µL form the artificial sebum was added the cell mixture in stages (every stage 50µL followed with mixing thoroughly). After that, the final mixture was poured in a petri dish. And the mites were introduced on the surface. Then petri dishes were incubated at 28 ˚C, with humidity between 50 to 80 %.	the other components	
28	Human carcinoma cell medium was consisting of 98 ml of RPMI medium mixed, in a 150 ml baker, with 2 ml of collage type 1, 0.5 g of bovine gelatine and 2 g of agarose gel. Then heated in the microwave for 7 time (20 second each) until the gel dissolve completely. When the mixture cooled down to37 ˚C, 300 µL of melatonin, 5 µL antibiotic was added and mixed thoroughly for 1 minute by vortex. Then 1 ml of the medium was poured in each petri dish. Prepare the first layer, 250 µL cell pellet (provided from Dr. Dash's laboratory) added to 250 µL human serum, then mixed the for 1 minute Before the medium become solid (37 ˚C), 100 µL of the first layer mixture was added to the top surface of the medium. Then, the medium kept for drying in safety cabinet (with the petri dish lid is partly opened). When the medium becomes solid, holes were made in the centre of the media (200 µm- 500 µm). Then it was returned to the safety cabinet to evaporate the medium from. When the holes are completely dried, 100 µL of human serum were added on the surface especially around the hole and keep for 30 min to allow the media to absorb the serum. Then another 100 µL of human serum and sterilised hair was used to force the serum to get inside the holes (one hair each hole). Then the medium was kept for 10 minutes at room temperature to allow the serum to inter inside the holes. After that, 200 µL of the human artificial sebum was added on the surface especially around the holes. Then a number of the mites was inoculated around the hole and incubate at 30 ˚C and humidity 70 %.	Although the mites molt, they were unable to live longer or produce new generation	96 hours
29	Human carcinoma cell medium in capillary tubes: In a 1.5 ml Eppendorf tube, 100 µL of melanoma cells were measured. Then we add to it 100µl RPMI media, 130 µl collage type 1, 150 µl Human serum, 15 µl Melatonin and 5 µl of antibiotic. Then mixed carefully with vortex for 1 minute. Sterilised capillary tubes were cut to 1 cm long. Then 2 % agarose gel were prepared and poured in petri dish. The capillary action was used to insert the medium inside the tubes then we dry it to make a thin layer of media on the wall. The same step was used to insert the human artificial sebum inside the tubes. Then the tubes placed horizontally at the surface of the gel. Then a number of the mites was inculcated around the hole and incubate at 30 ˚C and humidity 70 %.	Mites were found outside and underneath the capillary tubes so it was difficult to follow up them each time of visualization.	hours